Characterization of a retinylmonophosphatase in the plasma membrane of mouse brain

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Retinylmonophosphatase (RMPase) activity in mouse brain paralleled the subcellular distribution of the plasma-membrane marker $\text{Na}^+ + \text{K}^+$ -dependent ATPase. The enzyme had a pH optimum between 5.5 and 7.0. The enzyme demonstrated linear kinetics with respect to time and both protein and substrate concentrations. RMPase was saturated by low retinyl monophosphate (RMP) concentrations and exhibited an apparent K_m of $4.6 \mu\text{M}$. The enzyme did not require MgCl_2 for activity, and in fact assays were routinely run in the presence of $10 \text{ mM-Na}_2\text{EDTA}$. In general, detergents inhibited the enzyme, with 0.05% Triton X-100 causing a 30% loss of activity. Phosphatidic acid was also inhibitory, but phosphatidylcholine and sphingomyelin stimulated phosphatase activity. RMPase was inhibited 35% by 5 mM concentrations of fluoride, phosphate or pyrophosphate. A series of other phosphorylated compounds, including glucose 6-phosphate, α -glycerophosphate, ATP, AMP, *p*-nitrophenyl phosphate and thiamin pyrophosphate, showed little or no inhibition. RMPase activity differed in several characteristics from that previously reported for dolichylmonophosphatase. It is concluded that RMP could play a distinct role in the plasma membrane.

INTRODUCTION

Retinyl monophosphate (RMP) is a phosphorylated derivative of vitamin A that has been shown to be synthesized by mammalian tissues (Barr & De Luca, 1974; Martin & Thorne, 1974; Frot-Coutaz *et al.*, 1976; Peterson *et al.*, 1976; Masushige *et al.*, 1978; Adamo *et al.*, 1979; Creek *et al.*, 1985a). De Luca and Wolf and their co-workers (e.g. De Luca, 1977; Wolf *et al.*, 1979) have proposed that RMP functions in a manner similar to that of the polyisoprenoid dolichyl monophosphate (DMP) in accepting and transferring hexose units to asparagine groups on glycoproteins during their synthesis. DMP is able to transfer mannose, glucose and glucosamine, whereas RMP is specific for mannose (De Luca, 1977; De Luca & Shapiro, 1981). RMP-mannose has been isolated and its donation of mannose to endogenous receptor proteins has been reported by De Luca and co-workers (De Luca, 1977; Shidoji *et al.*, 1981) and confirmed by Frot-Coutaz *et al.* (1981). From these data it would appear that the basic difference between the actions of DMP and RMP is that DMP donates mannose to dolichol-*P-P*-oligosaccharides (to elongate the oligosaccharide) whereas RMP solely transfers mannose directly to the protein moiety.

Despite the accumulated evidence, however, a distinct role for vitamin A in protein glycosylation remains unsettled. It is possible, for example, that retinol is artifactually competing for enzymes designed for dolichol (Lennarz, 1975; Parodi & Leloir, 1979; De Luca *et al.*, 1982). Stoll *et al.* (1985) have shown that a single enzyme catalyses the formation of the mannosylphosphoryl derivatives of dolichol and retinol in both rat liver and Chinese-hamster ovary cells.

Thus, although its synthesis by mammalian tissues is not in doubt, a physiological role for RMP has yet to be proven. A characterization of RMPase could provide insight as to this possible role. In the present study we chose brain as a source of RMPase activity because its low concentrations of vitamin A would not interfere with the RMPase assay and also because it possesses a high activity of mannosyl-retinylphosphate synthetase (De Luca *et al.*, 1979).

MATERIALS AND METHODS

Chemicals

ATP (A5394), DMP (D5885), fatty acid-free bovine serum albumin (fraction V powder; A6003), phosphatidic acid, phosphatidylcholine, retinol and sphingomyelin were obtained from Sigma Chemical Co. SDS was purchased from Bio-Rad Laboratories. Thin-layer silica-gel chromatographic plates (no. 13179 without fluorescent indicator) were from Eastman Kodak.

