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DIMERIZATION OF BOVINE PAS IV IS AN ARTIFACT OF THE SOLUBILIZATION PROCEDURE

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

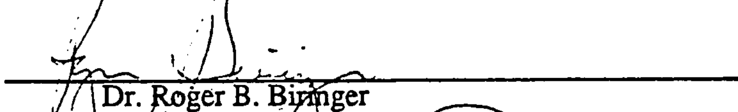
By

Sundari Suresh

August, 1990

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Dr. Dale E. Greenwalt



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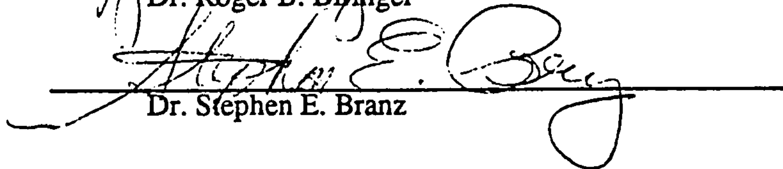

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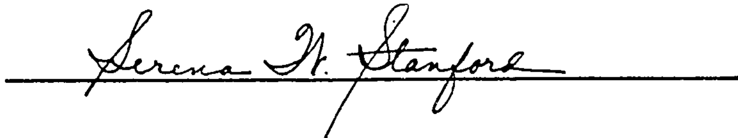

ABSTRACT

DIMERIZATION OF BOVINE PAS IV IS AN ARTIFACT OF THE SOLUBILIZATION PROCEDURE

by Sundari Suresh

Bovine PAS IV is a hydrophobic, membrane-bound glycoprotein (molecular weight, 78kDa) isolated from bovine milk-fat-globule membrane. Recent studies suggest that PAS IV may function as a thrombospondin receptor in mammary epithelial cells. Characterizing the structure of PAS IV could give valuable insight into its function. The present research was undertaken to determine the quaternary structure of PAS IV. Cross-linking of solubilized PAS IV with disuccinimidyl suberate and glutaraldehyde yielded dimers. In the native membrane-bound form, PAS IV did not cross-link as a dimer. It did not cross-link even after the removal of:

(i) the neighboring proteins in the membrane; and

(ii) the N-linked oligosaccharides.

Either of these factors could possibly interfere with the cross-linking reaction. Also, oxidation of MFGM did not yield a dimer. It is probable that the solubilization procedure alters the orientation of PAS IV such that it exists as a dimer.

ACKNOWLEDGEMENTS

I would like to record my sincere gratitude to Dr. Dale Greenwalt for his sustained encouragement and guidance over the past two years. I would also like to thank Dr. Biringer and Dr. Branz for serving on my committee.

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LIST OF ABBREVIATIONS

BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indoyl phosphate
βME	β-mercaptoethanol
BSA	bovine serum albumin
DMF	dimethyl formamide
DMS	dimethyl suberimidate
DMSO	dimethyl sulfoxide
DSP	dithiobis(succimidyl propionate)
DSS	disuccinimidyl suberate
DSST	disulfosuccinimidyl tartarate
DTBP	dimethyl-3,3'-dithiobispropionimidate
kDa	kilodaltons
MFGM	milk-fat-globule membrane
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
Tris	tris(hydroxymethyl) aminomethane

1. INTRODUCTION

Every living cell is enveloped by a cell membrane which is composed of lipids, proteins and carbohydrates. The lipid in the membrane is present as a bilayer and is a barrier to the passage of inorganic ions and hydrophilic molecules into or out of the cell. Access to these ions or molecules is vital for cell function. Proteins are key players in transport functions and in transmembrane signaling. Water-soluble signaling molecules—including neuro-transmitters, protein hormones, and growth factors—bind to specific cell surface receptors on their target cells. These receptors are membrane proteins which bind the signaling molecule with high affinity and convert this extracellular event into an intracellular signal that alters the behavior of the target cell. Receptor proteins normally have a transmembrane structure; that is, they have a hydrophobic region which enables them to span the lipid bilayer.

Transport proteins are also membrane-bound and often have multiple membrane spanning regions. Some transport proteins form aqueous channels that permit solutes of appropriate size and charge to cross the bilayer by simple diffusion; these are called channel proteins. Others, called carrier proteins, bind the specific molecule to be transported and transfer it across the membrane by undergoing a reversible conformational change.

Bovine PAS IV is a membrane-bound glycoprotein (molecular weight, 78kDa) isolated from bovine milk-fat-globule membrane (MFGM). It constitutes 2-5% of the MFGM protein and has been partially characterized. It is very hydrophobic as indicated by the following observations:

1. It is recovered in the detergent phase of Triton X-114 extracts of MFGM.
2. When bound to the membrane, PAS IV is resistant to proteinases. After solubilization it is readily degraded.

