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A Photoactivable Phospholipid Analogue That Specifically Labels Membrane Cytoskeletal Proteins of Intact Erythrocytes†

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ABSTRACT: A radioactive photoactivable analogue of phosphatidylethanolamine, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine ([^{14}C]AzPE), was synthesized. Upon incubation with erythrocytes in the dark, about 90% of [^{14}C]AzPE spontaneously incorporated into the cells; of this fraction, about 90% associated with the membrane, all of it noncovalently. Upon photoactivation, 3–4% of the membrane-associated probe was incorporated into protein. Analysis of this fraction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as well as extraction of labeled membranes with alkali or detergent, showed that the probe preferentially labeled cytoskeletal proteins. [^{14}C]AzPE appears to be a useful tool for the study of lipid–protein interactions at the cytoplasmic face of the plasma membrane of intact cells.

Molecular analysis of cell structure is heavily dependent on the use of probes which interact with cells in a specific, predictable manner; the site of interaction of probes on or within the cell, monitored by using radioactivity, fluorescence, absorption, electron spin resonance, or other detectable signals, provides information on the molecular constitution and topography of the cell. Photoactivable probes offer the unique advantage that they remain inert until they reach their targeted site, thus reducing nonspecific labeling. In studies of cell membranes, hydrophobic probes of this sort have been used to identify hydrophobic regions of membrane proteins (Bayley & Knowles, 1980; Brunner, 1981; Pradhan & Lala, 1987), and hydrophilic probes have been used to label surface proteins (Staros et al., 1974; Dockter, 1979). Although photoactivable phospholipid analogues have been synthesized for use as amphipathic probes (Bisson et al., 1979; Ross et al., 1982; Burnett et al., 1985), they have been used chiefly to study the interactions of integral membrane proteins with phospholipids; none have been applied to peripheral membrane proteins, which lie in proximity to the membrane. A variety of investigations would profit from the availability of a photoactivable phospholipid probe which could specifically label and thus identify proteins lining the cytoplasmic side of the plasma membrane of intact cells.

The human erythrocyte provides a simple cell in which to test the efficacy of such a probe. In particular, the known arrangement of phospholipids in the erythrocyte membrane facilitates the design of a probe with the desired specificity. Phosphatidylcholine (PC)[1] and sphingomyelin reside chiefly in the outer leaflet of the erythrocyte plasma membrane, whereas phosphatidylethanolamine (PE) is more abundant in and phosphatidylserine (PS) is restricted to the inner leaflet of the bilayer (Bretscher, 1973; Op den Kamp, 1979). Targeting a phospholipid-based probe to the internal surface of the plasma membrane, therefore, requires that the photoactivable phospholipid have either an ethanolamine or a serine as the headgroup.

The probe must also possess several other important characteristics. First, it should spontaneously partition into the plasma membrane from the aqueous medium. Since attachment of an aromatic moiety directly to the C2 position of the glycerol backbone facilitates transfer of phospholipids into cells (Bisson & Montecucco, 1985), attachment of an aromatic photoactivable group at this point should enhance uptake. Second, once inserted into the plasma membrane, the probe should migrate to its cytoplasmic side. If the photoactivable group is small, the analogue will resemble a lysophospholipid, which is translocated across the bilayer more readily than the parent phospholipid (Bergmann et al., 1984). In addition, the erythrocyte membrane is equipped with an activity which transports aminophospholipids from the outside to the inside of the membrane (Seigneuret & Devaux, 1984; Zachowski et al., 1986). If this activity applies to a PE-derived probe, net inward movement of the molecule would be favored. Third, if the proteins in apposition to the inner surface of the membrane are to be labeled, the photoactivable moiety should be

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[1] Abbreviations: AzBA, 2-azido-4-nitrobenzoic acid; AzPC, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phosphocholine; [^{14}C]AzPE, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

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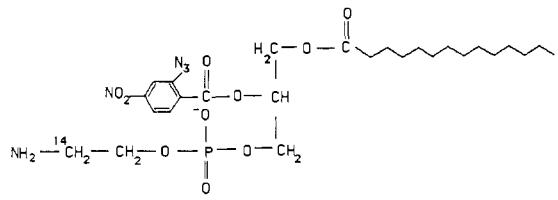

FIGURE 1: 2-(2-Azido-4-nitrobenzoyl)-1-myristoyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine ([^{14}C]AzPE).

presented at the interface of the membrane with the cytoplasm once the probe reaches the inside of the cell. Since the carbonyl group at the C2 position of the glycerol backbone is elevated out of the membrane (Strenk et al., 1985), a small and reasonably hydrophilic group attached at this carbonyl should position itself near the aqueous interface.

