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A Rat Liver Lysosomal Membrane Flavin-Adenine Dinucleotide Phosphohydrolase

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Abstract: An enzyme that hydrolyzes flavin-adenine dinucleotide (FAD) was found to be present in rat liver lysosomal membrane prepared from Triton WR-1339 filled lysosomes (tritosomes) purified by flotation on sucrose. This FAD phosphohydrolase (FADase) exhibited optimal activity at pH 8.5 and had an apparent Km of approximately 3.3 mM. The activity was decreased 50~70% by dialysis against EDTA and this was restored by Zn^{2+}. Mg^{+2}, Hg^{+2}, and Ca^{+2} ions inhibited the enzyme, but F^- and molybdate had no effect. The enzyme was also inhibited by *p*-chloromercuribenzoate (pCMB), reduced glutathione and other thiols, cyanide, and ascorbate. The presence of ATP, ADP, AMP, α-β-methylene ATP, AMP-*p*-nitrophenyl phosphate (PNP), GMP, and coenzyme A (CoA) decreased the activity on FAD, but pyrimidine nucleotides, adenosine, adenine, or NAD^+ were without effect. Phosphate stimulated the activity slightly. FAD phosphohydrolase activity was separated from ATPase and inorganic pyrophosphatase activities by solubilization with detergents and polyacrylamide gel electrophoresis and by linear sucrose density gradient centrifugation suggesting that the enzyme is different from ATPase, inorganic pyrophosphatase, and soluble lysosomal FAD pyrophosphatase. Paper chromatography showed that FAD was hydrolyzed to flavin mononucleotide (FMN) and AMP which were further hydrolyzed to riboflavin and AMP by phosphatases known to be present in lysosomal membranes. Incubation of the intact lysosomes with pronase showed that the active site of FAD phosphohydrolase must be oriented to the cytosol. The FAD hydrolyzing activity was detected in Golgi, microsome, and plasma membrane, but not in mitochondria or soluble lysosomal preparations.

Key words: flavin-adenine dinucleotide phosphohydrolase, liver, lysosome, membrane, rat.

Lysosomes are cellular organelles that function in the degradation of extracellular materials and intracellular components. These functions are accomplished by a unique membrane that sequesters numerous enzyme hydrolases utilized for degradation and modification of these substances and allows for the release of digestion products through exo- and endocytosis. The lysosomal membranes rapidly and bidirectionally communicate with other membranes from other organelles including Golgi, microsomes, and the plasma membrane by membrane fusion. Nevertheless, each structure can be distinguished by a combination of cytologic, biochemical, and physical criteria (Steinman *et al.*, 1983).

Two models (mannose-6-phosphate dependent and mannose-6-phosphate independent) for lysosomal enzyme transport have been proposed (Lemansky *et al.*, 1985; Murray *et al.*, 1985; Von Figura and Hasilik, 1986). However, it is not well understood how the lysosomal membrane and enzymes are formed and maintained during the rapid and extensive flow of membrane occurring throughout the vacuolar membrane system in organisms. Several lysosomal membrane proteins from various sources have been identified (Reggio *et al.*, 1984; Chen *et al.*, 1985; Lewis *et al.*, 1985), and several lysosomal diseases due to lysosomal protein defects have been reported (Klionsky and Emr, 1990; Alroy *et al.*, 1991; Gieselmann, 1995; Mahuran, 1995).

We have discovered an enzyme with FAD hydrolyzing activity in purified rat liver lysosomal membranes. Properties of this enzyme were characterized and compared to other phosphate releasing activities and cycling of the enzyme in membrane flow was checked.

Materials and Methods

Materials

FAD, pyrophosphate, nucleotides, NAD^+, detergents,

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CoA, bovine serum albumin, Tris, EDTA, and acid phosphatase were purchased from Sigma Chemical Co. (St. Louis, USA). Bicinchoninic acid was purchased from Pierce Chemical Co. (Rockford, USA). All other chemicals were of reagent grade quality.