RMP synthesis

RMP was chemically synthesized by a modification of the methods of Bhat *et al.* (1980) and Tsukida & Saiki (1982). Bistriethylamine phosphate was obtained as follows. A 6.1 ml portion of 85% (v/v) H_3PO_4 was added to 25.6 ml of acetonitrile. Triethylamine (28.4 ml) was added slowly over a 15 min period with constant stirring at room temperature. The mixture was then placed in an ice bath until initial crystals formed. Complete crystallization was allowed overnight at 4°C . Filtered crystals were washed with 25 ml of cold acetonitrile, dried on filter paper and stored desiccated for several months at 4°C .

Abbreviations used: DMP, dolichyl monophosphate; DMPase, dolichylmonophosphatase; RMP, retinyl monophosphate; RMPase, retinylmonophosphatase; $\text{Na}^+ + \text{K}^+$ -ATPase, $\text{Na}^+ + \text{K}^+$ -dependent ATPase.

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Retinol was then chemically phosphorylated as follows. A 50 mg portion of retinol dissolved in 6.25 ml of acetonitrile was mixed with 0.8 ml of trichloroacetonitrile. Over an 8 min period 100 mg of bistrisethylaminephosphate previously dissolved in 8 ml of acetonitrile was added. The reaction was allowed to proceed for 30 min with constant stirring at 40 °C on a hot-plate. After being cooled to room temperature the solution was neutralized with 2 drops of conc. NH_3 and centrifuged at 23 500 *g* in a Sorvall SS-34 rotor for 10 min. The supernatant was diluted to 200 ml with 99% (v/v) methanol and applied to a 2.5 cm $\times$ 13 cm DEAE-cellulose column. Retinol was eluted with 150 ml of 99% methanol, and oxidized by-products were removed with an additional 50 ml of 1 mM-ammonium acetate in 99% methanol. RMP was eluted with 150 ml of 30 mM-ammonium acetate in 99% methanol. Triethylamine (0.75 ml) was added, and the solution was concentrated to 50 ml in a 37 °C water bath under a constant stream of N_2 . This stable methanolic solution was then stored at -20 °C. Identification of RMP was made by applying a 5 μ l portion of the methanolic solution to a thin-layer silica-gel chromatographic plate. The plates were developed in methanol/ethyl acetate/water/triethylamine (100:90:10:1, by vol.), in which RMP has R_F 0.32 as observed under u.v. light. For calculating RMP concentration a molar absorption coefficient of 52480 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 325 nm was used (Morris & Redfearn, 1969). This value was determined for retinol dissolved in ethanol and may be slightly different for RMP in hexane.

Cell fractionation

All sucrose solutions were buffered with 5 mM-Hepes, pH 7.5. Brain tissues from mature Swiss Webster mice were homogenized in 10 vol. of 0.25 M-sucrose with five passes of a Potter-Elvehjem homogenizer. The homogenate was fractionated into crude nuclear (Sorvall SS-34 rotor; 600 *g* for 10 min), crude mitochondrial (Sorvall SS-34 rotor; 17 300 *g* for 20 min), microsomes (Sorvall T-865.1 rotor; 102 000 *g* for 60 min) and cytosol (microsomal supernatant) as previously described (O'Fallon & Chew, 1984). Nuclei were further purified by suspending the crude nuclear fraction in 0.8 M-sucrose containing 1 mM- MgCl_2 and centrifuging in a Sorvall T-865.1 rotor at 67 000 *g* for 30 min.

The crude mitochondrial fraction (7 ml) was layered over a gradient containing 3 ml of 0.5 M-sucrose and 6.5 ml each of 0.6 M-, 0.8 M-, 1.0 M- and 1.2 M-sucrose and further fractionated by another centrifugation (Sorvall AH-627 rotor; 64 400 *g* for 120 min) into cytoplasm, synaptic vesicles, myelin, membranes, synaptosomes and mitochondria as previously described (Harding & O'Fallon, 1979; O'Fallon & Chew, 1984). All subcellular fractions were frozen at -20 °C.

Assays

All assays were conducted under conditions where enzyme activity was linear with respect to both time and protein concentration.

