Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 89-95

# pH-DEPENDENT CONVERSION OF LIVER-MEMBRANOUS ALKALINE PHOSPHATASE TO A SERUM-SOLUBLE FORM BY n-BUTANOL EXTRACTION

Akira Miki, Tatsuya Kominami and Yukio Ikehara*

Department of Biochemistry, Fukuoka University School of Medicine
Nanakuma, Jonan-ku, Fukuoka 814-01, Japan

Received October 31, 1984

SUMMARY : Alkaline phosphatase released from rat liver plasma membrane under usual conditions was electrophoretically not identical with a soluble form in serum which was derived from the liver. The liver-membranous alkaline phosphatase, however, was converted to the serum-soluble form when the liver plasma membrane was treated with n-butanol under the acidic conditions lower than pH 6.5. Such pH-dependent conversion of the enzyme was not observed in plasma membrane of rat ascites heaptoma AH-130 cells. The converting activity for alkaline phosphatase was detected not only in plasma membrane but also in lysosomal membrane of rat liver. 

Alkaline phosphatase is a membrane-bound glycoprotein and one of markers for plasma membrane, and easily inducible in response to various stimuli. Its induction in liver leads to a marked elevation of the enzyme activity in serum (1-3), indicating that the enzyme is released from membranes and appears as a soluble form in serum. In vitro experiments have shown that alkaline phosphatase is released from membranes by treatment with n-butanol (4), bile acids (5,6) and phosphatidylinositol-specific phospholipase C (PI-PLase C) isolated from bacteria (7,8). Recently, we have characterized the hepatic alkaline phosphatase released by these treatments in comparison with the serum-soluble form derived from the liver, demonstrating that the electrophoretically identical form with serum-soluble one is obtained by treatment with PI-PLase C but not with others (9,10). The results suggest the involvement of phosphatidylinositol in attachment of alkaline phosphatase

* To whom all correspondence should be addressed.

Abbreviations used: Hepes, N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; PI-PLase C, phosphatidylinositol-specific phospholipase C.

0006-291X/85 $1.50

to membranes and a possible role of PI-PLase in the mechanism by which the hepatic enzyme appears as a soluble form in serum. However, all the preparations of PI-PLase C so far tested for release of alkaline phosphatase are those isolated from bacteria (7,8,10,11). If PI-PLase C is involved in release of the membrane-bound enzyme, an endogenous activity for the conversion of the membranous form to the serum-soluble one must be identified in tissues. It has been reported that there exist in mammalian tissues several types of PI-PLase including lysosomal, cytosomalic and membrane-bound ones (12). None of them, however, has been tested as a candidate responsible for the releasing mechanism of alkaline phosphatase.

In the present paper we report that the conversion of the liver-membranous alkaline phosphatase to the serum-soluble form occurs in the plasma membranes which are treated with n-butanol at acidic pH lower than pH 6.5. Such converting acitivity is also detected in the lysosomal membranes.

## MATERIALS AND METHODS

Preparation of Plasma Membranes and Lysosomes. Male Wistar rats, weighing 350-450 g, were used. Rats were pretreated for induction of liver alkaline phosphatase by bile duct ligation and colchicine injection (1 mg/kg body weight) (3,13) and starved for 20-24 h before they were killed. Liver plasma membrane was prepared according to the method of Ray (14). Liver lysosomes were prepared as described previously (15) and separated into membranes and contents by centrifugation after sonication in a hypotonic solution. Highly purified lysosomal membranes were also obtained from the liver according to the method of Ohsumi et al.(16). Hepatoma plasma membrane was isolated from ascites hepatoma AH-130 cells which had been transplanted in and harvested from male Donryu rats (17,18).

Butanol Extraction of Alkaline Phosphatase from Plasma Membranes. Plasma membrane suspensions (3-6 mg/ml) were adjusted to the indicated pH with appropriate buffers (final concentration, 50 mM) as shown in the figure legends, mixed with n-butanol (finally 25%) and stirred at 25°C for 60 min. The mixture was then centrifuged at 32,000 x g for 30 min, and the resulting aqueous phase was taken and used for the enzyme assay and electrophoretic analysis.

Release of Alkaline Phosphatase from Plasma Membranes by Phospholipase C. Alkaline phosphatase was released from plasma membranes of rat liver and ascites hepatoma AH-130 cells by treatment with PI-PLase C purified from Bacillus cereus, as described previously (10).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 8.6 according to Davis (19) with some modifications (17). Samples containing 0.5% Triton X-100 were subjected to electrophoresis in 7.5% polyacrylamide gels containing 0.1% Triton X-100. Gels were stained for alkaline phosphatase activity as described previously (17).