The molecule 2-(2-azido-4-nitrobenzoyl)-1-myristoyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine ([^{14}C]AzPE) (Figure 1) meets all of these criteria. It possesses an ethanolamine headgroup and an aromatic photoactivable moiety attached directly at the C2 position. We present here analyses of the interaction of this probe with erythrocytes, and particularly its specificity for peripheral proteins lining the cytoplasmic face of the membrane.

Materials and Methods

Chemical Synthesis. All chemicals and solvents used were of reagent grade. 2-Amino-4-nitrobenzoic acid was purchased from Aldrich and recrystallized from water (mp 268 °C). 1-Myristoylglycerophosphocholine was purchased from Sigma, carbonyldiimidazole from Aldrich, and radiolabeled ethanolamine from Amersham.

2-Azido-4-nitrobenzoic acid (AzBA) was synthesized from 2-amino-4-nitrobenzoic acid according to the procedure of Bisson and Montecucco (1981). All operations involving the azido compound were conducted in the dark or using a safelight. 2-(2-Azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phosphocholine (azidophosphatidylcholine; AzPC) was synthesized by coupling AzBA to 1-myrisotylglycerophosphocholine according to the procedure of Warner and Benson (1977). Briefly, the imidazole derivative of AzBA was prepared in freshly distilled DMSO by stirring under a stream of dry nitrogen a mixture of AzBA (11.22 mg, 56.109 μmol) and carbonyldiimidazole (10 mg, 61.72 μmol). The resulting azidobenzoylimidazolide was then added to a mixture of 1-myristoylglycerophosphocholine (12.5 mg, 25 μmol) and sodium dimethylsulfenamide (ca. 440 μmol). The final product, AzPC, was purified by elution from a silica gel column in $CHCl_3/CH_3OH$ (9:1 v/v) with a 60% yield. Purity was assessed by TLC ($CHCl_3/CH_3OH/H_2O$, 65:36:4) or normal-phase HPLC; a single product was seen. The UV spectrum of the product was also recorded and matched that reported (Bisson & Montecucco, 1981).

Transphosphatidylation of AzPC to azidophosphatidylethanolamine (AzPE) using [2-^{14}C]ethanolamine (50 mCi/mmol) was done by the procedure of Smith et al. (1978) followed by purification on a silica gel column with a 38% yield (48.2 mCi/mmol). The product was analyzed by TLC (s/s $CHCl_3/CH_3OH/H_2O$, 65:36:4; R_f 0.36) on plastic-backed silica gel plates. The plate was sliced, and the slices were counted for radioactivity, showing the compound to be greater than 98% pure. When stored under nitrogen dissolved in $CHCl_3/CH_3OH$ (9:1 v/v) at −20 °C in the dark, [^{14}C]AzPE was stable for up to 2 months. ([^{3}H]AzPE, synthesized by similar methods, was stable only up to 2 weeks.) Prior to incubation with cells, solvent was completely evaporated, and the residue was dissolved in ethanol.

Incorporation of [^{14}C]AzPE into Erythrocytes. Freshly collected heparinized human blood was centrifuged at 500*g* at 4 °C, the buffy coat removed, and the erythrocyte pellet washed several times in phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM KCl, 7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, pH 7.4, and 5 mM dextrose).

AzPE dissolved in 10 μL of ethanol was added to 10^9 erythrocytes in 1 mL of PBS, and the suspension was gently vortexed and incubated at 37 °C in the dark. Following incubation, samples to be photolyzed were irradiated for 10 min in a Rayonet Mini Reactor, RMR-500, equipped with four medium-pressure mercury lamps (300 nm). In some experiments, reduced glutathione (50 mM) was added to the suspension and incubated at 37 °C for 5 min prior to photolysis.

Analysis of Probe Location. Unphotolyzed or photolyzed samples were centrifuged at 4 °C at 500*g*, and an aliquot of the supernatant was counted in a Beckman LS 5801 scintillation counter. The pellet was washed 3 times with 10 volumes of PBS containing 0.5% BSA (w/v) and lysed in 10 volumes of hypotonic buffer (4.66 mM Na_2HPO_4 and 0.34 mM NaH_2PO_4, pH 8.0, containing 0.1 mM EGTA and 1 mM PMSF); the membranes were pelleted by centrifugation at 15000*g* at 4 °C for 20 min and washed 5 more times with the same buffer. Both the pellet and the supernatant were extracted 4 times with 1.5 volumes of solvent ($CHCl_3/CH_3OH$, 2:1 v/v), and aliquots from these extractions were counted. The aqueous fraction from photolyzed samples was concentrated for analysis by SDS–PAGE using a Centricon-10 microconcentrator.

Photolabeled membranes were treated with alkali by the method of Steck et al. (1976). Briefly, membranes were incubated with 0.1 M NaOH on ice for 1 min and then centrifuged for 30 min at 15000*g* at 4 °C. The supernatant containing the proteins released by the treatment was neutralized by adding 0.1 M HCl dropwise, concentrated by using a Centricon-10 microconcentrator, and analyzed by SDS–PAGE.

