RECONSTITUTION AND MODULATION OF AN α-MANNOSIDASE BY PHOSPHOLIPIDS

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The "high mannose" and complex oligosaccharide chains of glycoproteins have been shown to play important roles in biological recognition. These two major types of asparagine-linked oligosaccharides are derived from a common, dolichol-linked precursor ($Glc_3Man_9GlcNAc_2$). After *en bloc* transfer to nascent peptides, the oligosaccharide may undergo limited processing to yield high mannose structures, or else it may be extensively modified to yield complex oligosaccharide chains (1). Formation of glycoproteins containing high mannose chains involves the removal of three glucose and zero to four mannose units, whereas complex chain formation entails the loss of three glucose and six mannose residues followed by the addition of *N*-acetylglucosamine, galactose, sialic acid, and fucose. The extent of α-mannosidase activity appears to be a controlling element in determining the final outcome of this pathway. We have recently reported the purification of a microsomal α-mannosidase from rabbit liver which specifically hydrolyzes the α-1,2-mannosyl-mannose linkages in a heptasaccharide with a high mannose type structure (2). The purified enzyme is optimally active at pH 5.3 and its dependence upon calcium ion levels and on the phospholipid environment suggests possible control mechanisms for enzyme activity.

METHODS

Standard reaction mixtures for assay of the α-mannosidase contained 4,000 cpm of [^{14}C]Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ-GlcNAcβ-GlcNAc, 80 mM cacodylate/acetate, pH 5.3, 2 mM $CaCl_2$, 0.2% Triton X-100, 50 μg of phosphatidylcholine (PC), and 0.23 to 1.3 μg of enzyme in a final volume of 0.1 ml in glass tubes. After an incubation of 3 h at 37°C, the mixtures were subjected to paper chromatography, the areas corresponding to mannose were excised and their radioactivity determined.

RESULTS AND DISCUSSION

Under these conditions, α-mannosidase was shown to be optimally active when zwitterionic phospholipid (PC) was present in excess of a phospholipid:Triton X-100 ratio of 0.14 (wt/wt). As the concentration of PC was decreased, enzyme activity rapidly diminished to a minimum level (13%) at a PC:Triton X-100 ratio of 0.06 or lower. Half-maximal activity was observed at a PC:Triton X-100 ratio of 0.1. There is ample evidence that phospholipid and detergent will co-exist in uniform mixed micelles when sufficient detergent is present. When saturating amounts of phospholipid are present the micelles become larger, ellipsoidal, and presumably contain patches of aggregated phospholipid (3). Thus the data are suggestive of a requirement by α-mannosidase for the presence of aggregates of zwitterionic phospholipid to obtain maximum activity under the above assay conditions.

This requirement has been further examined by substitution of soybean PC by phosphatidylethanolamine (PE), sphingomyelin (SM), lyso-PC, and several synthetic diacylphosphorylcholines. α-Mannosidase had > 90% activity in the presence of 50 μg of PE, SM, dipalmitoyl- and didecanoyl-PC. The percent of maximum activity obtained with 50 μg of dihexanoyl-PC and lyso-PC was 13 and 57%, respectively. A 10-fold increase in dihexanoyl-PC (500 μg) only increased the activity to 26% of that of the control. Studies with short-chain diacylphosphorylcholines have previously shown that compounds containing

