Regulation of Rat Liver Epoxide Hydrolase by Tightly Bound Phosphoinositides

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Rat liver microsomal epoxide hydrolase (styrene oxide hydrolase) has three tightly bound phosphoinositides in its native state. Two methods of purification yielded an enzyme with apparent homogeneity. When dried films of this enzyme were repeatedly extracted with chloroform/methanol, the lipid-free films still contained bound inositides. Further extraction of the enzyme film using acidified chloroform/methanol gave three phosphoinositides per enzyme of 50,000 Da. Thin layer chromatography of the protein-bound inositides showed two to be phosphatidyl inositol and one to be phosphatidyl inositol 4-phosphate. Physical and chemical evidence suggests covalent binding between inositides and enzyme. After incubation at pH 9.2 for 16 hr at 4°C followed by chromatofocusing, an inositide-free, active enzyme was obtained. This inositide-free enzyme had much higher epoxide hydrolase activity than the inositide-bound enzyme. The enzyme activity was not inhibited by the presence of phosphatidyl inositol and phosphatidyl inositol 4-phosphate. These results suggest that the attachment of inositides is critical for the difference in styrene oxide hydrolase activity in low-ionic strength buffer between the native, inositide-containing enzyme (low activity) and the inositide-free enzyme (higher activity).

INTRODUCTION

Mammalian microsomal epoxide hydrolase (EC 3.3.2.3) catalyzes the formation of transdihydrodiols from a variety of epoxides, some of which are carcinogens (*1*). The rat liver enzyme has been purified to homogeneity by several techniques (*2-4*). The existence of multiple forms of the rat liver enzyme was indicated in a study which showed two distinct classes of activity could be separated using a non-ionic detergent solubilized preparation from rat liver microsomes, first purified by diethyl aminoethyl cellulose (DE-52) chromatography and then separation achieved on carboxymethyl cellulose (CM-52) chromatography at pH 6.5 (*5,6*). The amino acid analyses of both forms were not significantly different, and both had the same apparent subunit molecular weight of 50,000 Da, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after the preparations were reduced with mercaptoethanol. However, the A form had different activities towards several epoxide substrates when compared to the B form (*5,6*). A post-translational modification of the enzyme could account for some of these characteristics.

The data presented here indicate that normal and phenobarbital-induced microsomal epoxide hydrolase contains three very tightly bound inositides in its native state. The evidence suggests covalent binding. The inositides can be removed leaving an active enzyme. Isolation and characterization of these two forms is described below.

METHODS

Materials: DE-52 and CM-52 cellulose were obtained from Whatman Separations Ltd. (UK). Chromatofocusing gel PBE 94, Polybuffer 76 and Octyl-Sepharose CL-4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Polystyrene beads for detergent absorption (Bio-beads SM-2), crystalline hydroxyapatite and the reagents for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA), except for the SDS which was Sequenal grade from Pierce Chemical Co. (Rockford, IL). Soybean L-(α-phosphatidylinositol (PtdIns), bovine brain L-(α-phosphatidylinositol 4-phosphate (PtdIns 4P), L-(α-phosphatidylinositol 4,5-diphosphate (PtdIns 4,5P), phenobarbital, Luberol PX and distearyl-phosphatidyl choline were obtained from Sigma Chemical Co., (St. Louis, MO). All other chemicals were analytical grade. The ultrafiltration unit and

ultrafilters (YM 30) were from Amicon Corp. (Danvers, MA). Male Sprague-Dawley rats (100± 10 g) were from SASCO (Portage, MA) and were fed a basal diet, ad lib. Phenobarbital, when used, was added to drinking water at 0.10% for 72 h before killing the rat.

Techniques for Enzyme Purification: Two different species of rat liver epoxide hydrolase were obtained as described below. Each procedure started with rat liver microsomes prepared as described earlier (*4*) and was initiated by solubilizing the microsomes at room temperature with Luberol PX (final concentration of Luberol = 1%) at a detergent to protein ratio of 4:1. The solution was made 10% in glycerol, which was a component of all buffers throughout the rest of the purification procedures. All subsequent operations were performed at 4°C. Ribosomes were removed from solubilized microsomes by centrifugation at 110,000 x g for 1.5 h and the supernate contained over 75% of the total microsomal enzyme activity. The solubilized enzyme preparation was dialyzed against at least 70 volumes of 5 mM KPO_4, <KPO4>, pH 7.25, with 0.05% Luberol and 10% glycerol, and then applied to a DE-52 column equilibrated with the same buffer (*5,6*). The breakthrough fraction contained over 90% of the applied enzyme activity, enriched 3-4-fold. After careful adjustment to pH 6.5 and dialysis versus 10 volumes of 5 mM KPO_4, <KPO4>, pH 6.5, containing 0.05% Lubreol and 10% glycerol, the DE-52 fraction was applied to a CM-52 column previously equilibrated with the same buffer. As reported (*5,6*), the breakthrough fraction contained the A form of the enzyme, which accounts for about 75% of the applied activity. This A-form of the enzyme was purified further either by chromatography on hydroxyapatite (*5,6*) or on Octyl-Sepharose (*5,6*), yielding essentially the same homogeneous preparation as estimated by analytical SDS-PAGE (single band at 50,000 Da) and amino acid analysis (complements of R.Wang).