Photolabeled membranes were treated with 0.5% Triton X-100 in 10 mM Tris-HCl, pH 7.4, for 15 min at 4 °C and then centrifuged at 15000*g* for 20 min. The pellet was washed 5 times with 10 mM Tris-HCl, pH 7.4, and then analyzed by SDS–PAGE.

SDS–PAGE Analysis of Membrane Proteins. Washed photolabeled membrane fractions, including those obtained from alkali and detergent extractions, were solubilized in SDS sample buffer and analyzed by SDS–PAGE on 5.6% gels using the Laemmli system (1970). The gels were then stained with Coomassie Blue and cut into 2-mm slices. Slices were solubilized overnight in 3% Protosol (Du Pont) at 70 °C and then counted in 10 mL of EcoScint (National Diagnostics).

Results

Uptake of [^{14}C]AzPE by Erythrocytes. The lysophospholipid-like character of AzPE should permit incorporation of the probe into cells by exchange through the aqueous medium. To determine the kinetics of probe uptake, a suspension of washed erythrocytes was incubated with [^{14}C]AzPE in the dark at 37 °C for varying periods of time, the cells were pelleted by centrifugation, and aliquots of the supernatant were counted to determine the fraction of the added probe bound by the cells. As seen in Figure 2a, after a brief lag, uptake of probe by erythrocytes was roughly linear with time to a maximum of 91% of added label incorporated after 2.5-h incubation. These kinetics are comparable to the 3–5 h required for uptake by human erythrocytes of exogenously added

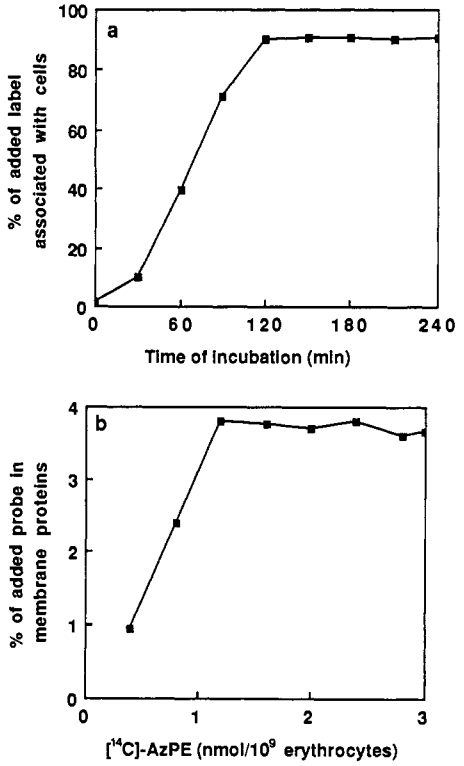

FIGURE 2: Association of [^{14}C]AzPE with erythrocytes. (a) Erythrocytes (10^9/mL) were incubated with [^{14}C]AzPE at 1.2 nmol/mL for increasing amounts of time in the dark at 37 °C. Cell-associated radioactivity was determined as the difference between the radioactivity added and that remaining in the supernatant after removal of cells by centrifugation. (b) Erythrocytes (10^9/mL) were incubated with increasing amounts of [^{14}C]AzPE (0.5–3 nmol/mL) for 2.5 h and then irradiated. The photolabeled cells were lysed, membranes were extracted with chloroform/methanol, and the aqueous phase was counted to determine the amount of probe in membrane proteins.

Table I: Distribution of [^{14}C]AzPE in Erythrocytes before and after Photolysis

	% of added probe[b]			
	membrane, chloroform/methanol		cytosol, chloroform/methanol	
treatment[a]	soluble	insoluble	soluble	insoluble
none	81.0 ± 3.8	0.7 ± 0.05	8.80 ± 0.04	0.07 ± 0.03
photolysis	78.4 ± 1.4	3.4 ± 0.9	0.06 ± 0.02	8.70 ± 1.1
GSH, photolysis	76.8 ● 1.6	3.2 ● 1.1	0.05 ± 0.02	8.20 ± 1.4

[a] For all samples, 10^9 erythrocytes/mL were incubated with 1.2 nmol/mL [^{14}C]AzPE at 37 °C for 2.5 h in the dark. Photolysis samples were then irradiated for 10 min, with or without reduced glutathione (GSH). Fractionation and extraction were performed as described under Materials and Methods. [b] Mean ± SD for five separate experiments.

lyso-PE, which [^{14}C]AzPE resembles (Bergmann et al., 1984).

