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Topography of multiple spanning transmembrane proteins – a photochemical approach

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Abstract- A photochemical approach to study the disposition of transmembrane proteins is described. It involves the use of hydrophobic phtoactivable reagents which on photolysis give rise to reactive intermediates like carbenes, capable of undergoing intermolecular insertion reactions. Two transembrane proteins responsible for the transport of anions and glucose in human erythrocytes have been studied using diazofluorene (DAF) as a photoactivable reagent. Isolation of these proteins and analysis of the DAF insertion site indicates several transmembrane segments. The relevance of the photochemical approach in view of limited information made available by biophysical methods is discussed.

One of the major problem associated with study of biological membranes is the availability of limited information on the structure of membrane bound proteins. Unlike soluble proteins wherein X-ray crystallography has provided complete three dimensional structure of proteins, the membrane-bound proteins are rarely amenable to X-ray crystallographic analysis (ref.1). These proteins carry out important functions like transport of material in and out of the cell and act as receptor for various hormones. To understand these important membrane associated processes it is critical to understand the structure of these proteins. The integral membrane proteins can be quite large with molecular weights ranging from ten thousand to several thousands. They contain both hydrophilic and hydrophobic amino acids, but unlike their soluble counterparts, they contain stretches of hydrophobic amino acids in their sequence which enable them to span the membrane hydrophobic matrix from once to several times. With an ever increasing number of sequences of integral membrane proteins being made available by gene cloning, the need for obtaining complete structural information on these proteins is being increasing felt. However none of the currently available biophysical techniques provide this information readily. The popularly used method at present is a theoretical model based on hydropathy indices, which provides a two dimensional model of the protein (ref.2). There have been very limited confirmation of such predicted models by any experimental technique and serious doubts regarding the validity of these theoretically predicted models in multiple spanning proteins have already been expressed in literature (ref.3).

The use of chemical reagents capable of modifying proteins have proved quite useful in studying protein structure. These reagents are elctrophilic in nature and react with various nucleophilic sites provided by side chain of polar amino acids like lysine, arginine, histidine and cysteine, found in proteins. However these reagents are of limited use to study the transmembrane segments of membrane-bound proteins, as these segments are composed largely of hydrophobic amino acids. The reagent of choice for studying the hydrophobic milieu should be capable of inserting into C-H bonds. A photochemical approach has been developed to study the topography of these complex proteins (ref.4,5). It involves the use of reactive intermediates like carbenes and nitrenes. The carbene or nitrene precursor is added to the membrane preparation and after a short period of incubation, it is photolysed. The resulting reactive intermediate then inserts into the neighboring molecule providing useful information on the disposition of membrane-bound proteins. While both carbene and nitrene precursors have been used, the former have been found to be much more effective specially for studies related to membranes. Currently there are three basic chromophores which have been used for these studies. Phenyl diazirine and subsequently adamantyl diazirine were among the first carbene based reagents used for labeling membrane hydrophobic core (ref.6). This was followed by trifluoromehylphenyl diazirine which was found to be more effective than phenyl diazirine (ref.7). We have reported

the use of diazofluorene and its analogs for photolabeling studies. In this article we summarize the results achieved with diazofluorene and its analogs with reference to membrane-bound proteins.

The photoactivable reagents used for labeling membrane hydrophobic core are either hydrophobic or amphipathic and easily partition into membranes. Reagents belonging to both of these categories have been prepared using diazofluorene (DAF) as the basic chromophore. We have tested several carbene precursors capable of undergoing intermolecular insertion reactions on photolysis. These results indicated that (DAF) is an effective hydrophobic photoactivable reagent. DAF readily partitioned into single bilayer vesicles prepared from phosphotidylcholine (PC) and on photolysis exclusively labeled the fatty acyl chains of PC. The insertion products were fluorescent where as DAF itself is nonfluorescent. This property provided a convenient method to monitor the insertion (ref.8). Further studies with DAF indicated that fluorenylidine generated from DAF in single bilayer vesicles equilibrates between the singlet and triplet states as products arising from both type of carbene states could be detected (ref.9). While fluorescence detection provided an effective method to monitor hydrophobic core labeling in artificial membranes, it was essential to synthesize a radioactive analog of DAF for more sensitive monitoring and for labeling membrane hydrophobic core in natural membranes. Towards this end 2-[^{3}H]diazofluorene and 2-[^{125}I]diazofluorene were synthesized (ref.10,11). Both these reagents were found to be effective in labeling the membrane hydrophobic core. Once the labeling in artificial membrane was established, we used human erythrocyte membrane as a model membrane as this is one of the well understood membrane system. DAF readily partitioned into erythrocyte membranes and exclusively labeled the integral membrane proteins (ref.11). Thus the anion transporter - Band 3, the glucose transporter - Band 4.5 and the sialoglycoproteins - PAS 1,2 and 3 were found to be the major proteins labeled by DAF as indicated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The extrinsic proteins like spectrin are not labeled significantly even though they are present in fairly high concentration in erythrocyte membranes. The labeling yields of ghost labeling varied from 50-60%. To get a further insight into the DAF labeling sites, the anion transporter and the glucose transporter were isolated from DAF labeled erythrocyte membranes and analysed for the insertion site by proteolytic digestion of these transporters.