Enzyme Assay and Protein Determination. Alkaline phosphatase activity was determined as described previously (17) and protein was determined by the method of Lowry et al.(20) with bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

We previously reported that simultaneous bile duct ligation and colchicine injection in rats caused a marked induction of alkaline phosphatase in liver (3,13), resulting in a rapid rise of the enzyme activity in serum (9). A role of bile acids has been strongly suggested for the mechanism by which the membrane-bound enzyme is released into bile, followed by its regurgitation into blood stream (5,21). On the other hand, extraction of membrane lipids with n-butanol has been widely used as a method for solubilizing, subsequently purifying, membrane-bound alkaline phosphatase from various tissues (4). However, all the preparations of alkaline phosphatase released from the liver-plasma membranes by treatment with bile acids or n-butanol at pH 7.5 were electrophoretically not identical with the serum-soluble form derived from the liver (9,10). In contrast, the hepatic alkaline phosphatase released by the bacterial PI-PLase C was found to be identical with serum-soluble form (10).

In attempts to identify an endogenous converting activity in tissues corresponding to the bacterial PI-PLase C action, we have found that the conversion of the liver-membranous alkaline phosphatase to the serum-soluble form occurs depending upon pH used during n-butanol extraction of the plasma membrane. As shown in Fig. 1, treatment of the plasma membrane with n-butanol under the acidic conditions lower than pH 6.5 (gels 1-5) released the liver alkaline phosphatase having the same mobility in electrophoresis as the serum-soluble form (designated as S), while the enzyme extracted under the conditions higher than pH 7.5 (gels 7-9, M) migrated more slowly than the S form. Electrophoresis with no addition of Triton X-100 in samples and gels confirmed the difference between the two forms, M and S; the M form did not migrate into gels at all, while the S forms from both the liver and serum migrated to the same position (data not shown). The results indicate that the M form still retains a hydrophobic nature of the membrane-bound enzyme, whereas both S forms are completely soluble in the buffers used.

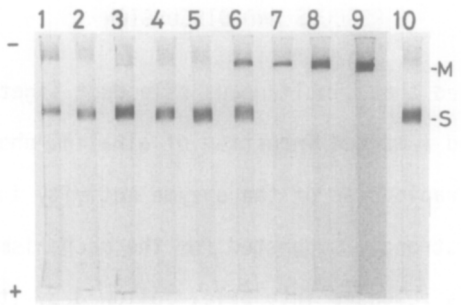


Fig. 1. Effect of pH on the conversion of alkaline phosphatase of liver plasma membrane during n-butanol extraction. Liver plasma membrane (5 mg/ml) suspended in buffers with the indicated pH containing 1 mM $MgCl_2$ were mixed with n-butanol (25%) and stirred at 25°C for 60 min, followed by centrifugation. The extracted samples were subjected to polyacrylamide gel electrophoresis, and gels were stained for alkaline phosphatase activity. Gels 1-3, samples treated at pH 4.0, 5.0, and 5.5, respectively, in 50 mM acetate buffer; gels 4 and 5, at pH 6.0 and 6.5, respectively, in 50 mM Mes buffer; gel 6, at pH 7.0 in 50 mM Hepes buffer; gels 7-9, at pH 7.5, 8.0 and 8.5, respectively, in 50 mM Tris-HCl buffer. Gel 10, serum-soluble form prepared as described previously (3). M and S denote the liver-membranous and serum-soluble forms, respectively, of the hepatic alkaline phosphatase.

Addition of Triton X-100 (0.5%) in place of n-butanol during the incubation at the low pH also caused a considerable conversion of the enzyme, though not complete as observed in the presence of n-butanol. However, in the absence of n-butanol or Triton X-100, incubation of the plasma membrane even at the low pH used as above caused no significant release nor conversion of alkaline phosphatase. Furthermore, no conversion was detected when the n-butanol extract which had been prepared at pH 8.5 was then adjusted to pH 5.5, followed by incubation at 25°C. Taken together, these results suggest that the conversion of the enzyme simultaneously requires the acidic conditions and some perturbation of the membranes, which may activate a possible converting factor like PI-PLase C in the membranes. In fact, Low and Zilversmit (22) suggested existence of PI-PLase C in kidney microsomes activated by n-butanol, although they did not describe requirement of the low pH for the reaction.