Citrate synthetase (EC 4.1.3.7). This mitochondrial-matrix marker was assayed by the method of Srere (1969). The 1.5 ml reaction mixture incubated at 30 °C contained Tris/HCl buffer, pH 8.1 (100 mM), 5,5'-dithiobis-(2-nitrobenzoate) (0.1 mM), acetyl-CoA (1 mM) and enzyme preparation. The absorbance at 412 nm was monitored

for 3 min to measure possible acetyl-CoA deacylase activity. The citrate synthetase reaction was then started by the addition of oxaloacetate (1 mM).

DNA. DNA was used as a nuclear marker. Its concentration in subcellular fractions was determined by the method of Giles & Myers (1965). To a sample of subcellular fraction in 2 ml of 10% (v/v) HClO_4 was added 2 ml of 4% (v/v) diphenylamine in acetic acid followed by 0.1 ml of 0.2% (v/v) acetaldehyde in water. After incubation at 30 °C overnight the absorbance difference at 595 nm and 700 nm was read against the 595 nm - 700 nm blank.

Lactate dehydrogenase (EC 1.1.1.27). This cytosolic enzyme was assayed as described by Possmayer *et al.* (1973). The incubation mixture contained $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 7.5 (100 mM), NADH (0.25 mM), 0.02 ml of a saturated ethanolic solution of rotenone, Triton X-100 (0.01%) and a suitable sample of enzyme preparation in a fixed volume of 3 ml. The decrease in absorbance at 340 nm produced by the addition of sodium pyruvate (0.2 mM) at room temperature was recorded continuously, and the initial linear portion of the slope was used to calculate the reaction rate.

$\text{Na}^+ + \text{K}^+$ -ATPase (EC 3.6.1.3). This plasma-membrane enzyme was assayed by the method of Forbush (1983). To 100 μ l of solution containing SDS (0.65 mg/ml), bovine serum albumin (1%) and imidazole/HCl buffer, pH 7.0 (25 mM), was added 50 μ g of subcellular fraction protein. The mixture was incubated at room temperature for 10 min and then diluted with 0.6 ml of bovine serum albumin (0.3%) in imidazole/HCl buffer, pH 7.0 (25 mM). This pretreated preparation (100 μ l) was transferred to assay tubes containing 0.5 ml of NaCl (144 mM) KCl (30 mM), MgCl_2 (4.8 mM), Na_2EDTA (1.2 mM) and Tris/HCl buffer, pH 7.5 (72 mM), with or without ouabain (1.2 mM). The reaction was started with 60 μ l of Na_2ATP (40 mM) and incubated for 20 min at 37 °C. The reaction was stopped with 1 ml of solution II [containing ascorbic acid (3%, w/v), HCl (0.5 M) and SDS (3%, w/v)] and then placed on ice for 10 min. After the addition of 1.5 ml of solution III [containing sodium arsenite (2%, w/v), disodium citrate (2%, w/v) and acetic acid (2%, v/v)] the tubes were rewarmed to 37 °C. After 10 min absorbance of the phosphomolybdate complex at 700 nm was measured.

Protein. The protein content of each fraction was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

RMPase. The standard 0.5 ml RMPase assay mixture contained chemically synthesized RMP (30 μ M), Na_2EDTA (10 mM), fatty acid-free bovine serum albumin (10 mg/ml), Mes/NaOH buffer, pH 6.0 (100 mM), and plasma-membrane protein (50 μ g/ml). The reaction mixture was incubated at 37 °C for 30 min and then stopped by vortex-mixing with 1 ml of 95% (v/v) ethanol. The mixture was neutralized with 0.5 ml of Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid]/NaOH buffer, pH 9.0 (100 mM), and vortex-mixed again. After standing in ice for 20 min the 2 ml mixtures were transferred to 16 mm $\times$ 125 mm screw-cap extraction

