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# TETRAMERIC ALKALINE PHOSPHATASE IN HUMAN LIVER PLASMA MEMBRANES

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Molecular weights of native membrane-bound alkaline phosphatase released by butanol and by nonionic detergents were more than twice that of the purified dimeric enzyme. Alkaline phosphatase released by phosphatidylinositol-specific phospholipase-C was of both high and low molecular weight: the former was a protomer of a single protein of the same molecular size as monomeric alkaline phosphatase. We conclude that the membrane-bound enzyme is probably a tetramer. © 1985 Academic Press, Inc.

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase [alkaline optimum, EC 3·1·3·1]) purified from human liver, one of the tissue-nonspecific alkaline phosphatases, is a glycoprotein of $M_r$ 130—180 k (1-4). This ectoenzyme is especially rich in the endothelial cells lining the blood sinusoids of human liver (5). A dimeric structure and $Zn^{2+}$ are essential for maintaining activity (6). Having identified certain chemical and kinetic differences between membrane-bound and purified alkaline phosphatase (7), we now determined the molecular size of the enzyme bound to the plasma membrane of human liver cells.

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Abbreviations: SDS, sodium dodecyl sulfate; NP40, Nonidet P-40; CHAPS, [3-(chloroamidopropyl)dimethylammonio]-1-propanesulfonate.

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## MATERIALS AND METHODS

Phospholipase-C (Bacillus cereus) type III, p-nitrophenylphosphate, naphthol AS—MX phosphate, NP40, and molecular weight markers for chromatography were purchased from Sigma Chemical Co. (St Louis, MO); Triton X-100, SDS, and CHAPS from Bio-Rad Laboratories (Richmond, CA); octyl-β-D-glycopyranoside from Calbiochem-Behring Corp. (La Jolla, CA); ethylaminoethanol from Aldrich Chemical Co. (Milwaukee, WI); Sepharose-6B, and molecular-weight markers for electrophoresis from Pharmacia Fine Chemicals AB (Uppsala, Sweden); 2.5–27% polyacrylamide gradient gels from Isolab (Akron, OH). All other chemicals were of highest reagent grade (Fisher Scientific, Fairlawn, NJ).

Plasma-membrane and enzyme preparations, and assays. Plasma membranes were isolated (7), and alkaline phosphatase was purified to homogeneity as judged by gel electrophoresis on SDS (8). Phosphatidylinositol-specific phospholipase-C was obtained by purification from a commercial preparation of nonspecific phospholipase-C (9). Alkaline phosphatase was assayed in 1 ml of medium containing p-nitrophenylphosphate (10 mM) and ethylaminoethanol (1.0 M), pH 10.3, at 30°C; the increase in absorbance at 404 nm was monitored in a spectrophotometer (Varian model 2200). Enzyme activity (U) was expressed as mol of p-nitrophenol released/min. Protein concentrations were determined using the Markwell modification of the Lowry procedure (10).

Solubilization of alkaline phosphatase. Aliquots of isolated plasma membranes (1 ml, containing 0.5 U of alkaline phosphatase activity) suspended in 125 mM sucrose, 50 mM KCl, 25 mM Tris HCl, 1.0 mM $MgCl_2$, 0.1 mM $ZnCl_2$ (pH 7.6), were incubated with detergent (range, 0–125 mM) for 1 h at 4°C with continuous gentle mixing, then centrifuged at 100,000 X g for 10 min for recovery of the enzyme. Detergents used were NP40, Triton X-100, β-octylglucoside (nonionic), CHAPS (zwitterionic), and SDS (ionic). When phosphatidylinositol-specific phospholipase-C was the solubilizing agent, 2 μg of phospholipase-C protein was added to the membranes in 1.0 ml of 0.25 M sucrose and 50 mM Tris HCl (pH 7.6), incubated for 1 h at 30°C, and centrifuged as before.

For butanol treatment, 1 ml of solvent added to 1 ml of membranes suspended in 0.25 M sucrose and 10 mM Tris HCl, pH 7.6, was gently mixed for 1 min then centrifuged for 10 min at 3500 X g. The bottom (aqueous) layer was divided into two aliquots; one was left unchanged and CHAPS detergent (up to 25 mM) was added to the other.

Molecular weight. This was determined for samples (0.05 U in 100 μl) of both solubilized and purified enzyme by electrophoresis in gradient polyacrylamide gels for 24 h at 150 V in 0.09 M Tris and 0.08 M borate buffer, pH 8.3 (1). Molecular weight markers were added to samples before electrophoresis. After electrophoresis, the gels were stained for activity in naphthol AS—MX phosphate (1 mg/ml) dissolved in 1.0 M ethylaminoethanol, pH 10.3, and viewed under UV light until band(s) of alkaline phosphatase became detectable (4). The gels were stained for protein with a rapid Coomassie blue R-250 stain (11).