To determine the cellular distribution of cell-associated probe, cells were labeled for 2.5 h in the dark, washed, lysed in hypotonic buffer, and centrifuged, and aliquots of the pellet (membrane fraction) and supernatant (cytosolic fraction) were counted for radioactivity. Approximately 90% of the cell-associated label was found in the membrane fraction and the balance in the cytosolic fraction (Table I). A similar distribution was obtained with samples taken throughout the kinetic curve shown in Figure 2a (data not shown). To ascertain whether the probe spontaneously associates with protein, both membrane and cytosolic fractions were extracted with chloroform/methanol. For both fractions, >99% of the radioactivity was extracted into the organic phase (Table I), implying that the probe had not been incorporated into protein.

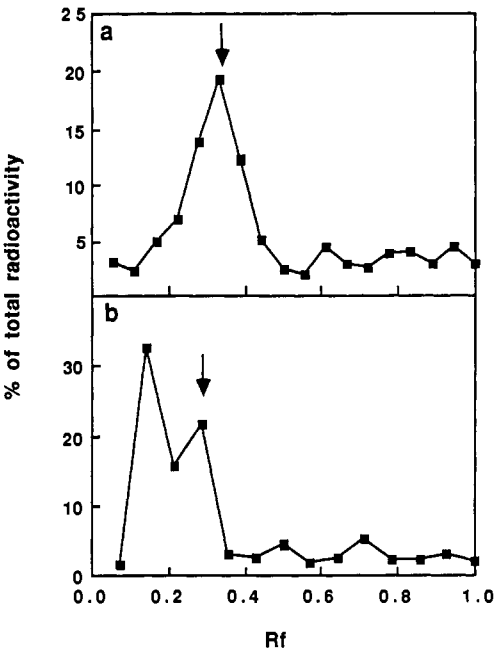

FIGURE 3: Thin-layer chromatograms of probe extractable from membranes with chloroform/methanol. Erythrocytes (10^9/mL) were incubated with probe (1.2 nmol/mL) for 2.5 h at 37 °C. Membranes from unirradiated cells (a) as well as irradiated cells (b) were extracted with chloroform/methanol, and the organic phase was analyzed by TLC. Arrows indicate the migration of authentic [^{14}C]AzPE.

Radioactivity in the organic phase from both membrane and cytosolic fractions comigrated with authentic [^{14}C]AzPE when analyzed by thin-layer chromatography (Figure 3a), indicating that the probe was not chemically modified during the incubation with cells. These results imply that radioactivity associated with protein following photoactivation arises entirely from cross-linking with the photoactivated species, with no contribution from any inherent chemical reactivity of the probe.

Covalent Insertion of [^{14}C]*AzPE into Erythrocytes.* Having shown that [^{14}C]AzPE can be specifically inserted into the plasma membrane of erythrocytes, remaining inert during incubations over several hours, the nature of the reactions induced when the incorporated probe is photoactivated by illumination with 300-nm light was investigated. Cells incubated with probe were illuminated and then fractionated into cytosolic and membrane fractions as above. As was the case with nonphotolyzed cells, approximately 90% of the label associated with photolyzed cells was found in the membrane fraction. After photolysis, however, the probe in both fractions was no longer entirely extractable with chloroform/methanol (Table I). In the cytosolic fraction, more than 90% of the probe was recovered in the aqueous fraction following extraction. Virtually all (98%) of the radioactivity in this aqueous fraction could be precipitated by addition of either acetone or TCA, suggesting that the probe was covalently inserted into protein.

In the membrane fraction of photolyzed cells, about 90% of the radioactivity remained chloroform/methanol extractable. When this material was examined by thin-layer chromatography, however, label comigrating with [^{14}C]AzPE was accompanied by several new species (Figure 3b), which may represent probe covalently cross-linked to phospholipids or to itself. In addition to this solvent-soluble material, approximately 4% of the radioactivity associated with the membranes of photolyzed cells was recovered in the aqueous fraction following solvent extraction, suggesting that the probe had been

Table II: Solubility of Label in Membrane Protein Fraction

treatment of photolabeled membrane	% of radioact.[a] in soluble fraction	% of radioact.[a] in insoluble fraction
NaOH	76.2 ± 1.5	23.9 ± 0.3
Triton X-100	21.2 ± 0.9	78.1 ± 2.1

[a] Amount present in the protein portion of the membrane prior to treatment. Mean ± SD for four separate experiments.

covalently inserted into protein. As in the case of cytosolic material, almost all (>98%) of the radioactivity in the aqueous fraction after extraction was precipitated with either acetone or TCA.