tubes. Hexane (5 ml) was then added to each and the tubes were vortex-mixed for 5 min on a multi-tube vortex-mixer. The tubes were then centrifuged at 400 *g* for 5 min. The hexane layer, now containing the retinol produced by RMPase activity, was transferred to a 12 mm × 75 mm culture tube and evaporated to dryness. For identification of retinol the contents of the tubes were redissolved in 20 μ l of hexane and 5 μ l portions were applied to a thin-layer silica-gel chromatographic plate. The plates were developed in cyclohexane/toluene/ethyl acetate (5:3:2, by vol.) (Groendijk *et al.*, 1980) and spots were observed under u.v. light. For quantification of retinol production, 1.2 ml of hexane was added to the dried tubes and the absorbance of retinol was measured at 325 nm. A molar absorption coefficient of 52480 M⁻¹·cm⁻¹ was used (Morris & Redfearn, 1969). This value was determined in ethanol and may be slightly different from that in hexane.

Thiamin pyrophosphatase (EC 2.5.1.3). This microsomal Golgi-apparatus enzyme was assayed by the method of Reith *et al.* (1972). The assay mixture at 37 °C was 0.4 ml in volume and contained Tris/maleate buffer, pH 7.0

(55 μ M), MgCl₂ (8 mM), thiamin pyrophosphate (3.6 mM) and enzyme preparation. The liberated P_i was measured by the method of Baginski *et al.* (1967). The enzymic reaction was quenched by addition of 2.0 ml of 2% (w/v) ascorbic acid/10% (w/v) trichloroacetic acid. After centrifugation, 0.5 ml of ammonium molybdate (1.0%, w/v) was added to 1.0 ml of supernatant followed by 1.0 ml of 2% (w/v) sodium arsenite/2% (w/v) disodium citrate. After 20 min absorbance of the phosphomolybdate complex at 700 nm was measured.

RESULTS

Distribution of RMPase activity

Mouse brain homogenates were separated into subcellular fractions and the amounts or activities of certain markers were measured in each fraction (Fig. 1). The markers used to monitor the composition of the fractions were the following: DNA for nuclei; Na⁺ + K⁺-ATPase for plasma membrane; citrate synthetase for mitochondria; thiamin pyrophosphatase for microsomal fraction; lactate dehydrogenase for cytosol. The activity

Fig. 1. Characterization of various subcellular fractions from mouse brain by using specific markers

Enzymes were assayed and DNA was determined as indicated in the Materials and methods section. The ordinate shows the relative specific activity of fractions (percentage of total recovered activity/percentage of total recovered protein), and the abscissa shows the relative protein content of the subcellular fractions. Subcellular fractions assayed were nuclei (N), plasma membrane (PM), mitochondria (M), microsomal fraction (Mc) and cytosol (C).

of RMPase paralleled that of the plasma-membrane marker $\text{Na}^+ + \text{K}^+$ -ATPase, which was distinctly different from the other subcellular fractions. Quite noticeable was the absence of RMPase from the cytoplasm.

Properties of RMPase

The plasma-membrane subcellular fraction was used for all subsequent studies on the characterization of RMPase.

pH optimum

The standard assay mixture with 100 μg of plasma-membrane protein/ml used but the buffer was varied. Assays were done in the presence of 0.1 M buffer, sodium acetate (pH 4–6), Mes/NaOH (pH 5.5–6.5), Hepes/NaOH (pH 7–8) or Ches/NaOH (pH 9–10). RMPase had maximum activity at pH 6.0 but exhibited similar activities at pH values from 5.5 to 7.0 (Fig. 2). The enzymic activity decreased rapidly at pH values below 5.5 and above 7.0.

Linearity of kinetics

The effect of plasma-membrane-protein concentration on RMPase activity was determined. Hydrolysis of RMP was directly proportional to protein concentrations from zero to 150 $\mu\text{g}/\text{ml}$ when the RMP concentration was 30 μM . Additionally, at a plasma-membrane-protein concentration of 50 $\mu\text{g}/\text{ml}$, retinol production from 30 μM -RMP was linear for 60 min. By using the standard assay conditions but various RMP concentrations a substrate concentration curve was obtained (Fig. 3). RMPase rapidly became saturated by low RMP concentrations, indicating a high affinity for its substrate. A Lineweaver-Burk plot revealed an apparent K_m of 4.6 μM (Fig. 3 inset).