Preparation of lysosomal membrane and other subcellular fractions

Lysosomes were purified from rat liver according to the flotation method of Wattiaux *et al.* (1963) modified by Trouet (1964) and Leighton *et al.* (1968). To prepare membranes, purified tritosomes were sedimented by centrifugation (44,000×*g* for 40 min), suspended in ice-cold distilled water, and dialyzed against water for 12 h with several changes to disrupt intact lysosomes. These membrane preparations were sedimented by centrifugation at 114,000×*g* for 60 min and then washed twice with 2 vol of 0.5 M KCl followed by a wash with cold distilled water. Membrane solubilization was accomplished by stirring for 30 min in 1% (v/v) Triton X-100 or 1 mg/ml lysolecithin in 25 mM Tris-acetate buffer, pH 7.4, on ice, followed by centrifugation at 189,000×*g* for 1 h. The resulting pellet was resuspended in 1% Triton X-100 or 1 mg/ml lysolecithin, and both supernatant solutions were combined. Solubilized membranes were diluted fourfold with 25 mM Tris-acetate buffer and then concentrated to about 5 to 10 ml (final concentration, 1~2 mg/ml) with the Amicon Diaflo apparatus equipped with a XM-50 membrane. All operations were performed at 0~4℃. These solubilized and concentrated membrane preparations were stable for several months when stored at −70℃. Other subcellular fractions were prepared according to methods described by Bergeron *et al.* (1982) for Golgi fractions, by Lindahl (1979) for mitochondria and microsomes, and by Armstrong and Newman (1985) for plasma membranes, and their lack of cross-contamination was verified by marker enzyme analyses.

Assays

Protein concentrations were determined spectrophotometrically with bicinchoninic acid as described by Smith *et al.* (1985) using bovine serum albumin in 0.25% (v/v) Triton X-100 or 0.1% (w/v) sodium dodecyl sulfate (SDS) containing 0.5 M Tris-HCl, pH 6.8, as standards. The hydrolysis of FAD to FMN and AMP was measured spectrophotofluorometrically as described by Ragab *et al.* (1968). This assay is based on the fact that FMN exhibits a fluorescence intensity threefold greater than that of FAD. Reaction mixtures (1 ml) contained 0.3 mM FAD, 100 mM Tris-acetate buffer, and other additions as described in the text. Possible quenching effects of these other additions on FMN fluorescence intensity were checked in each of these assays and data were then adjusted. A unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol FMN per minute at 37℃. The hydrolyses of NAD and CoA were assayed by incubations at pH 8.5 followed by measurement of inorganic phosphate released after further incubations with acid phosphatase at pH 5 using the phosphate assay of Fiske and Subbarow (1925). The hydrolysis of nucleoside phosphates and pyrophosphate was also measured by inorganic phosphate analysis under the same conditions as for FAD hydrolysis. *p*-Nitrophenyl phosphate was the substrate for acid phosphatase assays according to the methods of Engstrom (1961) with some modifications. Reaction mixtures contained 1.5 mM EDTA, 50 mM citrate buffer, 1 mM *p*-nitrophenyl phosphate and protein in volumes of 1 ml. Incubations were at 37℃ for desired periods followed by additions of 4 ml 0.1 M NaOH to stop the reaction and develop the color. Blanks contained water instead of protein solution. Absorbances were read at 410 nm. One unit of enzyme activity is defined as the activity producing 1 μmol of inorganic phosphate from the substrate per min under the desired conditions. Arylsulfatase was assayed by the method of Chang *et al.* (1981).

Gel electrophoresis

For the separation of FAD phosphohydrolase activity from other activities, non-denaturing polyacrylamide gel electrophoresis was first performed with 0.1 mg/ml lysolecithin. Separating gels were prepared in 1 ml of Tris-HCl, pH 7.5, 1 ml of 28% polyacrylamide, 2 ml of catalyst, and 0.1 mg/ml lysolecithin. Stacking gels were prepared in 0.1 ml of 0.33 M Tris-phosphate buffer, pH 5.5, 0.1 ml of polyacrylamide (28%), 0.1 ml of riboflavin, 0.5 ml of 40% (w/v) sucrose, and 0.1 mg/ml lysolecithin. Crystals of sucrose and 10 μl of tracking dye were added to lysolecithin-solubilized tritosomal membrane proteins (about 0.2 ml). After the lysolecithin solubilized tritosomal membrane proteins were carefully applied to the tops of the gels, electrophoresis was performed at 4℃, 25 mA per tube gel, until the tracking dye reached the ends of the gels. The gels were sliced into 2 mm sections and these were cut in half. This was accomplished by sucking the gels into the barrel of a 1 ml tuberculin syringe with the end removed and a cap containing a piece of platinum wire attached in such a way to extend across the end of the gel. As the gel was forced from the tube, it was split down the middle and 2 mm segments were sliced off. The sliced gels were assayed for the desired enzyme activities. To improve the mobility of native proteins in the non-denaturing gel electro-

phoresis, polyacrylamide slab (1.5 mm thick) gel electrophoreses were performed according to Newby *et al.* (1979) using 5.5% gel in the presence of 0.1% (v/v) Triton X-100 with 0.03% (w/v) sodium deoxycholate in the upper buffer. Although FAD phosphohydrolase activity was decreased approximately 40~50% in the presence of sodium deoxycholate, the mobility and separation of proteins were greatly improved if the ionic detergent was present in the upper buffer.

