PURIFICATION AND PROPERTIES OF NUCLEOTIDE PYROPHOSPHATASE FROM RAT LIVER PLASMA MEMBRANES

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1. Introduction

Various nucleotides suffer a rapid cleavage of their pyrophosphate bonds when added to the isolated hemoglobin-free perfused rat liver [1, 2]. This capacity was attributed to a nucleotide pyrophosphatase activity residing in the plasma membranes of the liver. Similar properties as well as a phosphodiesterase-type activity and a pyrophosphate liberation from ATP in isolated rat liver plasma membranes were described [3–9]. These different activities were supposed to belong to a single enzyme [8, 9]; a pure enzyme, however, was necessary to prove this point.

This report deals with the solubilization and purification to apparent homogeneity of a protein from rat liver plasma membranes which catalyzes the cleavage of a variety of nucleotides with the liberation of the respective nucleoside-5′-monophosphates. It is essentially free of 5′-nucleotidase and phosphatase activities. The substrate and inhibitor specificity of the enzyme show it to be both a nucleotide pyrophosphatase and a phosphodiesterase.

2. Experimental procedures

Plasma membranes from livers of Wistar rats were isolated according to Ray [10]. A discontinuous gradient (total vol 1780 ml) in a Beckman zonal rotor Type Ti 15 was applied consisting of 600 ml 37% (w/w), 600 ml 41% (w/w) and 400 ml 45% (w/w) sucrose. 180 ml of the crude membrane suspension isolated from 80 g liver wet weight were placed in the outer edge of the rotor. After 4 hr at 127,000 *g* the membranes banded at the interface of 37% and 41% sucrose; they were collected, washed free of sucrose, and suspended in 20 mM Tris-HCl buffer pH 8.8 containing 0.5 mM $CaCl_2$ (standard buffer).

The purity of the preparation was monitored by assays of cytochrome oxidase [11], glucose-6-phosphatase [12], acid phosphatase [12], lactate dehydrogenase [13], 5′-nucleotidase [14], and phosphodiesterase [15].

Solubilization and purification of the enzyme was attempted by several methods. Best results were obtained when 160 mg of plasma membrane protein were suspended in 52 ml 50 mM Tris-HCl buffer pH 8.8 containing 10 mM $CaCl_2$, 0.2% Triton TX-100, and 16 mg of a crude pancreatic lipase (steapsin) [16], and kept 4 hr at 37° with occasional shaking. Unsoluble protein was sedimented at 100,000 *g* (1 hr) and the clear supernatant was dialyzed against standard buffer for 12 hr at 5°. The dialysate was applied to a DEAE cellulose column (2.5 × 20 cm) equilibrated with 20 mM Tris-HCl buffer pH 8.6 containing 0.5 mM $CaCl_2$. The enzyme was eluted by a linear gradient (0–0.5 M NaCl) in the same buffer. The fractions containing nucleotide pyrophosphatase activity were concentrated by pressure dialysis to a vol of 2 ml. Further purification was obtained by preparative polyacrylamide gel electrophoresis at pH 9

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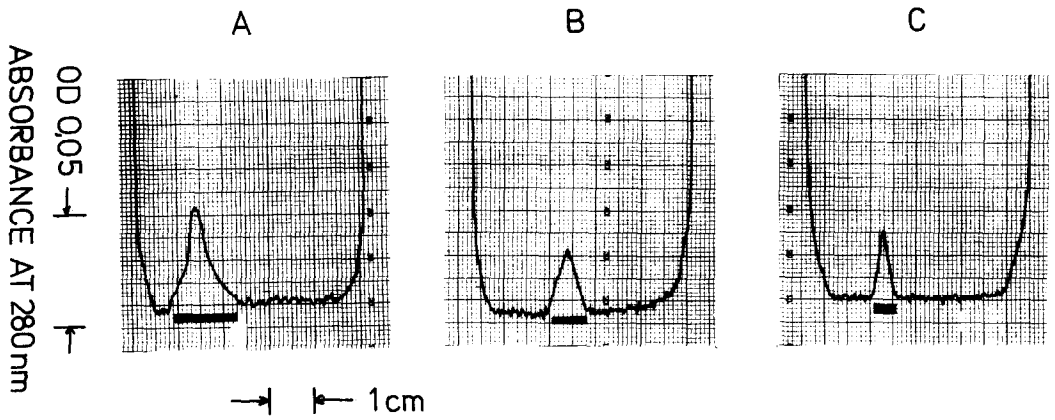

Fig. 1. Polyacrylamide gel electrophoreses of the purified enzyme at pH 7.5, 9.2 and 10.3. Absorption at 280 nm was scanned with a Gilford spectrophotometer 2400 equipped with a linear transport. The enzymic activity (indicated by bars) was recorded in the same gels after spraying with standard buffer and substrate by scanning at 405 nm.

Table 1
Purification of the nucleotide pyrophosphatase from rat liver plasma membranes.