To optimize conditions for the covalent labeling of membrane proteins, varying amounts of probe were incorporated into cells and then measurements made of the amount of radioactivity remaining in the aqueous phase after solvent extraction of photolabeled membranes. While adding more probe increased protein labeling over a broad range of probe concentrations, maximum incorporation as a fraction of probe added was achieved at 1.2 nmol of [^{14}C]AzPE per 10^9 erythrocytes, or approximately 1 molecule of probe for every 300 molecules of phospholipid (Figure 2b). When the time dependence for covalent protein labeling was measured, no significant increase in insertion was observed after 10 min of photolysis (data not shown). On the basis of these findings, subsequent experiments were carried out with 10^9 erythrocytes incubated with 1.2 nmol of probe for 2.5 h in the dark at 37 °C, followed by 10 min of photolysis.

Location of [^{14}C]AzPE in Cells. Although these data indicate that the probe is capable of labeling proteins, the location from which the label is inserted is not clarified. The presence of the PE headgroup in the phospholipid analogue would suggest that the probe should be on the inner surface of the bilayer. To determine whether this was in fact the case, advantage was taken of the highly electrophilic nature of the nitrenes generated by photoactivation, which are very easily scavenged in a reducing environment. Cells incubated with probe were photolyzed in the presence of exogenously added reduced glutathione, a membrane-impermeant reducing agent. Under these conditions, externally located probe will be quenched (Bayley & Knowles, 1978) and therefore unable to label external membrane proteins. As seen in Table I, addition of the reduced glutathione had no effect on labeling of either lipid or protein, suggesting that the labeling observed results from probe located in the internal leaflet.

The nature of the proteins labeled by the probe was addressed first by using differential extraction procedures to separate integral membrane from cytoskeletal proteins. Two such procedures were used: NaOH extraction of membranes, which solubilizes cytoskeletal proteins into the extract, leaving the integral proteins in pelletable form in the membrane; and Triton X-100 extraction, which solubilizes integral membrane proteins, leaving behind the cytoskeletal proteins in the form of a sedimentable "Triton shell". As shown in Table II, alkali extraction of photolyzed membranes resulted in 80% of the radioactivity appearing in the soluble extract (cytoskeletal fraction), whereas 80% of the radioactivity pelleted following Triton extraction of labeled membranes. Together, these data suggest that [^{14}C]AzPE labeling displays a high preference for cytoskeletal over integral membrane proteins.

SDS-PAGE Analysis of Membrane Proteins. Because the nature of the cytoskeletal proteins in erythrocytes is well established, this apparent preference of [^{14}C]AzPE for the cytoskeleton can be tested directly by molecular analysis of the labeled proteins. To this end, protein fractions of membranes

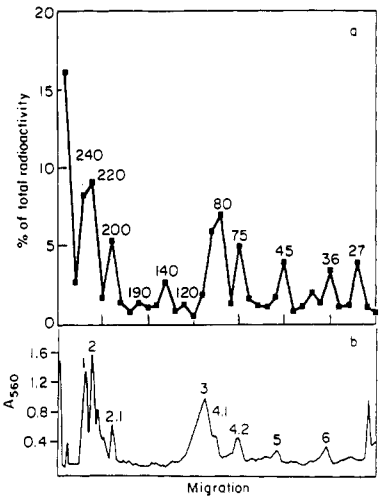

FIGURE 4: Electrophoretic separation of membrane proteins from erythrocytes photolabeled with [^{14}C]AzPE. Membranes from cells labeled by using the standard conditions presented in the text were delipidated by extraction with chloroform/methanol. The protein fraction was separated by SDS-PAGE; gels were stained, then sliced, and counted. (a) Radioactivity profile; numbers above peaks indicate molecular weights in $\times 10^{-3}$; hatch marks indicate gel slice number as in Figures 5 and 6. For reference, the gel slices corresponding to spectrin contained 400 cpm, and those to band 6 contained 36 cpm, the latter more than twice background. (b) Scan of Coomassie-stained gel for comparison. Band numbers are indicated above peaks.

from photolabeled cells were analyzed by SDS-PAGE. As shown in Figure 4a, photolysis resulted in the appearance on gels of several radioactive bands, each reproducibly observed in at least five different experiments. Among these is a large amount of radioactivity at the top of the gel, accompanied by a corresponding band stained with Coomassie Blue (Figure 4b), presumably representing cross-linked protein aggregates. To eliminate the possibility that these aggregates were cross-linked by the photolysis procedure itself, cells were irradiated in the absence of probe. Under these conditions, the aggregates did not form, indicating that the presence of the probe is required for their appearance.

In addition to the high molecular weight species, many radioactive bands which migrate into the gel are also observed after photolysis, most of which comigrate with bands stained by Coomassie Blue (Figure 4b). In Table III, the molecular weights of these labeled products are compared with those of known erythrocyte membrane proteins. This comparison reveals a labeled product corresponding in molecular weight to each of the abundant cytoskeletal proteins. In marked contrast, the integral membrane proteins band 3 and band 4.5 were poorly labeled. Since band 3 (95K) is easily the most abundant erythrocyte membrane protein (1.2×10^6 copies per cell), the absence of a peak of radioactivity in the 95K region of the gel is strong evidence that the probe has a high relative specificity for peripheral, over integral, membrane proteins.