Sucrose density gradient centrifugation

Twenty-five hundredths percent Triton X-100-solubilized tritosomal membrane proteins in 5% (w/v) sucrose were centrifuged at 50,000 rpm in a SW 50.1 rotor for 12 h in a linear 10~20% sucrose gradient containing 0.25% Triton X-100 and 25 mM Tris-acetate buffer, pH 7.4. Ten drop fractions were collected from the bottoms of the centrifuged tubes by means of a peristaltic pump. Each fraction was diluted with 25 mM Tris-acetate buffer, pH 7.4 to 1 ml, and aliquots of these were assayed for the desired enzyme activities.

Paper chromatography

Products of FAD hydrolase activity were identified by ascending paper chromatography in *n*-butanol-acetic acid-water (4:1:5) solvent. Spots were detected by fluorescence in ultraviolet light.

Results

Effects of pH and substrate concentration on FAD phosphohydrolase activity in membrane preparations

Initial studies were performed by measuring phosphate released from FAD degradation products by the action of phosphatases present in the lysosomal membrane (Chung *et al.*, 1980). The results showed that the FAD hydrolyzing activity had two pH optima: pH 5 at low substrate concentration, pH 8 at high substrate concentration. Further studies in which FAD phosphohydrolase was assayed by fluorescence revealed that the pH 5 peak was not enzyme catalyzed. Fig. 1 shows that FAD was apparently hydrolyzed non-enzymatically at acid pH. The quenching of riboflavin fluorescence by pH is also shown in Fig. 1. Therefore, when pH profiles without enzyme were substracted from enzyme-catalyzed pH profiles, a revised profile of FAD hydrolysis measured by fluorescence showed a sharp peak at pH 8.5 (Fig. 1). The pH curve suggested that the membrane FAD phosphohydrolase was different from the soluble lysosomal pyrophosphatase known to hydrolyze FAD since the soluble enzyme had an acid pH optima (Ragab *et al.*, 1968). The apparent K_m for hydrolysis

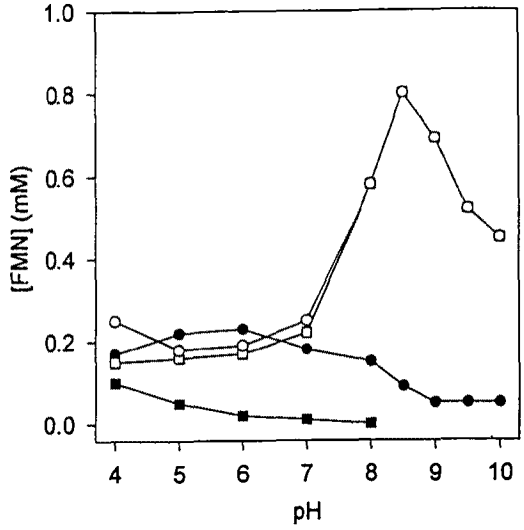

Fig. 1. The pH profile of FAD hydrolysis by lysosomal membrane. Reaction mixtures contained 0.1 M Tris-acetate buffer, 1 mM FAD and 10 μg of tritosomal membrane protein. Enzyme activities were assayed by fluorescence measurements (Materials and Methods) during incubation for 20 min at 37℃. ○, actual pH curve; ■, pH FAD quenching by buffer; □, substrated pH curve from FAD quenching by buffer; ●, quenching of riboflavin by pH.

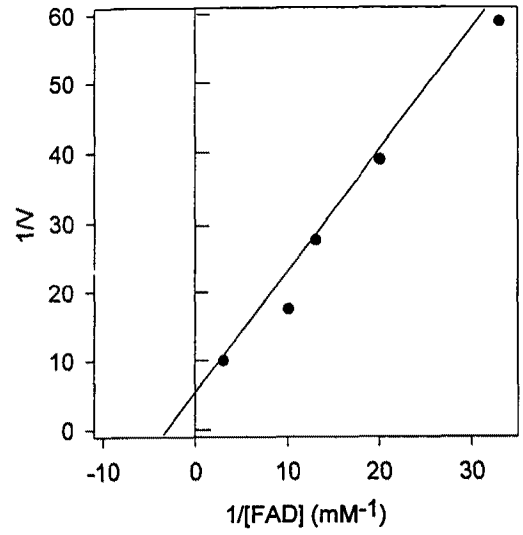

Fig. 2. A double reciprocal plot of FAD phosphohydrolase activity against FAD concentrations. Reaction mixtures were the same as those described in the legend to Fig. 1.

of FAD by intact membrane was 3.3×10^{-3} M (Fig. 2).