Previously we reported that rat ascites hepatoma AH-130 cells had a high specific activity of alkaline phosphatase, which was about 50-fold higher than that of the normal liver (17). In spite of such high activity, no significant activity was detected as a soluble form in ascitic fluid where the hepatoma cells grew, indicating that the enzyme seems not to be easily released

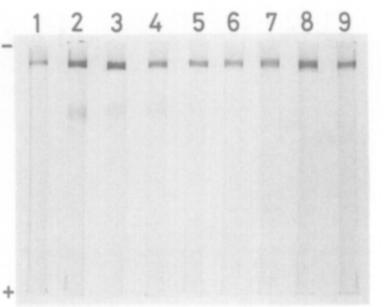


Fig. 2. Effect of pH on the conversion of alkaline phosphatase of hepatoma plasma membrane during n-butanol extraction. Plasma membranes of AH-130 cells (3.5 mg/ml) were suspended in buffers and treated with n-butanol under the same conditions as in Fig. 1. The extracted samples were analyzed by gel electrophoresis. Gels 1-9, samples treated at the same pHs as indicated in Fig. 1, respectively.

from the hepatoma membranes. When the hepatoma plasma membranes were treated with n-butanol under the same conditions as the liver membranes were done (Fig. 1), the enzyme was hardly converted to a soluble form, as shown in Fig. 2, in contrast with the results obtained for the liver enzyme (Fig. 1). It is unlikely that little conversion is due to a structural difference between the two enzymes. The hepatoma alkaline phosphatase has been demonstrated to be identical with the liver enzyme in enzymological and immunological properties (18,23). In addition, treatment of the plasma membranes with the bacterial PI-PLase C caused a complete conversion of the hepatoma enzyme to a soluble form as observed for the liver one (Fig. 3) [a relatively slow mobility of the hepatoma enzyme is due to a lower content of sialic acid residues in its oligosaccharide chains (23)]. Thus, the difference in the conversion (Fig. 1 vs. Fig. 2) is ascribed to lack of the endogenous converting activity in the hepatoma membranes. This may explain the reason why the hepatoma enzyme is not released as a soluble form into ascitic fluid in vivo and requires a detergent for its solubilization throughout all the purification steps after n-butanol extraction (18,23). On the other hand, lack of the converting activity and enrichment of alkaline phosphatase in the hepatoma membranes have an advantage for characterization of the activity involved in the conversion, because the membranes could be used only as a substrate for the reaction system.

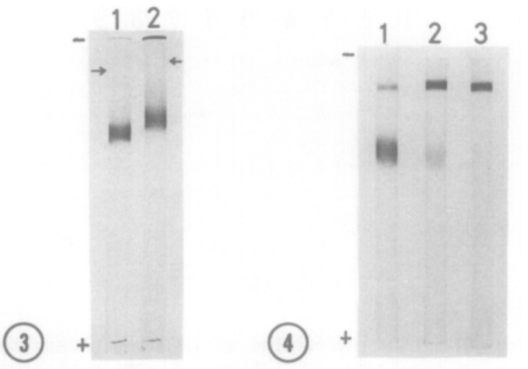


Fig. 3. Conversion by PI-PLase C of membranous alkaline phosphatase of liver and hepatoma. Plasma membranes of liver and AH 130 cells were suspended in 50 mM Tris-HCl buffer (pH 7.5) and incubated at 37°C for 90 min in the presence of PI-PLase C purified from Bacillus cereus (0.1 unit/ml), followed by centrifugation at 105,000 x g for 60 min. The supernatants were analyzed by polyacrylamide gel electrophoresis for alkaline phosphatase, as described in Materials and Methods. Gel 1, liver alkaline phosphatase; gel 2, hepatoma alkaline phosphatase. Arrows indicate the positions of membranous forms of each enzyme.

Fig. 4. Converting activity for alkaline phosphatase in subfractions of liver lysosomes. Hepatoma plasma membranes (3 mg/ml) suspended in 50 mM Mes buffer, pH 6.0, were treated with n-butanol at 25°C for 60 min in the presence or absence of lysosomal membranes (5.5 mg/ml) or contents (5.8 mg/ml) prepared from liver. The extracts were analyzed by polyacrylamide gel electrophoresis, followed by staining for alkaline phosphatase activity. Gel 1, sample treated in the presence of lysosomal membranes; gel 2, extract in the presence of lysosomal contents; gel 3, extract with no addition.