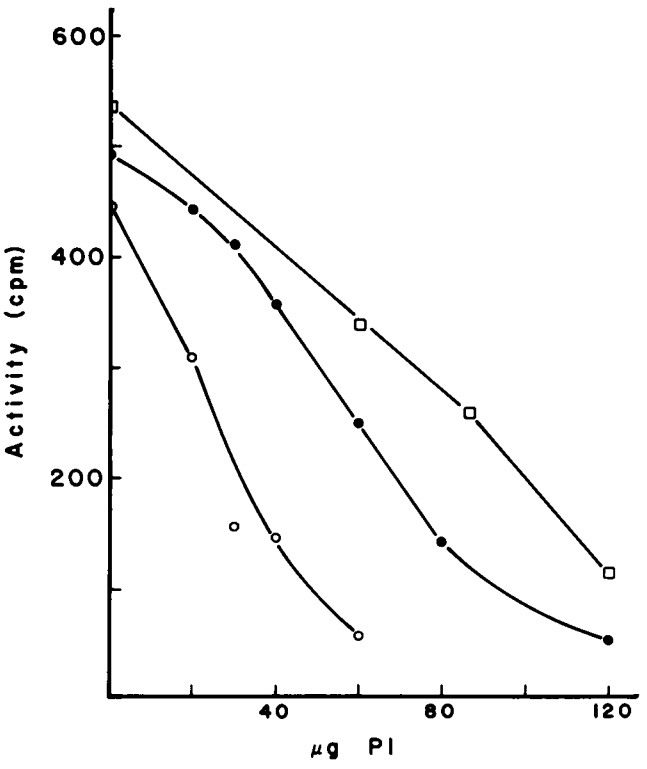

FIGURE 1 Inhibition of α-mannosidase by phosphatidylinositol (PI) at three concentrations of phosphatidylcholine. Reaction mixtures contained 1.0 μg of protein. All other reaction conditions were as described above except that phosphatidylinositol was added as indicated in the presence of 30 μg (○), 60 μg (●), and 90 μg (□) of phosphatidylcholine.

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C-6 or shorter chains do not form extended bilayers in aqueous solution even at concentrations above their critical micelle concentrations but instead form small micelles (4). The properties of lyso-PC also do not favor aggregates of this phospholipid in mixed micelles with detergent.

In contrast to the stimulatory action of zwitterionic phospholipids, enzyme activity was inhibited by negative phospholipids even in the presence of optimal PC. As shown in Fig. 1, activity in the presence of three different concentrations of PC was determined at increasing concentrations of phosphatidylinositol (PI). 50% inhibition of the enzyme occurrred at PI:PC ratios of 1.0, 1.02, and 0.93, respectively, in the presence of 30 μg, 60 μg, and 90 μg of PC; almost 90% inhibition of activity was observed at a PI:PC ratio of 2.0 in all three cases.

In summary, α-mannosidase appears to require a hydrophobic surface containing closely packed zwitterionic head groups, and the dispersion of these charge groups with either detergent or by dilution with negatively charged groups leads to a decrease in activity. Although the levels of PI which cause significant inhibition of the α-mannosidase in vitro are considerably higher than the amount of PI present in the membranes of golgi or endoplasmic reticulum, preliminary studies indicate that the enzyme is much more sensitive to PI in the absence of Triton X-100. Thus fluctuations in the phospholipid microenvironment of the α-mannosidase could markedly influence the activity of the enzyme.

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RECONSTITUTION OF THE MEMBRANE-BOUND FORM OF DOPAMINE-β-HYDROXYLASE

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In the adrenal medulla and extradrenal chromaffin tissue, chromaffin granules are involved with the biosynthesis, storage, and secretion of catecholamines. Dopamine-β-hydroxylase (DBH), which converts dopamine to norepinephrine, exists as both a soluble and membrane-bound enzyme in the granule. The two forms of hydroxylase purified by others have appeared identical in physical composition.

We report here the purification of both forms of DBH which appear to be different based on gel filtration chromatography and reconstitution into phospholipid-bilayer membranes. A comparison of the enzymatic activity of reconstituted membranous enzyme with soluble enzyme is also presented.

METHODS

Chromaffin granule membranes were isolated from fresh bovine adrenal medullas (1). Extraction of membranes with octyl glucoside (2%, 30 min) was followed by DEAE-cellulose chromatography (10–100 mM sodium phosphate gradient, pH 6.8, 1% octyl glucoside). The eluted hydroxylase was then chromatographed on Sepharose CL-6B (50 mM HEPES, pH 7.4, 100 mM NaCl). Concentration of fractions was done using an Amicon PM30 membrane (Amicon Corp., Lexington, MA). The soluble form of the enzyme was purified from the granule lysate by DEAE-cellulose chromatography (50 mM sodium phosphate pH 6.8, 200 mM NaCl) and gel filtration as above.

Enzyme assays were as described by Wallace et al. (2) in 0.2 M sodium acetate, pH 5.0 (during purification), or in 50 mM MES, pH 6.0 (kinetic experiments). The reconstitution procedure is described in the legend to Fig. 1. All reagents were obtained from Sigma Chemical Co., St. Louis, MO, except ^{14}C-phosphatidyl choline from New England Nuclear, Boston, MA.

RESULTS

The data from a typical purification of bovine adrenal DBH are shown in Table I. Activity of the enzyme is dependent on associated phospholipid and therefore the specific activities shown are only estimates. Four to five molar equivalents of phospholipid are associated with the final fraction of purified hydroxylase as determined by phosphate analysis and semiquantitative thin-layer chromatography.

Hydroxylase purified from the soluble contents of the granules was compared to the purified membranous hydroxylase on sodium dodecylsulfate polyacrylamide gel electrophoresis. Under reducing conditions both forms