Effect of ions on the enzyme preparation

Several ions known to be stimulators or inhibitors of other FAD hydrolyzing enzymes from various sources were tested on tritosomal membrane FAD phosphohydrolase activity. Table 1 shows that Mg^{2+}, Hg^{2+}, and Ca^{2+} inhibited and Zn^{2+} stimulated the enzyme

Table 1. Effect of ions upon enzyme activities

Ion	Molar concentration		
	10^{-2}	10^{-3}	10^{-4}
Mg^{2+}	−23.58	−22.20	−10.78
Hg^{2+}	−83.58	−60.47	−17.91
Zn^{2+}	+38.97	+19.91	+12.79
Ca^{2+}	−15.17	−8.53	−8.53
F^-	+9.24	+3.55	−7.11
MoO_4	+2.13	−7.82	−8.53

Results are shown as percentage stimulation (+) or inhibition (−) compared to the control. Reaction mixtures contained 0.1 M Tris-acetate buffer, pH 8.5, 1 mM FAD, 10 μg of tritosomal membrane protein and ions described above. No ion showed quenching of fluorescence. Incubation periods were 20 min at 37℃.

Table 2. Effect of dialysis against EDTA, water and Zn^{2+} on FAD phosphohydrolase activity

No. experiments	Dialysis against	Time (h)	% inhibition
2	EDTA	4	50~70
1	Water	8	48
1	Zn^{2+}	4	1

The enzymes were dialyzed against EDTA (1 mM) for 4 h, water for 8 h and Zn^{2+} (1×10^{-2} M) for 4 h sequentially. Aliquots were assayed after each dialysis. Reaction mixture and conditions were as described in Material and Methods. Incubation periods were 20 min at 37℃.

activity. An interesting observation was that F^- and molybdate, which are inhibitors of acid phosphatase and acid pyrophosphatase (Kumar *et al.*, 1965; Ragab *et al.*, 1968), had no significant effect on the enzyme. Since none of the known FAD hydrolyzing enzymes from various sources has been shown to be activated by Zn^{2+}, further studies on the effect of this metal were performed. A membrane preparation was dialyzed against 1 mM EDTA for 4 h, and aliquots were then removed and assayed. The remaining membranes were dialyzed against water for 8 h to remove EDTA and then dialyzed against Zn^{2+} for 4 h. Table 2 showed that the activity was decreased about 50~70% by dialysis against EDTA, and this was restored by dialysis against Zn^{2+}.

Effects of thiols, thiol reagents, nucleosides and nucleotides on the enzyme activity

p-Chloromercuribenzoate, reduced glutathione and cysteine at 10^{-3} M, 10^{-4} M, and 2×10^{-3} M respectively, inhibited the enzyme activity by approximately 40~60%. Arsenite and sulfite stimulated the activity slightly (Table 3). The activity was also sensitive to ascorbate and dithiothreitol, and 1 mM EDTA inhibited about 75% (Table 3). Even though this data cannot be used to determine binding mechanisms at the catalytic site of the enzyme in crude preparations, the results show that the effect of these chemicals on tritosomal membrane FAD phosphohydrolase was different from that of other enzymes known to hydrolyze FAD (Kumar *et al.*, 1965; Ragab *et al.*, 1968). The enzyme activity was also tested in the presence of other nucleo-

Table 3. Effect of some agents on enzyme activities

Chemical	Concentration (mM)	Effect
pCMB	1	−37.6
Cyanide	0.1	−13.2
Sulfite	1	+15.4
GSH	2	−37.7
Cysteine	2	−63.2
Arsenite	1	+5.2
Ascorbic acid	4	−31.6
Dithiothreitol	12	−77.2
EDTA	1	−74.9

Reaction mixtures were the same as those described in the legend to Table 1, except for the above substances. Incubation periods were 15 min at 37℃. No quenching of fluorescence was observed by the chemicals.