As an alternative confirmatory method, the molecular weight of alkaline phosphatase solubilized by 17 mM NP40 was determined with chromatography on Sepharose-6B equilibrated in 50 mM Tris—HCl and 1.7 mM NP40, pH 7.6. A Pharmacia K 16/100 column was employed at 8 ml/h; bed volume was 175 ml. Molecular weights were calculated from a standard curve of Log $M_r$ vs $K_{av}$, using molecular-weight markers. Markers and samples were run in triplicate.

Purification and SDS-polyacrylamide gel electrophoresis of high-$M_r$ alkaline phosphatase. Enzyme (1.0 U in 2 ml) solubilized with 12.5 mM NP40, was applied to a 7 ml phosphonic acid-Sepharose column (12) which had been equilibrated in 10 mM Mes, 6.25 mM NP40, 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, pH 6.0. The column was washed with 100 mM NaCl in equilibration buffer, and the alkaline phosphatase was selectively eluted with 25 mM β-naphthyl phosphate in equilibration buffer. Fractions containing enzyme activity were pooled; three 200 µl aliquots were subjected to gradient gel electrophoresis, and the band of alkaline phosphatase of high $M_r$ was cut from each. The three gel slices were suspended in 1.0 ml of 10 mM Tris, pH 7.6, then homogenized in a Potter—Elvehjem homogenizer with a Teflon piston. The homogenate was lyophilized, and the powder was iodinated with $Na^{125}I$ (specific activity, 17 Ci/mg) (13) and dialyzed against 10% methanol in deionized water to remove any free $^{125}I$. The resulting radio-labelled gel slurry was denatured in SDS 1% (w/v) (90° C, 1 h) and subjected to discontinuous electrophoresis on SDS-polyacrylamide gel (14); protein bands in the gel were electrophoretically transferred with a Trans blot cell (Bio-Rad Laboratories) onto a nitrocellulose sheet (15), and the sheet was autoradiographed (16) on X-ray film (Kodak XAR-5).

## RESULTS

Solubilization and $M_r$ of alkaline phosphatase. NP40 and Triton X-100 were the most efficient detergents for solubilizing alkaline phosphatase from plasma membranes (Fig. 1); higher concentrations of CHAPS and β-octylglucoside were necessary for complete solubilization. Gradient gel electrophoresis revealed that only NP40, Triton X-100, and β-octylglucoside (nonionic) liberated alkaline phosphatase of homogeneous $M_r$ (Table 1); CHAPS- and SDS-solubilized enzyme ran as a smear on top of the gradient gels. Butanol treatment released alkaline phosphatase from membranes, but the enzyme entered the gel only when CHAPS detergent was present; however, its $M_r$ was similar to that of the form solubilized by the nonionic detergents (Table 1). Phosphatidylinositol-specific

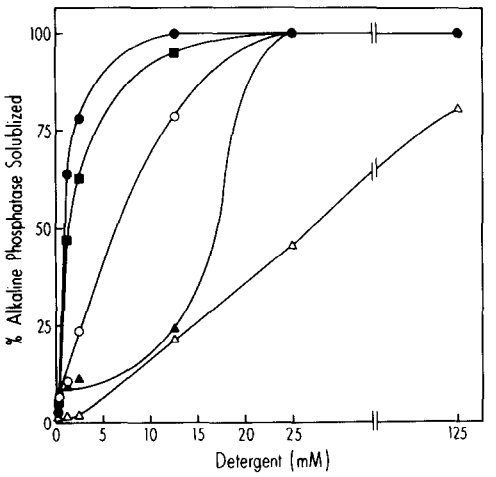



Figure 1. Solubilization of alkaline phosphatase by detergents. Membrane preparations were solubilized for 1 h at 4°C with NP40 (●), Triton X-100 (■), CHAPS (○), β-octylglucoside (▲) or SDS (△), then centrifuged at 100,000 X g for 10 min. Alkaline phosphatase activity in the supernatant fluid was expressed as a percentage of the activity in the suspension before centrifugation.

phospholipase-C released alkaline phosphatase of both high and low molecular weights (Table 1 and Fig. 2b).

Stability of high-$M_r$ alkaline phosphatase. Precipitation of the enzymes released by detergent and by phospholipase-C in 75% acetone converted the high-$M_r$ enzyme to the low-$M_r$ form (Fig. 2c). In NP40, the high-$M_r$ form was stable at pH 5.0-

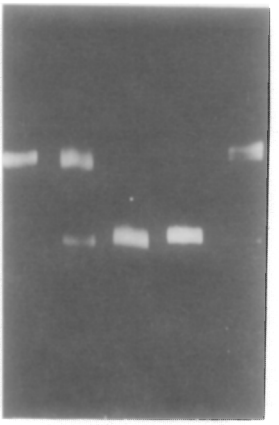
TABLE 1. RELATIVE MOLECULAR WEIGHTS OF PURIFIED AND DETERGENT-SOLUBILIZED ALKALINE PHOSPHATASES

| Form of enzyme | | $M_r$ x $10^{-3}$ |
|---|---|---|
| Purified | | 176 ± 13 |
| Released by: | NP40 | 411 ± 38 |
| | Triton X-100 | 417 ± 37 |
| | β-Octylglucoside | 567 ± 40 |
| | Butanol* | 380 ± 32 |
| | Phospholipase-C | 350 & 173 |

$M_r$ were determined by gradient polyacrylamide (2.5 27%) gel electrophoresis.