Effect of detergents

The effect of various detergents on RMPase activity was determined (Table 1). None of the six detergents tested stimulated RMPase activity. To the contrary, SDS, Triton X-100, Nonidet P-40 and Lubrol PX significantly inhibited RMPase activity when used at concentrations between 0.05 and 0.20%. The detergents Tween 80 and

Fig. 2. Effect of pH on RMPase activity

The standard assay with 100 μg of plasma-membrane protein/ml was used but the buffer was varied. Buffers used were sodium acetate (○), Mes/NaOH (●), Hepes/NaOH (△) and Ches/NaOH (▲).

Fig. 3. Effect of substrate concentration on RMPase activity

Assays were incubated for 30 min at 50 μg of plasma-membrane protein/ml and 0–30 μM -RMP.

Table 1. Effect of detergents on RMPase activity

Plasma membrane was assayed for RMPase activity under standard conditions but in the presence of various detergents. The activities are percentages of control assays incubated without detergents.

Detergent added	Activity (% of control)	
	Concn.	
	0.05%	0.20%
SDS	75	36
Triton X-100	72	59
Tween 80	100	104
Nonidet P-40	67	45
Lubrol PX	85	69
CHAPS	90	85

CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonic acid} exerted little or no effect on RMPase activity.

Specificity for RMP

As indicated in Table 2, 5 mM concentrations (167-fold excess over RMP) of ATP, AMP, cyclic AMP, glucose 6-phosphate, α -glycerophosphate, fructose 1,6-bisphosphate, thiamin pyrophosphate, NADP⁺ and *p*-nitrophenyl phosphate did not significantly decrease the amount of RMP hydrolysed to retinol. However, both P_i and PP_i decreased RMPase activity by 35%.

In Expt. I of Table 3 0.05% Triton X-100 was added to the RMPase assay mixture in order to solubilize the phospholipids used in the study. Although this Triton X-100 inhibited RMPase by 30% (Table 1), DMP does not serve as a substrate for DMPase unless it is present (Rip *et al.*, 1981). The phospholipids sphingomyelin and phosphatidylcholine (both 6.7-fold excess over RMP) stimulated RMPase activity, whereas phosphatidic acid inhibited it. In this experiment DMP at a 6.7-fold excess over RMP had no effect on the amount of RMP hydrolysed to retinol. In Expt. II of Table 3 the ratio of DMP to RMP was increased to 10:1 but again there was no competition from DMP, indicating a much higher affinity of the enzyme for RMP.

Table 2. Effect of various phosphorus-containing compounds on mouse brain plasma-membrane RMPase activity

The control assay mixture contained 30 μ M-RMP, 0.1 mM-Mes/NaOH buffer, pH 6.0, 10 mg of fatty acid-free bovine serum albumin/ml, 10 mM- Na_2EDTA and 100 μ g of plasma-membrane protein/ml. Competing substrates were added at 5 mM concentrations. Duplicate assays were incubated at 37 °C for 30 min.

Metabolite added	Activity	
	(nmol of retinol formed/assay)	(% of control)
None (control)	1.98	100
P_i	1.31	66
PP_i	1.33	67
<i>p</i> -Nitrophenyl phosphate	1.84	98
Fructose 1,6-bisphosphate	1.74	88
NADP ⁺	1.94	98
ATP	1.78	90
AMP	2.04	103
Cyclic AMP	2.06	104
α -Glycerophosphate	1.90	96
Thiamin pyrophosphate	1.78	90
Glucose 6-phosphate	1.76	89

Table 3. Effect of various phospholipids on plasma-membrane RMPase activity

In Expt. I the control assay mixture contained 0.05% Triton X-100 in addition to the standard constituents (see Table 2). In Expt. II the control assay mixture contained the standard constituents plus 0.05% Triton X-100 but only 10 mM-RMP.