Four radioactive protein bands, with molecular weights of 190K, 140K, 120K, and 27K, did not correspond to any abundant erythrocyte membrane proteins. These peaks might arise by proteolysis of known proteins, although they were not observed in the Coomassie Blue patterns, nor were their levels reduced by inclusion of various combinations of protease inhibitors. To show that these proteins are also cytoskeletal elements, the NaOH-soluble and -insoluble fractions of photolabeled membranes were analyzed by SDS-PAGE. As shown in Figure 5a, most of the radioactivity in the NaOH-insoluble fraction was found at the position of the high molecular weight aggregate, suggesting that cross-linked proteins account for the 20% of label not solubilized by NaOH; none

Table III: [^{14}C]AzPE Labeling of Known Erythrocyte Membrane Proteins

protein (band no.)	identity	mol wt ($\times 10^{-3}$)	cytoskeletal (C)/ integral (I)	labeling	no. of probe molecules/g of protein ($\times 10^3$)
1	α-spectrin	240	C	+	} 55
2	β-spectrin	220	C	+	
2.1	ankyrin	200	C	+	33
3	anion transporter	95	I	-	0.3
4.1		80	C	+	238
4.2		70	C	+	44
4.5	glucose transporter	60	I	-	1
5	actin	45	C	+	20
6	glyceraldehyde-3-phosphate dehydrogenase	35	C	+	10

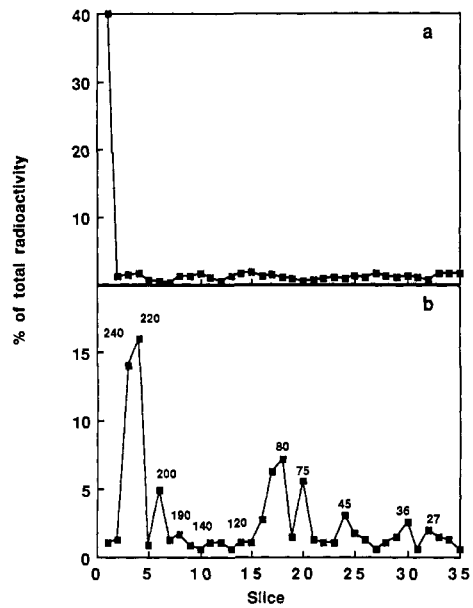

FIGURE 5: Electrophoretic separation of alkali-treated photolabeled membranes. Membranes were treated with 0.1 M NaOH as described under Materials and Methods and fractions analyzed by SDS-PAGE. (a) Alkali-insoluble fraction; (b) alkali-soluble fraction.

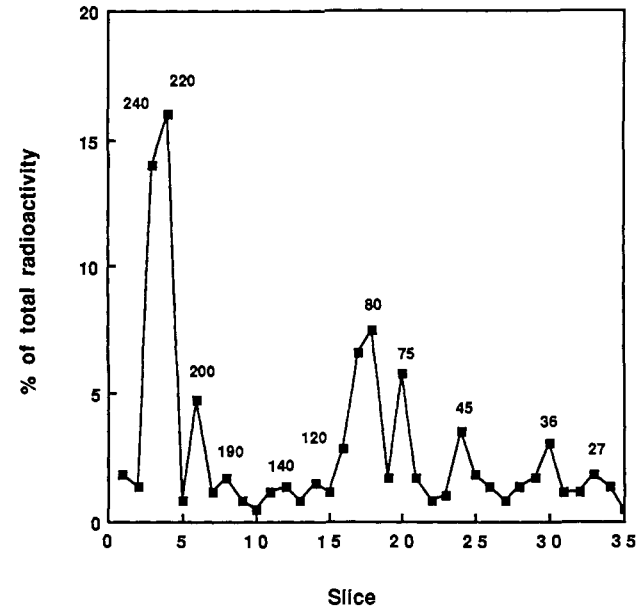

FIGURE 6: Electrophoretic separation of Triton X-100 shells from photolabeled erythrocytes. Membranes were extracted with 0.5% Triton X-100, and the insoluble fraction was analyzed by SDS-PAGE.

of the other labeled proteins found in whole membranes (Figure 4a) were present in this fraction. A similar analysis of the NaOH-soluble (cytoskeletal protein) fraction (Figure 5b) produced a profile closely resembling that obtained with whole membranes, including the four bands not corresponding to abundant cytoskeletal proteins. Analysis of photolabeled membranes extracted with Triton X-100 revealed that the residual Triton shells contained all of the peaks found in the unfractionated membrane (Figure 6), including the four additional proteins. Together, these results confirm the specificity of the probe for cytoskeletal proteins and further suggest that the four unknown proteins labeled by [^{14}C]AzPE originate in the cytoskeleton.