Requirement of the acidic pH for the conversion suggests a possible involvement of the lysosomal PI-PLase C which has been relatively well characterized in the liver (24,25). Using the hepatoma plasma membranes as a substrate, we examined the converting activity in lysosomes isolated from rat liver. In fact, lysosomes had the converting activity, most of which was found in a membrane fraction of lysosomes (Fig. 4). Such localization of the activity is in contrast with that of the acid PI-PLase activity identified in liver lysosomes, which exists exclusively as a soluble protein (24,25). Furthermore, although the lysosomal PI-PLase activity was shown to be strongly inhibited by divalent cations and cationic drugs (24), the converting activity for alkaline phosphatase was not affected at all by these agents (data not shown). These results indicate that the converting activity found in lysosomes was not exerted by the lysosomal PI-PLase so far characterized (12,24,25).

Demonstration of the endogenous converting activity in the liver plasma membrane will give a clue to the mechanism for occurrence of the hepatic

alkaline phosphatase in serum. However, it requires further investigation to determine whether the converting activity demonstrated here is really due to another type of PI-PLase or not. A functional relationship of its localization in the lysosomal membrane also remains to be solved.

ACKNOWLEDGEMENTS : This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

1. Kaplan, M.M. and Righetti, A.(1970) J. Clin. Invest. 49, 508-516.
2. Pekarthy, J.M., Short, J., Lansing, A.I. and Lieberman, I.(1972) J. Biol. Chem. 247, 1767-1774.
3. Ikehara, Y., Mansho, K. and Kato, K.(1978) J. Biochem. 84, 1335-1338.
4. Morton, R.K.(1954) Biochem. J. 57, 595-603.
5. Vyvoda, O.S., Coleman, R. and Holdsworth, G.(1977) Biochim. Biophys. Acta 465, 68-76.
6. Billington, D., Evans, C.E., Godfrey, P.P and Coleman, R.(1980) Biochem. J. 188, 321-327.
7. Ikezawa, H., Yamaguchi, M., Taguchi, R., Miyashita, T. and Ohyasu, T.(1976) Biochim. Biophys. Acta 450, 154-164.
8. Low, M.G. and Finean, J.B.(1977) Biochem. J. 167, 281-284.
9. Kominami, T., Oda, K. and Ikehara, Y.(1984) J. Biochem. 96, 901-911.
10. Kominami, T., Miki, A. and Ikehara, Y.(1984) Manuscripts submitted.
11. Shukla, S.D., Coleman, R., Finean, J.B. and Michell, R.H.(1980) Biochem. J. 187, 277-280.
12. Michell, R.H.(1975) Biochim. Biophys. Acta 415, 81-145.
13. Oda, K. and Ikehara, Y.(1981) Biochim. Biophys. Acta 640, 398-408.
14. Ray, T.K.(1970) Biochim. Biophys. Acta 196, 1-9.
15. Kato, K. and Ikehara, Y.(1975) Methods in Biochemstry 10, 509-524.
16. Ohsumi, Y., Ishikawa, T. and Kato, K.(1983) J. Biochem. 93, 547-556.
17. Ikehara, Y., Takahashi, K., Mansho, K., Eto, S. and Kato, K.(1977) Biochim. Biophys. Acta 470, 202-211.
18. Ikehara, Y., Mansho, K., Takahashi, K. and Kato, K.(1978) J. Biochem. 83, 1471-1483.
19. Davis, B.J.(1964) Ann. N.Y. Acad. Sci. 121, 404-427.
20. Lowry, O.H., rosebrough, N.J., Farr, A.L. and Randall, R.J.(1951) J. Biol. Chem. 193, 265-275.
21. Barnwell, S.G., Godfrey, P.P, Lowe, P.J. and Coleman, R.(1983) Biochem. J. 210, 549-557.
22. Low, M.G. and Zilversmit, D.B.(1980) Biochemistry 19, 3913-3918.
23. Kawahara, S., Ogata, S. and Ikehara, Y.(1982) J. Biochem. 91, 201-210.
24. Irvine, R.F., Hemington, N. and Dawson, R.M.C.(1978) Biochem. J. 176, 475-484.
25. Matsuzawa, Y. and Holtetler, K.Y.(1980) J. Biol. Chem. 255, 646-652.