The inositide-depleted enzyme was obtained using the DE-52 breakthrough fraction which was concentrated 20-fold by Amicon ultrafiltration. After adjustment to pH 9.2 using ethanolamine, the concentrated fraction was dialyzed against 20 volumes of 50 mM ethanolamine-acetate containing 0.05% Luberol for 16 h at 4°C. This dialysate was slowly applied to a solid support column of polybuffer-exchanger, PBE-94, previously equilibrated with the pH 9.4 buffer. Chromatofocusing was accomplished by elution with Polybuffer 76.

Identification and Quantitation of Inositol: Solutions containing enzyme forms were (a) depleted of detergent by shaking with polystyrene beads at 4°C, (b) extensively dialyzed against 5 mM ethylenediamine-tetraacetate at pH 7.0, (c) dialyzed against water and (d) finally dried under nitrogen. The resulting films were extracted three times with chloroform /methanol, 2:1, which removed all loosely bound phospholipids, including inositides. Finally the film was hydrolyzed in 4N HCL under nitrogen for 4 h at 100°C. These conditions resulted in quantitative recovery of inositol standards. Inositol was identified and quantitated in the hydrolysate as its aldital-hexaacetate derivative by gas-liquid chromatography and mass spectroscopy using xylose and inositol derivatives as standards. Analyses were run on a Tracor 222 gas chromatograph equipped with an automatic digital integrator, flame ionization detector, and a glass column (1.9 m x 4 mm inside diameter) packed with 3% OV-225 on 80/100 Supelcoport (Supelco Inc.; Bellefonte, PA). The mass spectrometer was a Kratos MS 25 system equipped with a glass capillary column (0.25 mm x 25 m) that was coated with 0.25 µ thick SE-30. The injector temperature was 220°C. and the oven was programmed to change from 220 to 310°C at 60/min. Mass spectra were recorded at an ionizing energy of 65 electron volts.

Other Analytical Techniques: Purified enzyme was dried under a stream of nitrogen and loosely associated phospholipids including the majority of inositides removed from the dried film by three successive extractions at room temperature with chloroform/methanol, 2:1. No inositides were detected in a fourth extraction with chloroform/methanol. The tightly bound inositides were isolated by two successive extractions with chloroform/methanol 2:1, containing 0.25% concentrated HCl at 37°C for 20 min for each extraction. The separated extracts were immediately neutralized with dry gaseous ammonia, the ammonium chloride filtered, and the solvent evaporated. Inositides were separated and identified by thin-layer chromatography on silica gel G plates in which the gel was impregnated with potassium oxalate (*7*). Lipid phosphorus was measured after hydrolyzing the eluted spot (*8*) using a colorimetric method (*9*). SDS-PAGE was performed according to the procedure described by Laemmli (*10*) and the gels were stained with Coomasse Blue. Antiserum to microsomal epoxide hydrolase was prepared in rabbits as described previously (*11,12*). Styrene oxide hydrolase was measured as reported previously (*3,5*). The microsomal form of the enzyme prefers a

monosubstituted epoxide such as styrene oxide (phenyl ethylene oxide). Buffer conditions were varied to give a low or high ionic strength in the incubations with enzyme.