Although photolysis results in efficient transfer of [^{14}C]-AzPE to all of the components of the cytoskeleton, a comparison of the radioactivity profile in Figure 4a with the scan of the Coomassie-stained gel in Figure 4b suggests that different members of the cytoskeleton label with different efficiency. To investigate this heterogeneity in relative labeling, the known specific activity of the [^{14}C]AzPE, combined with the number of cells used and the number of copies of each protein per cell, was used to calcualte the number of probe molecules associated with each protein species, as shown in Table III. If labeling of the cytoskeleton were completely nonspecific within this compartment, the specific activity of all of the proteins would be the same. Instead, values vary over 20-fold, from very low for actin and glyceraldehyde-3-phosphate dehydrogenase to high for band 4.1. These results suggest that [^{14}C]AzPE labeling is sensitive to some property of individual cytoskeletal proteins, such as proximity to the bilayer.

Discussion

The goal of this study was to design and test a probe for labeling proteins lining the inner side of the plasma membrane. To achieve this goal, we synthesized a transferable, radioactive phospholipid analogue bearing a photoactivable group esterified directly to the C2 position of the glycerol backbone. Our collective data indicate that this probe, [^{14}C]AzPE, spontaneously partitions into the erythrocyte membrane, is chemically inert in the absence of light, is transferred to the inner leaflet of the membrane, and upon photolysis specifically labels cytoskeletal proteins.

Other investigators have used photoactivable phospholipid analogues to label erythrocyte membranes (Schroit & Madsen, 1987; Zachowski et al., 1987). Their probes employed photoactivable groups separated from the glycerol backbone by a spacer. Since the hydrophobic spacer should tend to anchor the photoactivable group in the membrane core, it is not surprising that these probes labeled either all of the major integral proteins (Zachowski et al., 1987) or selected ones such as band 3 (Schroit & Madsen, 1987). Although the specific pattern of labeling observed thus differed somewhat depending upon the exact structure of the probe, efficient labeling of known cytoskeletal proteins was not observed.

Montecucco et al. (1986) used a probe similar to the one reported here, the only difference being that the headgroup

was choline. As with our probe, this PC analogue labeled interfacial proteins, i.e., proteins proximal to the polar headgroup of the phospholipids. [^{14}C]AzPE combines this property with a headgroup which is concentrated at the inner leaflet, and specifically labels cytoskeletal proteins. Of these, band 4.1 was the most heavily labeled. This protein binds spectrin tetramers (Tyler et al., 1979, 1980) and actin (Cohen & Korsgren, 1980), and the resulting ternary complex may be anchored directly to the inner membrane leaflet via band 4.1 (Sato & Ohnishi, 1983; Rybicki et al., 1988; Shiffer et al., 1988). If so, band 4.1 may be strongly labeled by [^{14}C]AzPE because it is physically more accessible to the probe than other members of the cytoskeleton. Indeed, band 4.1 has both acidic and basic segments (Leto & Marchesi, 1984), and the latter may be able to bind [^{14}C]AzPE directly, just as they are able to bind PS (Rybicki et al., 1988; Shiffer et al., 1988; Cohen et al., 1988).

In addition to known cytoskeletal proteins, labeling of four other protein species (190K, 140K, 120K, and 27K) was observed. These proteins appear to be cytoskeletal, since they were extractable with sodium hydroxide and remained insoluble in Triton X-100. Since these bands did not stain with Coomassie Blue, they are probably present in low copy numbers. Yet as seen in Figure 4, these regions of the gel had almost the same amount of radioactivity as did band 6 (500 000 copies per cell), making it likely that these proteins are labeled more heavily than some of the known cytoskeletal proteins. If labeling efficiency is a function of physical accessibility, the high specific activity of these proteins would suggest that they may directly interact with the bilayer. It must be cautioned, however, that these species could represent cytoskeletal proteins of lower molecular weight cross-linked by the probe.

The poor labeling of band 3 is somewhat surprising, since it implies that even the large cytoplasmic domain of the molecule is not efficiently labeled by the probe. There are several explanations which might account for this low reactivity. Many proteins are associated with the cytoplasmic portion of band 3 [reviewed by Low (1986)], including several of the cytoskeletal proteins labeled by the probe. These proteins may mask potential labeling sites. Another possibility is that the highly elongated cytoplasmic domain of band 3 extends away from the bilayer, leaving only a few amino acids at the interface of the membrane and cytoplasm accessible to the probe. Whatever the actual explanation, the effect is to enhance the apparent specificity of the probe for cytoskeletal over integral membrane proteins.