Table 4. Effect of some substances and phosphodiester compounds upon intact membrane FAD phosphohydrolase activity

Effector	Effect
ATP	−48.9
ADP	−23.4
AMP	−44.7
Adenosine	−5.0
Adenine	−11.2
NAD^+	−7.1
CoA	−43.7
Pyrophosphate	+19.3
GMP	−44.0
CMP	−2.0
TMP	−14.0
UMP	−17.0
Adenosine-2′ & 3′-monophosphoric acid	−5.0
AMP-PNP	−71.9
α,β-methylene ATP	−71.2

Results are shown as percentage simulation (+) or inhibition (−) compared to the control. Enzyme was assayed as described in the legend to Table 1, except for above compounds. Concentrations of effectors are all 50 mM. Incubation periods were 20 min at 37℃. None of the compounds showed quenching of FMN fluorescence.

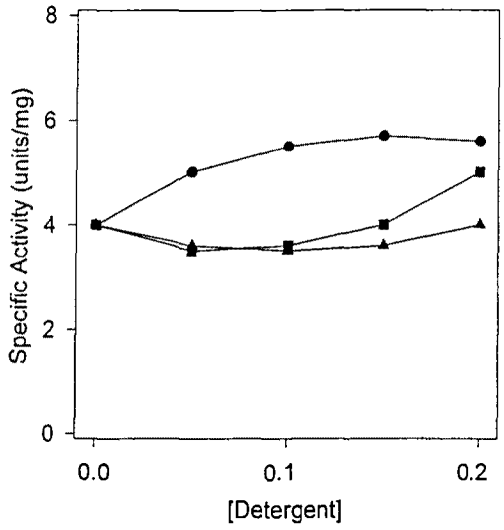

Fig. 3. Effect of lysolecithin, Zwittergent, and Triton X-100 on tritosomal membrane FAD phosphohydrolase activities. Enzyme activities were assayed at pH 8.5. Incubation periods were 20 min at 37℃. Reaction mixtures are described in Materials and Methods. ●, Triton X-100 (%); ■, Zwittergent (mg/ml); ▲, lysolecithin (mg/ml).

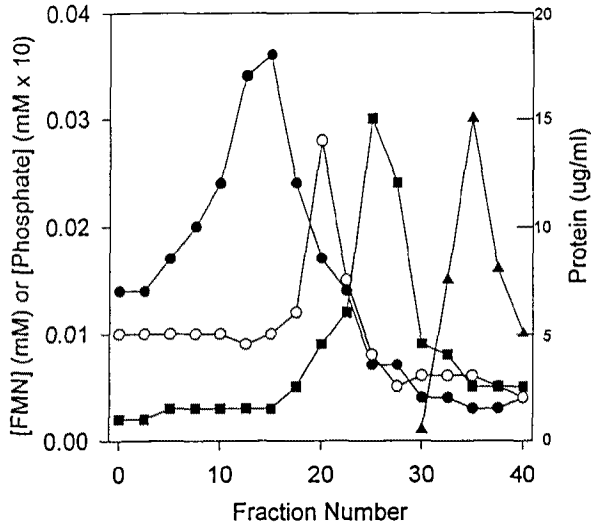

Fig. 4. Sucrose density gradient centrifugation of Triton X-100 solubilized membrane FAD phosphohydrolase, ATPase, and pyrophosphatase. Activities of enzymes were assayed at their pH optimum as described in the legend to Fig. 4. The incubation periods were 30 min at 37℃. ●, FAD phosphohydrolase; ■, ATPase; ○, pyrophosphatase; ▲, protein assay.

tides and pyrophosphodiester compounds which have partial structural similarity to FAD. ATP, ADP, AMP, AMP-PNP, α-β-methylene ATP, and coenzyme A inhibited; and adenosine, adenine, and NAD^+ had no significant effect on the FAD phosphohydrolase activity. Pyrophosphate stimulated the activity slightly (Table 4). Table 4 shows that GMP decreased the activity about 44% but CMP, UMP, TMP, and adenosine-2′ and 3′-monophosphoric acid had no effect. These results suggest that the mechanism of binding of FAD to the enzyme catalytic site may occur through phosphate bond and purine components.

Separation of FAD phosphohydrolase activity from other phosphohydrolase activities