RESULTS

Rat liver microsomal styrene oxide hydrolase from normal or phenobarbital-induced rats was purified to apparent homogeneity as shown by a single band at 50,000 Da on stained SDS-PAGE. This was shown on the enzyme purified by hydroxyapatite or Octyl-Sepharose as the final chromatographic step. Inositol was identified and quantified on the purified enzyme in several separate experiments. The derivatized inositol peak had two ionized mass-numbers characteristic of inositol hexaacetate: 168 and 210. A small amount of the acetate ester of mannose also was noted. Upon analysis for inorganic phosphate of the inositide fraction isolated from native enzyme protein, it was calculated that the enzyme contained about three molecules of inositide per enzyme subunit (Table 1). Phosphate analysis of the native enzyme's inositides after thin-layer chromatographic separation and elution of the spots corresponding to authentic inositide standards showed ≈2 mol PtdIns and ≈1 mol PtdIns 4P per mol enzyme subunit. Next, protein-bound inositides were labeled with ^{32}P by administering 5 mCi of $^{32}P_i$ to a phenobarbital-treated rat 5 h before killing. The enzyme, purified through the hydroxyapatite step, was radioactive. Immunoprecipitation of the enzyme followed by 10% SDS-PAGE in 6 M urea, showed a single radioactive band at 50,000 Da as revealed by autoradiography. The binding between inositides and enzyme, using the ^{32}P labeled enzyme, was also shown to be very tight, because this purified preparation still contained appreciable radioactivity after three successive chloroform/methanol extractions, which was the standard pre-extraction technique used to quantitate inositides. Despite this resistance to chemical or physical dissociation, inositides were extracted quantitatively from the enzyme using acidified chloroform/methanol. After extraction, the protein residue had only 5-10% of the original radioactivity. Thin layer chromatographic analysis of the acidified extract showed that 53% of the radioactivity was present in a spot corresponding to authentic PtdIns 4P, while, the radioactivity present in the spots corresponding to PtdIns and PtdIns 4,5P was 22 and 25%, respectively.

The chromatofocusing profile of the enzyme is presented in Fig. 1. The enzyme eluted at pH 8.2 and showed only a single band on analytical SDS-PAGE. This protein contained only a trace of inositides, 0.3 mol/mol enzyme subunit, and was apparently homogeneous. It had the highest activity of any preparation in low or high ionic strength buffer (Table 2.). Another major catalytic difference was observed between inositide-containing and inositide-depleted enzyme, viz., the former required higher ionic strength buffer for maximal activity (Table 2.). The inositide-containing enzyme's activity was 1,200 nmol styrene oxide hydrolized/min/mg protein in high ionic strength buffer, but only 35 nmol styrene oxide hydrolized/min/mg protein in low ionic strength buffer (Table 2.).

In several experiments, an attempt was made to convert the inositide-depleted form to behave in the manner of the inositide-containing form by reconstituting the deficient protein with 2 mol PtdIns and 1 mol PtdIns 4P per enzyme subunit (phosphatides added in phosphatidyl choline containing liposomes). This preparation still behaved like the inositide-free enzyme.

DISCUSSION

This study demonstrates (a) three inositides are tightly bound to rat liver microsomal epoxide hydrolase, (b) these inositides can be removed almost completely by incubation at pH 9.2 followed by chromatofocusing, and (c) the enzyme

TABLE 1. Tightly-bound inositide content of rat liver microsomal epoxide hydrolase.

Enzyme Form	Purification Method	Inositol Content (mol/mol enzyme)	Number of Experiments
Inositide depleted	Chromatofocusing[a]	0.3 ± 0.1	1
Inositide containing	Hydroxyapatite[a]	2.7 ± 0.3	1
Inositide containig	Octyl Sepharose[a]	2.5 ± 0.5	2
Inositide containing	Hydroxyapatite[b]	3.0 ± 0.1	2

[a] From two determinations on the preparation from the livers of rats fed phenobarbital. Duplicate assays, mean ± SDM.
[b] From two determinations on the preparation from control livers. Duplicate assays, mean ± SDM.

TABLE 2. Activity of inositide-containing and inositide-depleted microsomal styrene oxide hydrolase.

Enzyme Form	Low Ionic Strength[a] Tris.HCl, 5.0 mM	High Ionic Strength[a] Tris.HCl, 250 mM	High Ionic Strength[a] KPO_4, 250 mM
Inositide-depleted	1200 ± 80	1600 ± 150	1500 ± 100
Inositide-containing	35 ± 5	670 ± 50	590 ± 50

[a] The average of three determinations, ± S.D.M. Activity is nmol styrene oxide hydrolized/min/mg protein.

depleted of inositides is more active as a styrene oxide hydrolase than the native enzyme, whether purified by hydroxyapatite or Octyl-Sepharose. On the other hand, phenobarbital induction of epoxide hydrolase caused no observable change in the amounts of tightly-bound inositides. The tight association of 2 PtdIns and 1 PtdIns 4P isolated along with the purified enzyme could occur through three hydrophobic protein pockets, which may bind the inositide fatty acids onto the protein. LeBaron and coworkers (*13*) suggested that the protein-associated inositides in membrane structures of white matter in the brain might act as bridges between proteins and closely associated lipids. Though further work is needed to establish the exact nature of linkage between inositides and microsomal epoxide hydrolase protein, the data clearly suggest that the association between them must be very tight. First, only acidified chloroform/methanol was capable of separating inositides from protein. Second, immunoprecipitation of the 32 P labeled enzyme followed by SDS/PAGE in 6M urea yielded radioactivity only at the 50,000 Da band position. Thus reduction with mercaptoethanol did not dissociate the inositides. And third, the ratio of inositide to enzyme subunit was repeatedly 3:1.