Metabolite added	Concn. (mM)	Activity	
		(nmol of retinol formed/ assay)	(% of control)
Expt. I			
None (control, 30 μ M-RMP)	—	1.43	100
Sphingomyelin	0.2	2.97	208
Phosphatidylcholine	0.2	1.84	129
Phosphatidic acid	0.2	0.67	47
DMP	0.2	1.52	106
Expt. II			
None (control, 10 μ M-RMP)	—	1.17	100
DMP	0.1	1.28	109

Effect of phosphate-free compounds

Some phosphate-free compounds were tested on RMPase activity (Table 4). RMPase did not require metal ions for activity as represented by MgCl_2 , since equal activity was obtained with 5 mM- MgCl_2 or 10 mM- Na_2EDTA . RMPase was sensitive to NaF, where the inhibition was 36% and 85% at fluoride concentrations of 5 mM and 25 mM respectively. The fluoride inhibition was specific for fluoride compared with chloride, as 25 mM-NaCl had no effect on RMPase activity.

Table 4. Effect of phosphate-free compounds on RMPase activity

The standard control assay mixture was used (Table 2) except that Na_2EDTA was absent when MgCl_2 was tested. Compounds were added at the indicated concentrations. Duplicate assays were incubated at 37 °C for 30 min.

Compound added	Concn. (mM)	Activity	
		(nmol of retinol formed/assay)	(% of control)
None (control)	—	1.98	100
MgCl_2	5	2.00	101
NaF	5	1.27	64
NaF	25	0.30	15
NaCl	25	1.96	99

DISCUSSION

RMPase shares certain characteristics with those reported for DMPase. In particular, DMPase is also associated with the plasma membrane, exhibits a similar pH-activity curve, is metal-ion-independent, and is inhibited by fluoride and P_i (Idoyaga-Vargas *et al.*, 1980; Wedgwood & Strominger, 1980; Burton *et al.*, 1981; Rip *et al.*, 1981; Belocopitow & Boscoboinik, 1982). However, there are also differences between RMPase and DMPase. DMPase possesses a cytoplasmic component (Rip *et al.*, 1981) that we do not find for RMPase. DMPase is drastically inhibited by sphingomyelin, phosphatidylcholine and phosphatidic acid (Rip *et al.*, 1981; Belocopitow & Boscoboinik, 1982). This is a striking contrast with RMPase, which is stimulated by sphingomyelin and phosphatidylcholine. DMPase has a requirement for Triton X-100, without which there is no enzymic activity (Rip *et al.*, 1981). RMPase, on the other hand, is inhibited 30% by 0.05% Triton X-100. Perhaps the strongest evidence that the two enzymes are different is that DMP does not inhibit RMPase activity.

RMPase also shows great specificity in relation to other phosphate compounds. It is not inhibited by any we have tested other than P_i , PP_i and phosphatidic acid. These data and the fact that RMPase is metal-ion-independent eliminates the possibility that it is a general phosphatase. Since RMPase is not inhibited by *p*-nitrophenyl phosphate, it cannot be acid phosphatase (EC 3.1.3.2). Whereas RMPase is inhibited by phosphatidic acid, phosphatidate phosphatase is not inhibited by P_i (Burton *et al.*, 1981). Thus RMPase exhibits an enzymic activity that appears to be specific for RMP. Furthermore, this is the first enzyme that has been demonstrated to be unique to RMP metabolism, in that formation of the mannosylphosphoryl derivatives of dolichol and retinol has been found to be catalysed by a single enzyme (Stoll *et al.*, 1985) whereas retinol kinase has not yet been characterized.

Poznyakov & Solov'eva (1979) have previously reported on an RMPase activity in the subcellular fractions of rat liver. They also found RMPase activity to be associated with the plasma membrane. However, as their assay mixture contained MgCl_2 , part of the enzymic

activity was probably due to non-specific phosphatase activity. This would explain why they observed RMPase activity in the cytoplasm. Unfortunately, these authors did not report on any specificity studies.

It has been found (Creek *et al.*, 1985b) that the endogenous mannose acceptor in microsomal preparations, previously thought to be RMP (e.g. De Luca *et al.*, 1982, 1984), was actually phosphatidic acid. This evidence, coupled with the fact that RMPase is a plasma-membrane enzyme, suggests that RMP may play a role in the plasma membrane rather than in the microsomal fraction.

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