Alkaline phosphatase was located by staining for activity with naphthol AS—MX phosphate. $M_r$ values are means ± SD (n = 5).

* Required 25 mM CHAPS to enter gradient gels.



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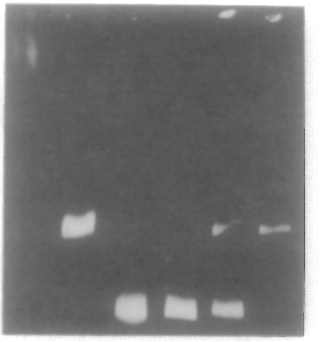

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Figure 2. Gradient gel electrophoresis of various forms of alkaline phosphatase showing size and stability. Gel a, solubilized with NP40; b, solubilized with phospholipase-C; c, solubilized with phospholipase-C, precipitated in 75% acetone, and redissolved in electrophoresis buffer; d, purified enzyme preparation; e, solubilized with NP40, then heated to 40°C for 24 h. Staining for activity followed electrophoresis.

Figure 3. Gradient gel electrophoresis of various forms of alkaline phosphatase showing detergent interactions. Gel a, enzyme solubilized with NP40 after removing detergent by Sepharose-6B chromatography; b, sample from gel a plus 12.5 mM NP40; c, purified enzyme; d, purified enzyme plus 12.5 mM NP40; e, purified enzyme plus NP40-solubilized enzyme. Staining for activity followed electrophoresis.

10.0 for 24 h but converted partly to the low-$M_r$ form after being heated at 40°C for 24 h (Fig. 2). Removal of detergent by gel-permeation chromatography blocked the enzyme's entry to the gels, but when the detergent was replaced, the high-$M_r$ species returned (Fig. 3). Detergent did not change the $M_r$ of either the purified enzyme (Fig. 3) or the detergent solubilized form treated with acetone.

$M_r$ values confirmed with Sepharose chromatography for the NP40-solubilized membrane-bound and purified enzymes were 476 k and 198 k, respectively.

Under denaturing conditions, electrophoresis of the purified high-$M_r$ species of the enzyme revealed only one protein band; $M_r$ was 70 k (Fig. 4).

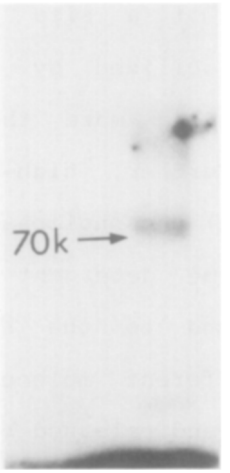


Figure 4. Autoradiography of purified high-$M_r$ enzyme after SDS-electrophoresis. ($M_r$ = 70 k)

## DISCUSSION

The native molecular weight of alkaline phosphatase purified from human liver has been reported in the range of 130-180 k, and the native enzyme has mostly been described as a dimer (1-4). The $M_r$ of 176 k that we determined is within this range, but $M_r$ values of the enzyme released by butanol and the nonionic detergents were more than twice as high (Table 1). We believe that the high-$M_r$ species represents a tetrameric conformation: heating at 40°C or acetone treatment dissociated the complex, which unlike the dimeric form, required detergents to remain soluble; also, it was a protomer of one type of protein subunit ($M_r$ 70 k) (Fig. 4) of the same molecular size (range 68-80 k) as the monomer of alkaline phosphatase from human liver (1-4).

It is unlikely that the high-$M_r$ species can be explained by an association between the detergent molecules and the dimeric enzyme, because neither the purified nor the acetone-treated enzyme had any affinity for detergent molecules: a dimeric

enzyme-detergent complex of a size similar to that of the alkaline phosphatase solubilized by β-octylglucoside would require the enzyme to bind more than twice its weight in detergent molecules. Further, high-$M_r$ alkaline phosphatase from the (detergent-free) phospholipase-C preparation obviates the possibility of enzyme detergent complexes. A residual amount of detergent bound to the high-$M_r$ enzyme probably accounts for the different molecular weights of enzyme solubilized by detergents and released by phospholipase-C.

Several reports have suggested the existence of tetrameric alkaline phosphatases. The *E. coli* enzyme dimer can form tetramers at pH 8.3 when excess $Zn^{2+}$ is present (17), and *Pseudomonas aeruginosa* contains small amounts of tetramer (18). Also, alkaline phosphatases from human placenta, pig kidney, chick kidney, and chick epiphyseal cartilage may have a tetrameric quaternary structure (19-22). In our studies, the enzyme operated as a tetramer in the plasma membrane and detergents were required to keep it soluble outside its membrane environment. Acetone precipitation probably disrupts the noncovalent and hydrophobic forces that stabilize the tetramer complex, causing dissociation; solubilization of the acetone pellet results in dimeric alkaline phosphatase soluble in aqueous media. The tetrameric conformation may explain some of the differences we have reported between the properties of the membrane-bound enzyme and of the purified form (7).

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