HUMAN ERYTHROCYTE ANION TRANSPORTER

The anion transporter, Band 3 in human erythrocyte membranes is a major integral protein accounting for around 25% by weight of the membrane protein fraction. Kapito & Lodish (ref.12) have reported the entire sequence of Murine Band 3 with a molecular weight of 102.5 kDa. On the basis of this sequence data, hydropathy plot and proteolytic fragments of Band 3 reported earlier, they have proposed a model for orientation of Band 3 in membranes (ref.12). According to this model there are eight to twelve transmembrane domains in the approximately 60 kDa membrane bound C-terminal part of the molecule whereas the balance of the N-terminal part protrudes into the cytoplasm. Band 3 in intact cells is cleaved by chymotrypsin at an extracellular site (Tyr 572) to give membrane bound sugar bearing 39.3 kDa C-terminal fragment (CH-35) and a membrane bound 63.2 kDa N-terminal fragment (CH-55). In ghost preparations the CH-55 fragment is further cleaved at an intracellular site (Tyr 372) to a membrane bound 22.3 kDa (CH-17) and a 40.9 kDa N-terminal cytoplasmic fragment (ref.13). The cytoplasmic fragment is dispensable as far as the function of anion transport is concerned, however the CH-17 and CH-35 transmembrane fragments are required for the anion transport. More recently Tanner et al(ref.14) have reported the complete 911 amino acid sequence of Band 3 from human erythrocytes and we refer to this amino acid sequence in this manuscript.

The [^{3}H]-DAF labeled erythrocyte ghosts were treated with chymotrypsin at 100 µg/ml and analysed by SDS-PAGE. This analysis indicated that radioactivity associated with Band 3 region originally decreased, while new peaks appeared at 57, 37(major), 17 and 15 kDa. Increase in concentration of chymotrypsin to 250 µg/ml led to appearance of major peaks only at 35 and 16 kDa and finally at 350 µg/ml only one peak corresponding to 17 kDa was observed (ref.11). These results indicated that Band 3 has been labeled by [^{3}H]-DAF at multiple sites in the hydrophobic domain and the chymotrypsin fragmentation can be conveniently followed by radioactive monitoring. Interestingly though the CH-17 fragment could be seen at higher concentration of chymotrypsin, the CH-35 fragment could not be observed. It is likely that at higher concentra-

tions of chymotrypsin (350 µg/ml) the extracytoplamic CH-35 fragment is cleaved to smaller fragments and is therefore not seen on the gel.
In order to obtain maximum number of [^{3}H]-DAF labeled fragments of Band 3, we decided to find an optimum concentration of chymotrypsin wherein the CH-35 carboxyl terminal fragment of Band 3 is not fully cleaved as observed at high concentrations of chymotrypsin. The most optimal concentration for this was found to be 200 µg/ml. Thus after base stripping, the [^{3}H]-DAF labeled ghosts were treated with chymotrypsin (200 µg/ml) and analysed. The use of 15% urea-SDS-PAGE system at this stage or after Triton X-100 solubilization gave very broad bands on radioactive monitoring, which made the analysis rather difficult. Considerable experimentation with various gel systems indicated that the use of Laemlli system with 10% SDS-PAGE gels gave the best results for analysing the Triton X-100 pellet and the supernatant. Using this system at this stage gave fairly sharp bands with apparent molecular weights which were closer to those predicted from the murine Band 3 sequence (ref.12). Thus using this electrophoresis system the DAF labeled ghosts after base stripping and chymotrypsin (200 µg/ml) treatment as mentioned above indicated two major peaks at 26 and 19 kDa and a few other small peaks in the high molecular weight region. For further analysis the chymotrypsin treated ghosts were solublised in Triton X-100 and centrifuged. The gel pattern for the pellet indicated major labeling in the 26 and 18 kDa peaks. On the other hand a similar analysis for the supernatant fraction gave a gel which indicated peaks at 96 and 42 kDa, possibly corresponding to uncleaved Band 3 and the CH-35 fragment respectively, and a major peak at 26 kDa. The 26 kDa fragment in our opinion arises from a chymotryptic cleavage of CH-35 fragment at position TYR-818 which would give rise to a sugar bearing 28.6 kDa fragment and a C-terminal 10.6 kDa fragment. The 28.6 kDa fragment appears in these gels with apparent molecular weights of 23 and 26 kDa, the variation in molecular weights being possibly a result of large sugar unit linked to this fragment. The 10.6 kDa fragment being a low molecular weight peptide is not seen in these gels as it may run too close to the tracking dye.

