INTERACTION OF MEMBRANOUS CYTOCHROME b_5 WITH ARYLAZIDOPHOSPHOLIPIDS

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1. Introduction

Many different water-soluble, lipid-insoluble reagents are available for labelling of the surface-exposed proteins in membranes. In contrast, reagents for labelling of proteins from within the lipid bilayer have proved much more difficult to design (reviewed [1,2]). One approach has been to synthesize derivatives of small organic molecules [3], the rationale being that these reagents will partition into the lipid, whereupon photoactivation, they can covalently insert into the buried portion of intrinsic membrane proteins. There are however, two major drawbacks with small, hydrophobic, photoaffinity probes as pointed out [4].

(i) Their location within the bilayer is hard to define.
(ii) The photogenerated nitrene or carbene groups are electrogenic and although these reagents partition mainly in the lipid phase, a significant amount could be present in the aqueous phase and thus react with hydrophobic pockets of surface-bound proteins.

More recently, several laboratories have synthesized phospholipids with an azido or diazirine group in one of the fatty acid tails [5,6]. Incorporation of the photoreactive group into a lipid should constrain it to the hydrophobic phase unless the modified fatty acid can loop back to the surface, for example, as proposed [4]. Unfortunately, there is no experimental work which bears on this possibility. Therefore as a preliminary to our work on mitochondrial inner membrane proteins we have studied the interaction of arylazidophospholipids with cytochrome b_5. This protein can be reassociated with lipids to form membrane vesicles which are a good model system in which to study protein–lipid interactions. The available data indicate that cytochrome b_5 sits in these membranes with its N-terminal hydrophilic domain out of the bilayer and a C-terminal hydrophobic domain intercalated among the lipids [7–9]. If the nitrene groups in our lipid molecules are constrained within the bilayer they should react with the C-terminal domain but not the N-terminal domain. On the other hand, if the photoaffinity group reacts from outside the membrane, we can expect labelling of the N-terminal hydrophilic domain.

2. Materials and methods

Calf-liver cytochrome b_5 was a kind gift of Dr T. Miller, Oregon State University. The preparation of 1-myristoyl, 2-[12 amino(4*N*-3-nitro-1-azidophenyl]-lauryl-*sn*-glycero-3-[^{14}C]phosphocholine (PL I) and of 1-palmitoyl 2-(2-azido-4-nitro)benzoyl-2*n*-glycero-3-[^{3}H]phosphocholine (PL II) will be detailed elsewhere (R.B., C.M., in preparation). The specific radioactivity was 177 Ci/mol and 3.9 Ci/mmol, respectively. Vesicles of egg lecithin (0.4%, w/w, in 10 mM phosphate buffer (pH 7.4)) were prepared by sonication under nitrogen in a bath sonicator. The vesicles contained 0.4% of phospholipid I and 0.1 or 0.2% of phospholipid II with respect to the total lipid. Cytochrome b_5 (100 μg) was incubated with 50 μl vesicles for 2 h at 37°C according to [8] and irradiated for 40 min at 0°C with a low intensity ultraviolet lamp. The protein was then precipitated with 90% acetone at −5°C and centrifuged at 6000 rev./min for 30 min.

The pellet was dissolved in 10 μl 1.5% Triton X-100, 25 mM Tris–HCl (pH 8.1). The refolded protein was reacted with chloro(-*N*-tosyl-L-phenylalanyl)methane-treated trypsin at a molar ratio of trypsin to cytochrome b_5 between 1:10 and 1:100 at 0–4°C for 2 days. The digestion was stopped by adding trypsin inhibitor 2:1, w/w, against trypsin. The active site titrant phenylmethylsulfonylfluoride was also added to 10^{-3} M final conc. The digestion of cytochrome b_5 with carboxypeptidase A or Y was performed essentially as in [10]. After acetone treatment and centrifugation, the cytochrome b_5 pellet was dissolved in 5 μl 1% Triton X-100, 50 mM Tris–acetate (pH 8.1) and then diluted 5 times with 0.1 M *N*-methylmorpholine acetate (pH 8.5) containing 0.1% NaN_3. Carboxypeptidase A (Worthington) was added at a molar ratio between 1:100 to 1:1000 (carboxypeptidase A to cytochrome b_5). Digestion was performed overnight at room temperature and was stopped by adding 1–10 *o*-phenanthroline (1 mM final conc.) and 2% sodium dodecyl sulfate (SDS). Polyacrylamide gel electrophoresis was by the procedure in [11] using 20% acrylamide and 0.67% *N,N'*-methylene bis(acrylamide). After destaining, the radioactive gels were sliced in 1 mm thick slices and counted.

3. Results

Cytochrome b_5 used in this study was essentially pure as judged by gel electrophoresis on 20% polyacrylamide gels in a buffer containing 0.1% SDS and 8 M urea (fig.1). The trace impurities were all of higher molecular weight than the hemoprotein.