Lysosomal membranes have been shown to contain 5′-nucleotidase (AMPase), ADPase, ATPase, pyrophosphatase, and glycerol phosphatase (acid phosphatase) activities (Chung *et al.*, 1980). It is possible that the FAD hydrolase activity was due to one of these phosphohydrolases. Several attempts were made to differentiate FAD phosphohydrolase from other phosphate-hydrolyzing activities. In order to separate FAD hydrolyzing activity from other phosphohydrolases, it was necessary to solubilize tritosomal membranes. Fig. 3 shows that Triton X-100, lysolecithin, and zwittergent were effective solubilizing agents for the FAD phosphohydrolase. Triton X-100 stimulated the activity, but lysolecithin and zwittergent had no apparent effects. Even though Triton X-100 solubilized most of the membrane proteins, this detergent was difficult to be removed from the preparations, and it interfered with protein assays. Since lysolecithin has a relatively low molecular weight and had no effect on lysosomal membrane FAD phosphohydrolase and protein assays, this detergent was occasionally used to solubilize the enzyme for polyacrylamide gel electrophoresis and other preparations. Non-denaturing polyacrylamide gel electrophoresis was performed with 0.1 mg/ml lysolecithin at 4℃. Gel sections (2 mm) sliced in half (see Methods) were incubated overnight in the presence of various substrates. FAD phosphohydrolase activity was separated from ATPase and pyrophosphatase activity in the same gel preparations (data not shown). Although the activities partially overlapped, the peaks were significantly separated.

Triton X-100 solubilized lysosomal membrane proteins were applied to the top of 10~20% (w/v) linear sucrose gradients and centrifuged for 12 h at 118,000 ×*g*. FAD phosphohydrolase activity was also separated from ATPase and pyrophosphatase activities by this technique (Fig. 4). These results suggest that the FAD phosphohydrolase was not due to the other known lysosomal membrane phosphohydrolases.

Products of FAD hydrolysis

Isobutanol-acetic acid-water (4:1:5) was used as a solvent for the paper chromatography of reaction mixtures containing FAD, NAD^+, $NADP^+$, and coenzyme A. After incubation with solubilized membrane prepara-

Table 5. The effect of pronase on intact lysosomal FAD phosphohydrolase and acid phosphatase activity

	FAD phosphohydrolase	Acid Phosphatase
Without pronase	100	100
With pronase	62	88

Intact lysosomes in 0.25 M sucrose were divided into 2 parts and then incubated with or without pronase (0.1 mg/ml) in 0.25 M sucrose for 5 min at 30℃. Reaction was stopped by chilling in ice and centrifugation. Pellets were washed with 0.25 M sucrose and then suspended in water. Suspensions were assayed for FAD phosphohydrolase and acid phosphatase as described in Materials and Methods. Incubation periods were 20 min at 37℃. Activities are shown as percentages.

Table 6. Distribution of FAD phosphohydrolase and other lysosomal marker enzymes in cellular organelles

	FAD phosphohydrolase	Acid phosphatase	Aryl-sulfatase
Lysosomal membrane	0.320	0.14%1%	0.045
Soluble fraction	0	0.18	0.380
Golgi	0.072	0.008	0.009
Plasma membrane	0.067	0.029	0.016
Microsome	0.040	0.010	0.008
Mitochondria	0	0.042	0.053

Enzyme activities and protein concentrations were determined as described under Materials and Methods. Results are reported as specific activities (μmol/min/mg). FAD phosphohydrolase assays were done only at pH 8.5.

tions, the reactions were stopped and spotted on the paper. RFs of FAD, FMN, riboflavin, AMP, and adenosine were 0.166, 0.367, 0.673, 0.226, and 0.433, respectively, in this system. Reaction mixtures containing FAD showed 3 spots corresponding to standards of riboflavin, adenosine and FAD, but none of other substrates showed detectable breakdown products in this assay (data not shown). In the presence of the acid phosphatase inhibitors tartrate or fluoride, FAD hydrolysis showed two major spots corresponding to AMP and FMN standards and only traces of adenosine and riboflavin (data not shown).

Orientation of the FAD phosphohydrolase in the membrane

The evidence that lysosomal membrane FAD phosphohydrolase is active at pH 8.5~9 suggested that the active site of the enzyme may not be toward the luminal side of the lysosome, which is acidic, since there was no detectable enzyme activity at acidic pH (Fig. 1). In efforts to determine if the FAD phosphohydrolase active site was situated on the outer membrane surface, intact lysosomes were briefly incubated with 0.1 or 0.2 mg/ml pronase in sucrose, and the activity was then compared with non-pronase treated lysosomes. The effect of the pronase treatment on intact lysosomes is shown in Table 5. These results are consistent with the active site of FAD phosphohydrolase oriented to the cytosol.

Organelle distribution of FAD phosphohydrolase activity

Since lysosome proteins are derived from the Golgi, microsome, and possibly plasma membrane, these organelles and mitochondria were isolated and assayed for FAD hydrolysis. Table 6 shows FAD hydrolyzing activity in these preparations. Golgi, plasma membrane, and microsome fractions showed detectable FAD hydrolyzing activity but no activity was detected in mitochondria and soluble lysosomal preparations.

