The Role of Phospholipid in the Multiple Functional Forms of Brain Monoamine Oxidase*

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The nature of phospholipid requirement and lipid-protein interactions for the multiple functional forms of monoamine oxidase was investigated by rebinding the purified phospholipid to a lipid-depleted brain mitochondrial preparation. It was found that phosphatidylinositol uniquely stimulated the monoamine oxidase A activity to 80% over that in the original intact mitochondria. Other negatively charged phospholipids, although not as potent, could fully or partially re activates the A or the B activity. Phosphatidylerine was relatively more effective in restoring the B activity. Phosphatidylcholine, a zwitterionic phospholipid, reconstituted 70% of the A activity but did not influence the B. More importantly, efficiency-gradient analyses indicated a distinct nature in the mechanism of lipid-protein interactions for the negatively charged and the zwitterionic phospholipids. The potency of the negatively charged phospholipid decreased sharply with increasing lipid molecules. No further stimulation could be detected when the lipid to protein ratio reached about 30 molecules of the negatively charged phospholipid for 100,000 daltons of membrane protein. The negatively charged phospholipid appeared to bind directly to the monoamine oxidase protein boundary with a high affinity. Phosphatidycholine might reassociate as the membrane fluid bilayer, which in turn modulated the monoamine oxidase A activity.

The oxidative deamination of biogenic monoamines is accomplished by two functionally different forms of monoamine oxidase (EC 1.4.3.4) (1). Monoamine oxidase A type preferentially deaminates serotonin and is more sensitive to inhibition by clorgyline. Monoamine oxidase B type deaminates phenylethylamine and is more sensitive to inhibition by deprenyl. The monoamine oxidase enzyme, an intrinsic membrane flavoprotein, has been localized in the outer mitochondrial membrane (2). Attempts to separate and purify the different types of monoamine oxidase activity from tissues have involved solubilization and/or lipid extraction procedures which invariably inactivate the A type activity preferentially (3-5). It has also been demonstrated that a number of the electrophoretically separable monoamine oxidase forms, treated with chaotropic agents to remove the phospholipids, converged to a single monoamine oxidase B type by the criteria of both polyacrylamide gel electrophoretic mobility and substrate-inhibitor specificity (6). Furthermore, that the multifunctional monoamine oxidase from rat and human liver may be a single gene product has been substantiated by immunochemical studies (3). The A and B type activities may reside within the same monoamine oxidase enzyme protein. The apparent specificities associated with the multiple functional forms of monoamine oxidase \textit{in situ} may depend upon the different natures of lipid-protein interactions in the membrane (7-9). Recently it was also suggested that distinct enzyme molecules may be associated with the monoamine oxidase A and monoamine oxidase B activities in rat hepatoma line, MH;Cg, and rat glioma line C2 using the technique of limited proteolytic digestion and peptide mapping (10). However, none of the observation has elucidated conclusively the molecular basis of the differences between the two monoamine oxidase types. It has not been clear whether these multiple functional forms represent different enzymatic proteins or the same enzymatic protein of identical conformation with a different spectrum of lipid association or the same enzymatic protein exists in the membrane as two conformational isomers which interact with the membrane lipid distinctly. Refolding of the bovine pancreatic trypsin inhibitor protein has resulted in substantial quantities of a metastable folded species which is identical with native protein but possesses a somewhat different conformation (11).

In this report, highly purified intact rat brain mitochondria were digested with phospholipase A and extensively washed with medium containing fatty acid-free bovine serum albumin. The procedure removed almost 70% of the phosphatides with concomitant inactivation of about 50% of both the monoamine oxidase A and monoamine oxidase B type activities (9). The interactions between the phospholipids and the exposed hydrophobic lipid-binding areas on the surface of the monoamine oxidase protein were examined systematically by adding back the various classes of purified phospholipids.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The chemicals used in this work were obtained as follows: $\beta$-[1,14C]phenylethylamine hydrochloride and $\beta$-hydroxy[14C]tryptamine binoxalate from New England Nuclear; phospholipase A$_1$ (porcine pancreas) from Sigma; purified phospholipids including bovine heart cardiolipin and phosphatidylcholine, bovine brain phosphatidylethanolamine and phosphatidylinserine, and bovine liver phosphatidylinositol from Avanti; other chemicals were obtained from standard commercial sources.