Incubation at pH 9.2 released active enzyme suggesting that mild alkaline hydrolysis was sufficient to release the inositides, so the enzyme could be separated by chromatofocusing. One candidate for such a direct linkage is an ester between an enzyme free-carboxylic acid group and a hydroxyl group on inositol. There is a precedent for an ester linkage between the hydroxyl groups of inositol and a carboxylic acid group in biological systems. Inositol ester derivatives of angelic acid (*14*) and indol acetic acid (*15*) have been reported in plants. Rabbit liver microsomal epoxide hydrolase has been sequenced (*16*), and this enzyme shares similar N-terminal sequence homology for at least 20 residues with the rat liver enzyme (*17*). Antiserum to either species cross-reacts with the other (*18*). It is not unreasonable to suppose that the same three hydrophobic regions which are found in the rabbit enzyme (*16*) also occur in the rat protein. If one includes residue #3 (glu) in the N-teminal hydrophobic sequence (pocket), there is also one glu as the only polar amino acid in the next two hydrophobic sequences in the rabbit enzyme (*16*). This makes glutamic acid's gamma carboxygroup a candidate for three covalently attached inositide, through an ester bond to inositol. Phosphatidyl inositol has been reported as a component of an anchor, attaching plasma membrane proteins to some mammalian outer membranes (*19*). A review lists 15 such proteins and describes the N-terminal anchor array as follows: the protein is attached to ethanolamine, which is attached to a glycan (mucin), which in turn is attached to inositides (*20*). The fatty acid esters of the inositide dissolve in plasma membrane phospholipid to complete the anchoring (*21*). The endoplasmic reticulum localized epoxide hydrolase has three inositides attached, but the only other moiety we observed was (about) one mannose per subunit, which had been observed previously (*22*).

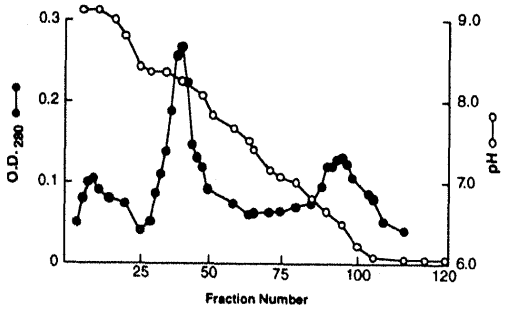

Figure 1. Chromatofocusing profile. The gradient (pH 9.2 to 6.0) was established using Polybuffer 76 and epoxide hydrolase activity was eluted between pH 8.3 and 8.0 (fractions 33-47). Inset: SDS-10% polyacrylamide gel electrophoretogram of purified, stained enzyme.

The isolation of an inositide-depleted enzyme allowed comparison of its catalytic behav-

ior with the native enzyme. The inositide-depleted enzyme's styrene oxide hydrolase activity (1,600 nmol/mg/min; Table 2) is 2-fold higher than the inositide-containing enzyme we isolated and purified through hydroxyapatite or Octyl-Sepharose chromatography (670 nmol/mg/min; Table 2). Thus rat liver microsomal epoxide hydrolase appears to be naturally regulated by bound inositides. Another difference we have quantitated is the partial requirement for higher ionic strength for maximal activity of the inositide-containing, purified enzyme. A report relates how phosphorylation of an associated inositide of sarcoplasmic reticulum Ca^{++}-transport ATPase activates this enzyme (*23*). Phosphaticlyl inositol activates a vascular smooth muscle calcium-dependent protease (*24*). Microsomes from mouse livers labeled *in vivo* with radioactive inorganic phosphate, provided SDS-PAGE patterns in which phosphorylation of protein bands (autoradiography) was resistent to chloroform/methanol, but was extractable with acidified chloroform/ methanol (*25*).

What these bound inositides' physiological role are, remains to be established. Phosphoinositides have an important regulatory role in interactions between membrane proteins (*26*). One role for inositides is as a reservoir for long chain fatty acids, whose metabolism results in prostaglandins and leukotrienes. Epoxide hydrolase may have associated inositides to attach epoxidized long chain fatty acids to accelerate hydrolysis to their dihydrodiols. For example, a major lipid metabolite, the epoxide of oleic acid, is a substrate for the microsomal enzyme, which converts it to its dihydrodiol, a major step in eliminating the oxidized product from the cell.

How the specific inositide distribution is arranged on the two CM-52 separable forms of microsomal epoxide hydrolase enzyme (*5,6*) and on the "Preneoplastic antigen" form(s) of the enzyme (*27*) remains to be investigated. Preliminary reports of inositides bound to rat liver epoxide hydrolase have been published (*28*).

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