In conclusion, [^{14}C]AzPE appears to be a reagent useful for specifically labeling cytoskeletal proteins. As such, it may prove valuable in a variety of applications. As shown here, it may identify cytoskeletal proteins present at only low copy numbers and thus not readily detectable by other means. The probe may also be used to study interactions between the membrane and cytoskeletal proteins. For instance, the cytoskeleton is abnormal or perturbed in erythrocytes from patients with a variety of diseases (Marchesi, 1985; Basu et al., 1988). The probe might be useful in assessing whether these abnormalities extend to perturbation of normal membrane–cytoskeletal interactions as well (Williamson et al., 1987). Finally, since an asymmetric distribution of phospholipids may exist in the plasma membrane of other cells besides erythrocytes (Op den Kamp, 1979), the probe may find use in labeling cytoskeletal elements and elucidating membrane–cytoskeletal interactions in other types of cells.

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F_1 ATPase from the Thermophilic Bacterium PS3 (TF_1) Shows ATP Modulation of Oxygen Exchange†

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ABSTRACT: The ATPase from the ATP synthase of the thermophilic bacterium PS3 (TF_1), unlike F_1 ATPase from other sources, does not retain bound ATP, ADP, and P_i at a catalytic site under conditions for single-site catalysis [Yohda, M., & Yoshida, M. (1987) *J. Biochem. 102*, 875–883]. This raised a question as to whether catalysis by TF_1 involved alternating participation of catalytic sites. The possibility remained, however, that there might be transient but catalytically significant retention of bound reactants at catalytic sites when the medium ATP concentration was relatively low. To test for this, the extent of water oxygen incorporation into P_i formed by ATP hydrolysis was measured at various ATP concentrations. During ATP hydrolysis at both 45 and 60 °C, the extent of water oxygen incorporation into the P_i formed increased markedly as the ATP concentration was lowered to the micromolar range, with greater modulation observed at 60 °C. Most of the product P_i formed arose by a single catalytic pathway, but measurable amounts of P_i were formed by a pathway with high oxygen exchange. This may result from the presence of some poorly active enzyme. The results are consistent with sequential participation of three catalytic sites on the TF_1 as predicted by the binding change mechanism.

The sequential participation of two or three catalytic sites during ATP hydrolysis by the F_1 ATPase leads to the prediction that when the medium ATP concentration is lowered a catalytic site should release bound ADP and inorganic phosphate (P_i)[1] slowly until another ATP binds at another catalytic site. Thus, if medium ATP is removed, or if the enzyme is exposed to substoichiometric amounts of ATP, tightly bound ATP, ADP, and P_i should be present at a catalytic site. Experimental evidence for this important characteristic was first reported by Moudrianakis (Adolfsen & Moudrianakis, 1976) for the enzyme from the bacterium *Alcaligenes faecalis* and has since been demonstrated for the mitochondrial (Gresser et al., 1982; Cross et al., 1982; Penefsky, 1985; Cunningham & Cross, 1988), chloroplast (Wu & Boyer, 1986), yeast (Konishi et al., 1987), and *Escherichia coli* (Wise et al., 1984) enzymes. However, in contrast to the behavior shown for these enzymes, the enzyme from the thermophilic bacterium PS3 (TF_1) when exposed to substoichiometric amounts of [α,γ-^{32}P]ATP did not retain [^{32}P]P_i or [α-^{32}P]ADP at catalytic sites after the enzyme was centrifuged through a Sephadex column (Yohda & Yoshida, 1987). This suggested that the TF_1 might not follow a sequential site mechanism. Such observations were important because in the binding change mechanism for ATP synthesis a similar sequential participation of catalytic sites is postulated. The mechanism is proposed to be general for all ATP synthases, and thus the result with TF_1 raised doubts about its validity.

Other considerations suggested that further exploration of the behavior of TF_1 might provide some clarification. In order for the release of products to be markedly promoted by ATP binding at an alternate site, it is not essential that the products be retained sufficiently long to pass through a Sephadex column. Retention for a time considerably longer than the turnover time when excess substrate was present would suffice. Another important characteristic demonstrated for the mitochondrial (Hutton & Boyer, 1979), chloroplast (Kohlbrenner & Boyer, 1983), and *E. coli* enzymes (Wood et al., 1987) is that when medium ATP concentration is lowered the bound ATP continues to undergo reversible interconversions to bound ADP and P_i. This behavior results in an increase in the number of water oxygens incorporated into each P_i formed. It thus seemed appropriate to use this approach to check whether, during hydrolysis of lowered concentrations of ATP by TF_1, ATP, ADP, and P_i might be transitorily tightly bound

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[1] Abbreviations: TF_1, CF_1, and MF_1, soluble ATPase portion of the ATP synthase from thermophilic bacterium PS3, chloroplasts, and mitochondria, respectively; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

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