Discussion

Rat liver lysosomal membrane contains a number of phosphohydrolase activities differing from their soluble counterparts with respect to pH optima and divalent cation requirements (Chung *et al.*, 1980). FAD phosphohydrolase from lysosomal membrane fractions described here showed different properties from its soluble lysosomal counterpart or other enzymes known to hydrolyze FAD.

First, lysosomal membrane FAD phosphohydrolase was most active at pH 8.5~9 with no detectable activity at pH 4~5 which is the optimum pH for the soluble enzymes (Ragab *et al.*, 1968).

Second, lysosomal membrane FAD phosphohydrolase was not stimulated by Mg^{2+}, or Ca^{2+}, and was inhibited by EDTA, and stimulated by Zn^{2+}. The soluble enzyme is not affected by divalent cations, including Zn^{2+}, nor is it inhibited by EDTA (Ragab *et al.*, 1968). However, the possibility exists that membrane-associated activities are simply adsorbed soluble enzymes, or soluble acidic forms that have been converted to membrane bound enzymes with alkaline pH optima by the action of certain lysosomal proteinases or glycohydrolases. For example, Goldstone *et al.* (1971) found that neuraminidase treatment changed the electrophoretic mobilities of several lysosomal enzymes from acidic to basic forms. Basic forms are more firmly bound to lysosomal membrane residues and are released by treatment with Triton X-100. However, there have been no reports of treatments resulting in changes in pH optima or in divalent cation requirements of soluble lysosomal enzymes. FAD phosphohydrolase activity was decreased by dialysis against 1 mM EDTA but restored

by dialysis against Zn^{2+} (Table 2) suggesting that the lysosomal membrane FAD phosphohydrolase requires Zn^{2+} for its function. None of the FAD degrading enzymes previously reported has been shown to require Zn^{2+} (Kornberg and Pricer, 1950; Deluca and Kaplan, 1958; Kumar *et al.*, 1965).

Third, FAD hydrolysis in the lysosomal membrane preparations was inhibited by several thiols, thiol reagents, nucleosides, and nucleotides (Table 3 and 4). However, these data cannot be directly interpreted with respect to substrate binding mechanisms, but can be used to suggest that the sensitivities of the lysosomal membrane FAD phosphohydrolase toward certain agents were different from those of other enzymes known to hydrolyze FAD. For example, the Mung bean enzyme was inhibited by sulfite and arsenite (Kumar *et al.*, 1965), but pCMB had no effect, while the lysosomal membrane enzyme was inhibited by pCMB and slightly activated by sulfite and arsenite (Table 3). The FAD phosphohydrolase described here is not an acid phosphatase as shown by the fact that the presence of tartrate and fluoride did not affect FAD hydrolysis (Table 1). However, FAD hydrolyzing enzymes have been reported as pyrophosphatases (Kornberg and Pricer, 1950; Yamawaki, 1956; Ragab *et al.*, 1968) or as ATPase (Dickson *et al.*, 1983). Several attempts were made to separate FAD phosphohydrolase in the crude lysosomal membrane preparations from other activities reported to hydrolyze FAD. Non-denaturing polyacrylamide gel electrophoresis of lysolecithin-solubilized lysosomal membrane showed that FAD phosphohydrolase activity was separable from ATPase and pyrophosphatase in the same gel preparations. Sucrose density gradient centrifugation experiments support these results. Finally, separation of FAD phosphohydrolase from ATPase and acid pyrophosphatase activities therefore showed conclusively that the FAD phosphohydrolase was not due to these enzymes. Thus, FAD phosphohydrolase in the crude lysosomal membrane as characterized here is different from soluble lysosomal acid phosphatase as well as membrane ATPase or pyrophosphatase.

Lysosomal membrane proteins are synthesized in endoplasmic reticulum and transported to Golgi for processing and sorting. FAD phosphohydrolase activity was found in Golgi, plasma membrane and microsome, but not in mitochondria and soluble lysosomal fractions (Table 6). However, the activities of these organelles were very low compared to that of lysosomal membranes. This could be explained by the small amount of the enzyme in Golgi and plasma membrane (shown by specific activities compared to lysosomal membrane) and possible cycling of this enzyme from these organelles. It has been shown that lysosomal contents and membrane components can be sorted from one another through rapid movement of vesicles in seconds to minutes (Herzog and Farquhar, 1977; Steinman *et al.*, 1983; Lippincott-Schwartz and Fambrogh, 1986) by different mechanisms (Klionsky and Emr, 1990).