Examination of the phospholipids by the two-dimensional thin layer chromatographic procedure of Horrock’s (12) (nonactivated Brinkmann silica gel G plates without fluorescent indicator using solvent system 1 (chloroform/methanol/ammonia, 65:25:4) and solvent system 2 (chloroform/methanol/acetic acid/water, 75:15:30:15:7.5) followed by visualization after spraying with 50% H$_2$SO$_4$, and charring at 180 °C) indicated that each compound was essentially pure.

**Enzyme Preparation**—Pure intact rat brain mitochondria were prepared by purifying crude mitochondrial fractions from rat brain (male Sprague-Dawley, weight 120-180 g) utilizing a discontinuous
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Ficoll density gradient (3% and 6%) procedure (13). The purification was determined by electron microscopy to be intact and contained at least 90% mitochondria with minute synaptosomal contamination (8). Depletion of Mitochondrial Phospholipids with Phospholipase A2 Digestion—Three volumes of intact rat brain mitochondrial suspension in an isotonic medium (0.25 M sucrose and 10 mM Tris, pH 7.4) was added to 1 volume of phospholipase A2 solution (porcine pancreas) at a ratio of 20 mg of mitochondrial protein/mg of phospholipase A2 protein. CaCl2 was added to give a final concentration of 10 mM. The mixture was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.08 M EDTA to a final concentration of 18 mM. The phospholipase A2-treated mitochondria were centrifuged at 40,000 × g. The pellet was then washed four times with a 10% solution of bovine serum albumin (fatty acid-free) in 30 mM Tris, pH 7.4, containing 5 mM dithiothreitol, 1 mM EDTA, and 0.25 mM sucrose to remove the liberated fatty acids and lyso-derivatives. The inactivation of the monoamine oxidase enzyme has been demonstrated to be independent of the production of these end products (9). It was also shown that the phospholipase A2 did not contain any significant proteolytic activity (9). The pellet was then washed twice again with a 30 mM Tris solution, pH 7.4, containing 5 mM dithiothreitol, 1 mM EDTA, and 0.25 mM sucrose. After washing, the pellet was divided into aliquots and stored at −80 °C. Inactivation of monoamine oxidase A type activity (using serotonin as substrate) and monoamine oxidase B type activity (using phenylethylamine as substrate) to about 50% was achieved after the lipase A2 digestion under the experimental conditions. The loss of enzymic activities was parallel to the hydrolysis of almost 90% mitochondria with minute synaptosomal contamination (8). A