For labelling experiments, cytochrome b_5 was incorporated into vesicles of egg lecithin containing either azidophospholipids I or II. After photolysis the protein was separated from lipid by acetone extraction followed by refolding in Triton X-100. The interaction of cytochrome b_5 with the modified lipids was then examined by SDS–polyacrylamide gel electro-

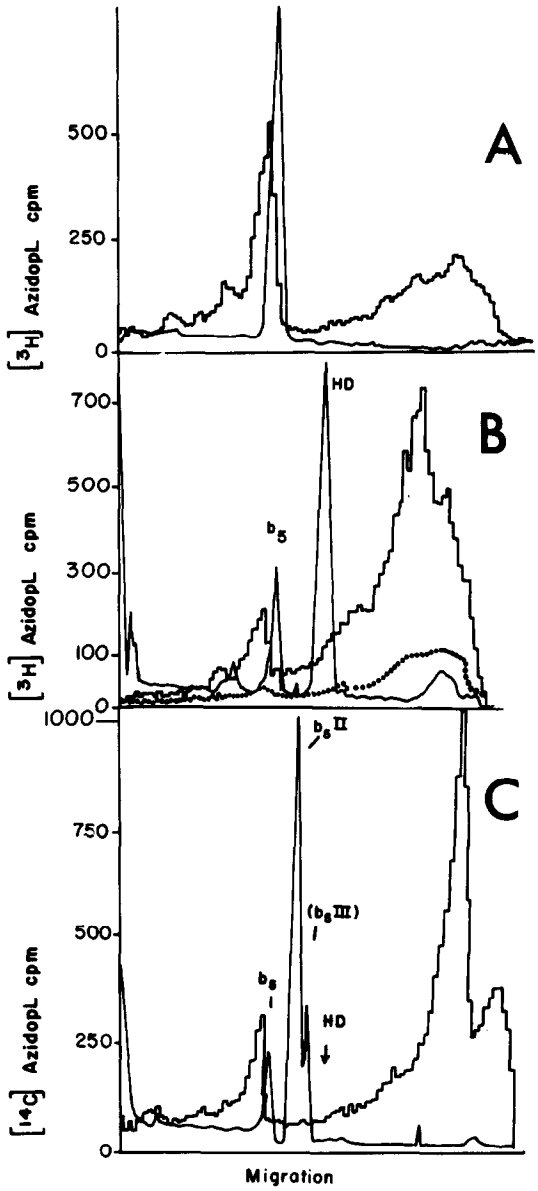

Fig.1. Labelling of cytochrome b_5 and cytochrome b_5 fragments with arylazidophospholipids. (A) Densitometric trace and labelling profile (barograph) of a gel containing cytochrome b_5 which has been reacted with phospholipid II. (B) Densitometric trace and labelling profiles of gels containing cytochrome b_5 reacted with phospholipid II and then subjected to mild trypsin digestion. The dotted and solid line barographs show the distribution of radioactivity before and after photoactivation of the probe respectively. HD is the hydrophilic domain. The hydrophobic domain runs close to free lipid. (C) Densitometric trace and labelling profiles of a gel containing cytochrome b_5 labelled with phospholipid I and then subjected to carboxypeptidase A treatment. b_5 II and b_5 III are fragments 1–115 and 1–106, respectively (see text). HD is the position at which the hydrophilic domain would run on this gel.

phoresis. Figure 1A shows the labelling of cytochrome b_5 by probe II which had been incorporated into the phospholipid micelles in the ratio of 1 mol:1000 mol of egg lecithin. Typically there were two peaks of radioactivity. One of them ran just behind the protein peak (visualized by Coomassie blue staining prior to slicing) with an apparent molecular weight equivalent to protein with one phospholipid molecule bound (i.e., 16 700 cf. 16 000 for unlabelled protein). The second peak of radioactivity ran close to the dye band in the position of free lipid. Labelling of cytochrome b_5 with probe I gave similar results (not shown).

Cytochrome b_5 can be cleaved at several well-defined positions in the sequence as summarized in fig.2 [10]. This made it possible to locate the site(s) of interaction with both arylazidophospholipids to specific segments of protein.

After prolonged incubation with trypsin, cytochrome b_5 was resolved into 2 fragments by SDS–polyacrylamide gel electrophoresis, the larger segment containing the N-terminal part of the molecule, the smaller fragment containing residues 91–133. As shown in fig.1B the labelling of cytochrome b_5 by probe II was restricted to the smaller fragment; there was no radioactivity associated with the larger domain. The same result was obtained with probe I.

Cytochrome b_5 is sensitive to carboxypeptidase as shown by the elegant studies of [10]. In the experiment shown in fig.1C, carboxypeptidase was used to remove the C-terminal 18 amino acids. This cleavage led to release of all the bound phospholipid, the radioactivity now being found in a broad peak close to the dye band but overlapping the peak of free lipid. This band presumably contained 'free' amino acids with bound phospholipid. The gel trace

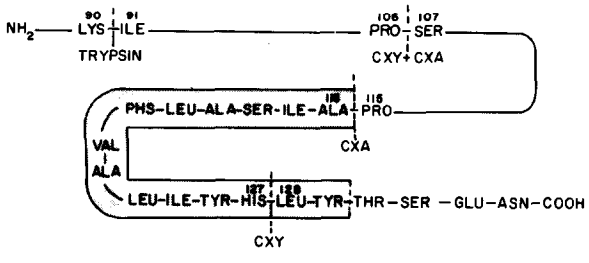

Fig.2. Fragmentation of cytochrome b_5 by carboxypeptidase as described [10]. The shaded area shows the fragment containing amino acids interacting with arylazidophospholipids.

in fig.1C is for probe I; identical results were obtained with probe II.