To determine the orientation of the active site of FAD phosphohydrolase, intact lysosomal activity was assayed with or without pronase. The results obtained in this experiment are consistent with a cytosolic orientation of the active site. This is also supported by the pH 8.5~9 optimum of the enzyme. This enzyme would probably be non-functional if the active site were oriented toward the luminal side of the lysosome where the pH is acidic. This suggests that the functional role of the FAD phosphohydrolase is directed toward the cytosol where it may be involved in vitamin B_2 (riboflavin) metabolism (FAD is hydrolyzed to FMN and AMP which are further hydrolyzed to adenosine and riboflavin) or it is possibly related to some structural function of the lysosomal membrane.

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References

Alroy, J., De Gasperi, R. and Warren, C. D. (1991) *Carbohydr. Res.* **213**, 229.

Armstrong, J. M. and Newman, J. D. (1985) *Arch. Biochem. Biophys.* **238**, 619.

Bergeron, J. M., Rachubinski, R. A., Sikstrom, R. A., Posner, B. I. and Paiement, J. (1982) *J. Cell Biol.* **92**, 139.

Chang, P. L., Rosa, N. E. and Davidson, R. G. (1981) *Anal. Biochem.* **117**, 382.

Chen, J. W., Murphy, T. L., Willingham, M. G., Pastan, I. and August, J. T. (1985) *J. Cell Biol.* **101**, 85.

Chung, C. H., Elliot, R. L. and Mego, J. L. (1980) *Arch. Biochem. Biophys.* **203**, 251.

Deluca, C. and Kaplan, N. O. (1958) *Biochem. Biophys. Acta.* **30**, 6.

Dickson, R. B., Beguinot, L., Hanover, J. A., Richert, N. D., Willingham, M. C. and Pastan, I. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5335.

Engstrom, L. (1961) *Biochem. Biophys. Acta.* **52**, 36.

Fiske, C. H. and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375.

Gieselmann, V. (1995) *Biochim. Biophys. Acta.* **1270**, 103.

Goldstone, A., Konecny, P. and Koenig, H. (1971) *FEBS Lett.* **13**, 68.

Herzog, V. and Farquhar, M. G. (1977) *Proc. Natl. Acad.*

Sci. USA **74**, 5073.
Klionsky, D. J. and Emr, S. D. (1990) *J. Biol. Chem.* **265**, 5349.
Komberg, A. and Pricer, W. E. (1950) *J. Biol. Chem.* **182**, 763.
Kumar, S. A., Appaji, R. N. and Vaidyanathan, C. S. (1965) *Arch. Biochem. Biophys.* **111**, 646.
Leighton, F., Poole, B., Beaufay, H., Bauhiun, P., Coffey, J. W., Fowler, S. and De Duve, C. (1968) *J. Cell Biol.* **37**, 481.
Lemansky, P., Gieselmann, V., Hasilik, A. and Von Figura, K. (1985) *J. Biol. Chem.* **260**, 9023.
Lewis, V., Green, S. A., Marsh, M., Vihko, P., Helenius, A. and Mellman, I. (1985) *J. Cell Biol.* **100**, 1839.
Lindahl, R. (1979) *Biochem. J.* **183**, 55.
Lippincott-Schwartz, J. and Fambrough, D. M. (1986) *J. Cell. Biol.* **102**, 1593.
Mahuran, D. J. (1995) *Clin. Biochem.* **28**, 101.
Murray, G. J., Jonsson, I. V., Sorrell, S. H., Ginns, E. I. and Tager, J. M. (1985) *Fed. Proc.* **44**, 709.
Newby, A. C. and Chrambach, A. (1979) *Biochem. J.* **177**, 623.
Ragab, M. H., Brightwell, R. and Tappel, A. L. (1968) *Arch. Biochem. Biophys.* **123**, 179.
Reggio, H., Bainton, D., Harms, E., Coudrier, E. and Louvard, D. (1984) *J. Cell Biol.* **99**, 1511.
Smith, P. S., Krolm, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76.
Steinmann, R. M., Mellman, I. S., Muller, W. A. and Cohn, Z. A. (1983) *J. Cell Biol.* **96**, 1.
Trouet, A. (1964) *Arch. Int. Physiol. Biochem.* **72**, 698.
Von Figura, K. and Hasilik, A. (1986) *Ann. Rev. Biochem.* **55**, 167.
Wattiaux, R., Wibbo, M. and Bandhuin, P. (1963) in Lysosomes, Ciba Foundation Symposium (De Reuck, A. V. S. and Cameron, M. P., eds.) pp. 176–[illegible]. Little Brown Co., Boston.
Yamawaki, S. (1956) *J. Biochem.* **43**, 683.