Table 1

<table>
<thead>
<tr>
<th>Average unsaturation per acyl group</th>
<th>Average chain length per acyl group</th>
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<tbody>
<tr>
<td>Phosphatidylserine</td>
<td>0.41</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>1.92</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1.95</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>2.08</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1.06</td>
</tr>
</tbody>
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incorporation of phospholipid into the monoamine oxidase-membrane system occurred only in the lipid-depleted state. In contrast to a similar average chain length, the degree of unsaturation varied among the acyl groups of the purified phospholipids (Table I). Activation of the lipid-depleted monoamine oxidase by phospholipid was a rapid event. The activity of the activated enzyme was linear with time for over 20 min at 37 °C. Effect of the Negatively Charged Phospholipids—Phosphatidylserine, cardiolipin, or phosphatidylinositol activated both the A and the B type activities in qualitatively and quantitatively distinct dose-dependent manners (Fig. 1, A–C). In the rat brain mitochondria, PI, PS, and CL constitute about 5%, 6%, and 14% of the total phospholipids (9). The maximum level of activation achieved in the presence of PS, CL, and PI was 0.73, 0.90, and 1.85 for the monoamine oxidase A type activity and 1.0, 0.9, and 1.0 for the monoamine oxidase B type activity, respectively (Fig. 1, A–C). 1.0 unit was normalized to represent 100% reconstitution to the original activity in the intact rat brain mitochondria. PS preferentially activated the B type activity (Fig. 1A). In the presence of 0.4 mg of PS/mg of protein, the monoamine oxidase B type activity was completely recovered, whereas 10-fold higher lipid concentrations were required to reconstitute 70% of the A type activity (Fig. 1A). CL activated both types similarly (Fig. 1B). The most dramatic effect was observed in the case of PI. At the level of 0.18 unit of PI, the original intact level of A type activity was regenerated (Fig. 1C). The concentration of 0.18 unit of PI was equivalent to the binding of 23 lipids to a lipid-depleted protein of an average molecular weight of 100,000 daltons if all of the liposomal lipids present were bound to membrane proteins. Furthermore, the monoamine oxidase A type activity could be stimulated to 80% over and above the original control value by PI. Although PI was not as effective with the B type, the B activity was 100% reconstituted at higher lipid concentrations (Fig. 1C). At high levels of PS or CL (~4 units), these phospholipids might be slightly inhibitory to the B type activity (Fig. 1, A and B). The efficiency gradient, in stimulating the functional activities of the lipid-depleted monoamine oxidase by the purified lipid, was analyzed using the first derivative plot of the corresponding dose-response curve (Fig. 2, A and B). The first derivative plots obtained from the negatively charged phospholipids indicated: 1) the efficiency gradient decreased extremely rapidly with increasing amounts of lipids (Fig. 2, A and B); 2) the potency dropped to zero level when more than 30 liposomal lipid molecules were available to each protein molecule (Fig. 2, A and B); 3) the negatively charged phospholipids might bind directly to the exposing hydrophobic surface of the lipid-depleted monoamine oxidase protein with a high affinity and a limited capacity; 4) the rank order of the

1 The abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
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A

FIG. 1. Effect of the negatively charged phospholipids on the activation of monoamine oxidase activity. ○, Serotonin as substrate; ×, phenylethylamine as substrate. The values represent the average and standard deviations of three determinations. The lipid-depleted mitochondria were mixed with varying amounts of freshly prepared liposomes made of purified phospholipids (0.07 M phosphate buffer, pH 7.4). The mixture was incubated at 37°C for 20 min prior to monoamine oxidase assay as described under "Experimental Procedures." The normalized specific activity represents the ratio of monoamine oxidase activity of the lipid-depleted mitochondria in the presence of liposomal phospholipids to that of the intact rat brain mitochondria. A, phosphatidylserine; B, cardiolipin; C, phosphatidylinositol.
relative potency toward stimulating the apparent A type activity for the first 5 lipid molecules present was found to be $\text{PI} \gg \text{CL} \gg \text{PS}$ (Fig. 2A); and 5) the corresponding sequence of descending potency toward the B type was $\text{PS} > \text{PI} = \text{CL}$ (Fig. 2B).

**Effect of the Zwitterionic Phospholipids**—The response of the lipid-depleted monoamine oxidase to the addition of purified phosphatidylcholine or phosphatidylethanolamine was markedly different from that observed in the case of the negatively charged phospholipids. In the rat brain mitochondria, PC and PE account for 75% of the total membrane phospholipids (9). PC was effective in restoring up to 70% of the original monoamine oxidase A type activity at relatively high lipid levels, but did not influence the B type activity (Fig. 3A). PE did not show any effect on the A or the B type activity over the entire range of lipid concentrations examined (Fig. 3B).

The efficiency gradient of the activation of the monoamine oxidase A type activity remained essentially constant up to the first 150 molecules of PC. At higher levels, when saturation occurred, the potency diminished to zero (Fig. 2A). PC, in contrast to the negatively charged phospholipids, interacted with the monoamine oxidase A enzymic protein with a relatively lower affinity and higher capacity.