The radiolabeling of the integral membrane protein also provides a sensitive monitoring procedure. Thus it is usually difficult to detect the sugar bearing CH-35 fragment of Band 3 by coomassie blue staining of SDS-PAGE gels. At best it appears as a fuzzy band. In the current study not only could CH-35 be easily detected but the base stripping followed by chymotrypsin treatment of the labeled ghost indicated that other sites in Band 3 possibly become accessible to chymotrypsin cleavage after removal of cytoskeletal proteins. This is not surprising as Band 3 is reported to be strongly associated with cytoskeletal proteins. Such an analysis could be difficult to achieve without recourse to radioactive analysis. We have suggested that the new chymotrypsin cleavage site of CH-35 is Tyr 836 as this tyrosine residue lies in proline rich region with Pro residues present at positions 816,816,820 and 823. Low et al (ref.15) have similarly proposed that the cytoplasmic domain of CH-65 has a hinge region which is rich in proline. These proline residues can disrupt the a-helical structure of the polypeptide and thus rendering it susceptible to proteolysis. Interestingly the other intracellular and extracellular proteolytic sites of Band 3 also exist in Pro rich region i.e between residues 351-371 and 548-568 respectively. Further chymotrypsin cleavage at Tyr 836 would split the parent 39.3 kDa fragment to 28.6 and 10.7 kDa fragments. The Triton X-100 solubilized labeled ghost on electrophoretic analysis indicate a peptide with apparent molecular weight close to 28.6 kDa, both in the pellet and supernatant fraction. Lack of solubilization by Triton X-100 indicates that the labeled peptide fragment observed by us still bears the sugar residues. Tanner et al (ref.14) have also recently reported the presence of the new proteolytic site mentioned above.

HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER

The entry of D-glucose in human erythrocytes is mediated via a carrier protein which facilitates diffusion of glucose into cells. The carrier protein - glucose transporter has been purified to near homogeneity (ref.16) and has been found to be a glycoprotein which runs as a broad band on SDS-PAGE corresponding to apparent M_r 55,000. The complete amino acid sequence of glucose transporter from human hepatoma cell line HepG2 was deduced from cDNA clones (ref.17). Based on these studies, a model comprising of twelve transmembrane fragments has been proposed. Structural studies on human erythrocyte glucose transporter have indicated that this protein is similar to the hepatoma protein (ref.17).

Several photolabeling studies involving the use of probes that compete for the glucose binding site in the glucose transporter have been reported

(ref.16,18). Most notable among these has been the glucose transporter inhibitor, Cytochalasin B which specifically labels the transporter protein (ref.16). However, no study involving the use of hydrophobic photoactivable reagents, which readily partition into the membrane hydrophobic core and label transmembrane fragments of integral membrane proteins has been reported for the erythrocyte glucose transporter though such reagents have been widely used to label other integral membrane proteins. Needless to say, the use of such probes would provide direct evidence for verification of the two-dimensional models proposed on the basis of theoretical predictions on the primary structure of transport proteins.

The glucose transporter from human erythrocytes was thus purified from [^{3}H]DAF photolabeled ghost membrane preparations by the n-octyl glucoside procedure (ref.16). Briefly, the labeled ghost were stripped off their peripheral proteins with 15.4mM NaOH containing 1.54mM DTT . The alkali stripped ghosts were solubilised in 50mM Tris-Hcl, 2mM DTT (pH 7.4) containing 46mM octyl glucoside. The detergent extract was centrifuged at 130,000xg for 1 hr. The supernatent was passed through a DEAE-cellulose column and the flow through fraction containing the glucose transporter was collected. The counts in the washings after alkali stripping of the labeled ghost pellet were found to be only 0.76% which is as expected since 2-[^{3}H]DAF labels only the hydrophobic regions of the membrane. Electrophoretic analysis of the flow through fraction showed a broad peak of apparent M_r 55,000 corresponding to the transporter with few contaminants in other regions of the gel.

The transporter was then trypsinised, delipidated and analysed on 15% SDS-PAGE. The radioactive profile showed the presence of two peaks around the 18 kDa region. Thus, tryptic digestion of the reconstituted glucose transporters indicated that the major peak corresponds to an apparent M_r of 18,000. This fraction has been shown by Cairns et al. (ref.19) to correspond to the sequence 270-456 and contains the glucose transport site. This fragment 270-456 is very hydrophobic and according to the proposed model contains five transmembrane fragments (ref.17). It is thus interesting to note that this hydrophobic fragment is strongly labeled. Interestingly this fragment also contains the glucose transport site and has been photolabeled by other glucose based photoprobes (ref.16,17). The presence of relatively small number of counts around the 35 kDa region indicated that this fragment is poorly labeled. In conclusion, the current study indicates that 2-[^{3}H]DAF effectively labels hydrophobic segments in the C-terminal tryptic peptide of the glucose transporter.

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