Purification step	Total protein (mg)	Total units (μmole/min)	Specific activity (μmole/min/mg)	Overall purification	Yield (%)
Liver homogenate	42,000	3950	0.09	–	100
Plasma membranes	160	415	2.6	28	10.5
Solubilization	48	230	4.8	51	5.8
Gradient elution from DEAE cellulose	11.6	139	12	128	3.5
Preparative gel electrophoresis, pH 9	1.6	115	72	770	2.9
Gel chromatography on Sephadex G-150	0.96	93	97	1030	2.4
Gel electrophoresis, pH 7	0.49	74	151	1580	1.9

[17], omitting a spacer gel. The fractions containing the activity were concentrated and lyophilized. The protein was dissolved in 0.2 ml of standard buffer and applied to a G-150 column (1 × 100 cm). Elution was performed with standard buffer and fractions of 2 ml were collected.

After concentration of the active protein polyacrylamide gel electrophoresis at pH 7.5 was performed according to Maurer [18]. The gel was scanned at 280 nm. The enzyme was recovered by electrophoretic elution [19].

Enzyme activity was measured according to Razzell [15]. Protein was determined by Lowry's method [20] or, in the later stages of purification, by UV-spectroscopy [21].

3. Results and discussion

Purified plasma membranes were obtained in a yield of 0.9 mg membrane protein/g liver wet weight. They were almost free of enzymes of other cell compartments as judged by the relative specific activities (ratio of the specific activity of isolated membranes to that of total liver homogenate) of the following enzymes: glucose-6-phosphatase, 0.5; acid phosphatase,

Table 2
Substrate specificity of the nucleotide pyrophosphatase.

Substrate	$K_m \times 10^4$	V_{max} (μmole/min/mg)	V_{max} relative to p-nitrophenyl-thymidine 5′-phosphate
p-Nitrophenyl thymidine 5′-phosphate	2.0	151	100
UDPG	5.0	120	80
UTP	3.3	22	14.6
ATP	7.8	17.5	11.7
UDP	–	1	0.6
ADP	–	0.8	0.5

Assays were run at 25° in the presence of 100 mM Tris-HCl buffer pH 8.9, 10 mM $MgCl_2$ and varying amounts of substrate. p-Nitrophenol formation was recorded at 405 nm [15] using 0.02 μg of purified enzyme and 0.1 μg membrane protein, respectively, in a total vol of 0.5 ml. For UDPG cleavage 0.015 μg of enzyme and 2 μg of membrane protein, respectively, in a total vol of 0.25 ml were used. UMP [23] and G-1-P [24] were assayed enzymatically after heat inactivation. The same procedure, but with 0.04 μg of purified enzyme was applied with ATP and UTP, and 0.4 μg with ADP and UDP (10 mM each). AMP [25] and PP_i [26] were assayed enzymatically. P_i was estimated according to Lowry and Lopez [27].

Table 3
Inhibitors of the nucleotide pyrophosphatase.

Inhititor	$K_i \times 10^4$	Type of inhibition
UMP	2.6	competitive
UDPG	1.8	competitive
α,β-Methylene ATP	2.5	competitive
AMP-amidat	3.0	competitive

p-Nitrophenyl thymidine 5′-phosphate was used as a substrate throughout. K_i values were calculated by the method of Dixon [28].

0.2; cytochrome oxidase, 0.07; lactate dehydrogenase, 0.2; 5′-nucleotidase, 24; phosphodiesterase, 26. These data agree with previously reported values [9, 10].

The purification procedure of the enzyme is summarized in table 1. The resulting material migrated in polyacrylamide gel electrophoreses at 3 pH values (7.5, 9.2 and 10.3) as a single symmetrical band (fig. 1). Based on the initial liver homogenate a 1580-fold purification was achieved which exceeds considerably earlier attempts [15, 22].

Solubilization and purification did not seem to affect the catalytic properties of the enzyme. The K_m values for UDPG and for p-nitrophenyl-thymidine 5′-phosphate were about the same with plasma membranes and with the pure enzyme: 4.5×10^{-4} and 5×10^{-4} M, respectively, for the former and 2.5×10^{-4} and 2×10^{-4} M, respectively, for the latter compound. Furthermore, the pH optima (9.0), the instability below pH 6, and the requirement for Mg^{2+} or Ca^{2+} were the same at all purification stages. EDTA inactivated the enzyme; a partial reactivation only was obtained by addition of Mg^{2+} or Ca^{2+}.

The enzyme acts both on pyrophosphate and phosphodiester bonds of nucleotides (table 2). The affinities of the substrates tested were similar but V_{max} values differed widely. All these compounds were hydrolyzed in a manner which yields a nucleoside 5′-monophosphate; the second cleavage product was glucose-1-phosphate (from UDPG) and PP_i (from ATP, UTP). A 5′-MP liberation from RNA (yeast), DNA (*E. coli*), and polyadenylate proceeded with less than 1% of the rate of p-nitrophenyl-thymidine 5′-phosphate cleavage. p-Nitrophenyl-deoxy-thymidine-3′-phosphate was not split, nor were 5′-nucleotide monophosphates and phosphate esters such as cyclic AMP and glycerol phosphate.