**DISCUSSION**

It has been generally recognized that lipid-protein interactions may play a key role in the structural and functional properties of the multiple forms of the membranous monoamine oxidase (6-9). The hypothesis that phospholipids may be involved as regulators of the functional activities and specificities by inducing the proper conformation in the monoamine oxidase enzymic protein is most stimulating. The precise molecular nature remains to be determined.

Our measurements on the effect of the lipid at the functional level demonstrate that the hydrophobic surface of the monoamine oxidase protein selectively interacts with the negatively charged phospholipids. Although PC may reconstitute a fraction of the original monoamine oxidase A activity, the negatively charged PS characterized with similar chain length and unsaturation could activate both the monoamine oxidase A and the monoamine oxidase B more effectively. Furthermore, the efficiency gradient analyses indicate that marked distinct mechanism of lipid-protein interactions may be involved for the PS and the PC. That the high initial potencies observed for all of the negatively charged phospholipids rapidly diminish to zero at the lipid to protein ratio of 30 strongly suggests a local and short ranged interaction of high affinity and low capacity. The negatively charged phospholipid may be involved as the bound lipid at the lipid-protein interface. The preference for the negatively charged lipid at the lipid-protein interface was also demonstrated at the structural level in the membranous Na+, K+-ATPase system by using the ESR technique which distinguishes between the bound lipid label and the bilayer component (19). Interestingly, the number of

![Figure 2](image_url)

![Figure 3](image_url)
lipid molecules in direct contact with the calcium-dependent ATPase, a rhodopsin monomer, and the trimeric bacteriorhodopsin have also been roughly calculated to be 25–30, 24–28, and 25–28, respectively (20–22). In contrast, PC, a zwitterionic phospholipid, may reassociate as the membrane fluid bilayer which in turn modulates the monoamine oxidase A type activity. Previously, we have demonstrated that the functional state of the monoamine oxidase A, but not that of the monoamine oxidase B, was dependent upon, in part, the fluidity of the bulk membrane bilayer region (8, 9).

The negatively charged phospholipids, the degree of unsaturation of the acyl moiety for the CL is comparable to the fluidity of the bulk membrane bilayer region (8). The PI, whereas that of the PS is considerably lower.

The effect described in this report is of particular interest. Turnover of PI has long been recognized to occur in many tissues in response to a variety of stimuli (23). Within neuronal tissue, PI which is roughly at 20–100 μg/ml represents 2–10% of the total phospholipid (24). Recently it has been suggested that PI may be a potent modulator of tyrosine hydroxylase activity (24).

Our data suggest that the chemical structure of the polar head group of the negatively charged phospholipid may be important to the lipid-protein interactions at the protein boundary in the region where the polypeptide emerges from the lipid bilayer. It is of interest in this regard that recent sequence data on glycophorin, histocompatibility antigens HLA-A and HLA-B2, the coat protein of the filamentous phage fd, and one of the smallest polypeptides of cytochrome oxidase indicated the presence of several arginines and lysines at the cytoplasmic end of a hydrophobic stretch at the COOH termini of these polypeptides (25–29).

If the multiple functional forms of brain monoamine oxidase play a role in regulating the putative neurotransmitters in vitro, it can be hypothesized that direct manipulation of the composition and distribution of the membrane phospholipids might be the underlying mechanism. Proteins which catalyze the exchange of phospholipids between membranes have been isolated from a number of sources and have been used to study the distribution and transbilayer movement of PC or PI in artificial and biological membranes (30–32). A mechanism for an enzyme-mediated flip-flop, i.e. enzyme synthesis and rapid translocation of PC by two methyltransferases, has also been studied and demonstrated in many biomembranes to be functionally important (33). It can be speculated that the properties of the negatively charged phospholipid associated with the enzymic protein at the lipid-protein interface may regulate, rapidly, the conformation of the active site which in turn influences the nature of substrate and inhibitor specificity of monoamine oxidase. In addition, the dynamic redistribution of PC might further affect the functional level of the monoamine oxidase A type activity.

Studies on the structural nature of the lipid-protein interactions in the monoamine oxidase system are currently under way.

REFERENCES