Carboxypeptidase Y has been used [10] to obtain a cytochrome b_5 derivative missing the 6 C-terminal amino acids. In several experiments with this protease we saw a shoulder running ahead of the major peak of unmodified cytochrome b_5 which could represent this minimally cleaved derivative. In these gels the peak of radioactivity was always broader than for unmodified protein indicating that label was contained in both the fragmented and 'native' cytochrome b_5.

4. Discussion

Cytochrome b_5 is one of the simplest and best characterized intrinsic membrane proteins. It can be separated into 2 domains after limited proteolysis of the protein [7–9].

(i) The domain involving the N-terminal 90 or so residues is globular, water-soluble, contains heme and is extrinsic to the lipid bilayer.

(ii) The domain containing the C-terminal 42 amino acids is insoluble in water and very hydrophobic in amino acid composition [7].

There are several lines of evidence to indicate that the C-terminal hydrophobic domain serves to anchor cytochrome b_5 in the membrane [8,10–14]. Results presented here show that arylazidophospholipids, with the photoreactive group close to the head group or the methyl terminus of the fatty acid chain, react with the C-terminal domain and not with the globular N-terminal domain of cytochrome b_5. These findings indicate that rearrangements of the azidophospholipids which lead to exposure of the reactive group at the surface of the membrane (as suggested [4]) do not occur or are infrequent enough that there is no appreciable labelling of protein in the aqueous phase. Arylazidophospholipids therefore have widespread application and should prove invaluable probes for the organization of subunits of mitochondrial inner membrane proteins such as cytochrome *c* oxidase [15].

Recent studies on cytochrome b_5 have been aimed at determining the portion of the hydrophobic tail needed for interaction with lipids. Dailey and Strittmatter [10] have used carboxypeptidase cleavage of soluble cytochrome b_5 to obtain three shortened cytochrome b_5 derivatives: b_5III, containing amino

acids 1–106; b_5II, 1–115; and b_5I, 1–128. Derivatives b_5II and b_5III but not b_5I were found to bind to phospholipid vesicles. Thus the binding site for lipids is localized close to the C-terminal end of the chain. A similar conclusion has been reached [12] based on chymotrypsin cleavage of membrane intercalated cytochrome b_5. This protease was found to cleave the protein at peptide bonds 97–98 and 129–130 leaving the fragment containing amino acids 98–129 within the bilayer and associated with lipids.

In our study two different arylazidophospholipids, one with a reactive group close to the head group and the other with its reactive group at the methyl end of the fatty acid chain, both reacted with cytochrome b_5 near the C-terminus and within the sequence involving residues 115–133. Taken together the above data indicate that the portion of cytochrome b_5 directly associated with lipid is small and could be as little as 14 (or less) amino acid residues. How such a small segment of chain would be arranged with respect to lipid is not clear and requires further experimentation.

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References

[1] Wallach, D. F. H. and Winzler, K. J. (1974) in: Evolving Strategies and Tactics in Membrane Research, Springer-Verlag, New York.
[2] Staros, J. V., Richards, F. M. and Haley, B. E. (1975) J. Biol. Chem. 250, 8174–8178.
[3] Klip, A. and Gitler, C. (1974) Biochem. Biophys. Res. Commun. 60, 1155–1162.
[4] Bailey, H. and Knowles, J. R. (1978) Biochemistry 17, 2414–2415.
[5] Chakrabarti, P. and Khorana, M. G. (1975) Biochemistry 14, 5021–5033.
[6] Stoffel, W., Därr, W. and Salm, K. P. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 453–462.
[7] Spatz, L. and Strittmatter, P. (1971) Proc. Natl. Acad. Sci. USA 71, 4565–4569.
[8] Robinson, N. C. and Tanford, C. (1975) Biochemistry 14, 369–377.
[9] Tajima, S., Enomoto, K. and Sato, R. (1978) J. Biochem. 84, 1573–1580.
[10] Dailey, A. H. and Strittmatter, P. (1978) J. Biol. Chem. 253, 8203–8209.
[11] Swank, R. T. and Munkres, K. D. (1971) Anal. Biochem. 39, 462.
[12] Ozols, J. (1979) Fed. Proc. FASEB 38, 472.
[13] Enomoto, K. and Sato, R. (1977) Biochem. Biophys. Res. Commun. 60, 1155–1162.
[14] Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
[15] Bisson, R., Montecucco, C., Gutweniger, H. and Azzi, A. (1979) Biochemical. Soc. Trans. 7, 156.