Compounds containing a 5′-nucleotide residue are competitive inhibitors (table 3) α,β-methylene-ATP being of special interest, as it is not hydrolyzed by both nucleotide pyrophosphatase and 5′-nucleotidase.

The enzyme is similar to the phosphodiesterases of various tissues described by Razzell [29] and others [22, 30–33]. It combines the properties of the phosphodiesterase and nucleotide pyrophosphatase activities described in plasma membranes [3–9]. Considering its action on physiological substrates (table 2) the term nucleotide pyrophosphatase seems the more adequate.

The biological function of this enzyme is difficult to assess at present. It was considered to participate in the intracellular degradation of nucleotides [5]. Its action on extracellular compounds in combination with 5′-nucleotidase and phosphatase [1, 2] envisages a role of nucleotide pyrophosphatase in the removal of nucleotides leaked from damaged cells into the extracellular fluid.

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References

[1] E. Bischoff, M. Liersch, D. Keppler and K. Decker, Hoppe-Seyler's Z. Physiol. Chem. 351 (1970) 729.
[2] M. Liersch, H. Grotelüschen and K. Decker, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 267.
[3] P. Emmelot, C.J. Bos, L. Benedetti and P.H. Rümke, Biochim. Biophys. Acta 90 (1964) 126.
[4] I. Liebermann, A.I. Lansing and W.E. Lynch, J. Biol. Chem. 242 (1967) 736.
[5] J.E. Franklin and E.G. Trams, Biochim. Biophys. Acta 230 (1971) 105.
[6] M. Erecinska, H. Sierakowska and D. Shugar, European J. Biochem. 11 (1969) 465.
[7] A.I. Lansing, M.L. Belkohde, W.E. Lynch and J. Liebermann, J. Biol. Chem. 242 (1967) 1772.
[8] J.R. Skidmore and E.G. Trams, Biochim. Biophys. Acta 219 (1970) 93.
[9] O. Touster, N.N. Aronson, Jr., J.T. Dulaney and H. Hendrickson, J. Cell. Biol. 47 (1970) 604.
[10] T.K. Ray, Biochem. Biophys. Acta 196 (1970) 1.
[11] D.C. Wharton and A. Tzagoloff, in: Methods in Enzymology, Vol. 10, eds. R.W. Estabrook and M.E. Pullmann (Academic Press, New York and London, 1967) p. 245.
[12] F. Appelmans, R. Wattiaux and C. De Duve, Biochem. J. 59 (1955) 438.
[13] H.U. Bergmeyer and E. Bernt, in: Methoden der enzymatischen Analyse, ed. H.U. Bergmeyer (Verlag Chemie Weinheim, 1970) p. 533.
[14] R.H. Michell and J.N. Hawthorne, Biochem. Biophys. Res. Commun. 21 (1965) 333.
[15] W.E. Razzell, in: Methods in Enzymology, Vol. VI, eds. S. Colowick and N.O. Kaplan (Academic Press, New York and London, 1963) p. 236.
[16] K.W. Bock, P. Siekevitz and G.E. Palade, J. Biol. Chem. 246 (1971) 188.
[17] Buchler, Instruction Manual, 1971, p. 3.
[18] H.R. Maurer, Discelectrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis (Walter de Gruyter, Berlin and New York, 1971) p. 44.
[19] H.R. Maurer, Discelectrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis (Walter de Gruyter, Berlin and New York, 1971) p. 119.
[20] O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[21] O. Warburg and W. Christian, Biochem. Z. 310 (1941) 384.
[22] M. Futai and D. Mizuno, J. Biol. Chem. 242 (1967) 5301.
[23] D. Keppler, J. Rudigier and K. Decker, Anal. Biochem. 38 (1970) 105.
[24] H.U. Bergmeyer and G. Michal, in: Methoden der enzymatischen Analyse, ed. H.U. Bergmeyer (Verlag Chemie, Weinheim, 1970) p. 1196.
[25] D. Jaworek, W. Gruber and H.U. Bergmeyer, in: Methoden der enzymatischen Analyse, ed. H.U. Bergmeyer (Verlag Chemie, Weinheim, 1970) p. 2051.
[26] J.C. Johnson, M. Shanoff, S.T. Bass, J.A. Boezi and R.G. Hansen, Anal. Biochem. 26 (1968) 137.
[27] O.H. Lowry and J.A. Lopez, J. Biol. Chem. 162 (1946) 421.
[28] M. Dixon, Biochem. J. 55 (1953) 170.
[29] W.E. Razzell and H.G. Khorana, J. Biol. Chem. 234 (1959) 2105.
[30] K. Kesselring and G. Siebert, Z. Physiol. Chem. 337 (1964) 79.
[31] L.H. Schliselfeld, J. van Eys and O. Touster, J. Biol. Chem. 240 (1965) 811.
[32] H. Ogawa, M. Sawada and M. Kawada, J. Biochem. Tokyo 59 (1966) 126.
[33] C.L. Harvey, K.C. Olson and R. Wright, Biochemistry 9 (1970) 921.