3. Amino acid analysis of purified PAS IV revealed a high percentage of nonpolar amino acids (Greenwalt & Mather, 1985).

Immunofluorescence techniques indicate that PAS IV is present in mammary epithelial and endothelial cells. In the heart, spleen, liver, and intestinal smooth muscle it is confined only to the capillary endothelial cells. However, it is absent in the capillary endothelial cells of lung, kidney, and brain (Greenwalt and Mather, 1985; Greenwalt, et al., 1985).

Although earlier thought to be a transport protein, recent studies indicate that PAS IV is a receptor. Greenwalt, et al. (1990) established that the endothelial form of PAS IV is CD36, an endothelial and platelet glycoprotein. The N-terminal sequences of human PAS IV, bovine PAS IV, bovine endothelial cell CD36, and human platelet CD36 (having molecular weights of 80, 78, 85 and 88 kDa, respectively) are homologous. All four of these proteins react with antiserum to human PAS IV. After deglycosylation, all four proteins have M_r values of approximately 57 kDa. This suggests that cell-specific processing gives rise to differently glycosylated forms of CD36.

CD36 binds to thrombospondin (McGregor, et al., 1989). Thrombospondin is an adhesive protein released by platelets upon activation and is important for the stabilization of platelet aggregates. It has also been found to be a component of the extracellular matrix of various cell types (Sage, et al., 1983). CD36, by interacting with thrombospondin on the activated platelet surface, may play an important role in the platelet aggregation process. CD36 also binds to erythrocytes infected with the mature *Plasmodium falciparum* parasite. Purified CD36 or antibodies to CD36 inhibited and reversed the binding of infected erythrocytes to cultured endothelial cells and melanoma cells *in vitro* (Ockenhouse, et al., 1989). Cerebral malaria is characterized by sequestration of erythrocytes infected with the

mature parasite. Therefore, the portion of the CD36 molecule that reverses cytoadherence may be useful therapeutically for rapid reversal of sequestration in cerebral malaria.

Leung et al. (1990) have shown that bovine PAS IV binds to thrombospondin. This observation, combined with the presence of very high levels of thrombospondin in human colostrum (Dawes, et al., 1987) suggests that PAS IV, like CD36, functions as a thrombospondin receptor in mammary epithelial cells. Characterizing the structure of PAS IV could give valuable insight into its function. As a step in this direction, the present research aims to determine the quaternary structure of PAS IV.

What is a quaternary structure ?

Every protein is characterized by four tiers of structure. The primary structure is the basic amino acid sequence of the polypeptide chain. Conformations of these amino acids with respect to each other refers to the secondary structure. α-helices and β-sheets are examples of secondary structures. Tertiary structure is the characteristic folding of the polypeptide chain or packing of the secondary structure. The "immunoglobulin domain" of immunoglobulins is an example of tertiary structure. In multimeric proteins, the **quaternary** structure is defined by how subunits pack, including orientation and interactions.

Review of previous work

Membrane proteins that are deeply embedded in the lipid bilayer cannot be easily manipulated like soluble proteins. Solubilization involves the disruption of the bilayer with detergents; these detergents render many physical methods difficult or impractical. Chemical cross-linking is a method that is applicable to both membrane systems and

detergent systems and has emerged as an important tool for studying associations between subunits of oligomeric membrane proteins (Peters & Richards, 1977).

Investigations of quaternary structure of oligomeric membrane proteins differ from similar studies of soluble proteins in another important respect: in membrane proteins the subunit interactions could be cytoplasmic or extracytoplasmic. Ideally, one would like to obtain this topological information along with the subunit proximity. Many membrane impermeant cross-linking agents have therefore been developed (Staros, 1988). Chemical cross-linking has been used to study the quaternary structure of a number of membrane proteins. A few of these studies are described in the following paragraphs.

Na^+K^+-ATPase is a membrane-bound enzyme responsible for catalyzing the active transport of sodium and potassium ions across the plasma membrane. The enzyme consists of two subunits, α and β, and these exist in an equimolar ratio within the same molecular complex, as indicated by cross-linking with DMS, glutaraldehyde and copper-phenanthroline complex (Craig & Kyte, 1980). Other workers have demonstrated that the enzyme is present as an $\alpha\beta$ heterodimer (Askari, et al., 1980). Studies to determine the smallest unit necessary for catalytic activity have also been undertaken (Craig, 1988; Huang, et al., 1988).

The mitochondrial NADH dehydrogenase complex consists of more than twenty polypeptide subunits. Interactions between subunits and the transmembrane organization of the enzyme have been revealed by cross-linking with DSST and DMS (Patel & Ragan, 1988; Patel, et al., 1988).

CTP phosphocholine cytidylyltransferase catalyzes a rate-limiting reaction in the synthesis of phosphatidylcholine. Cross-linking with DSP or DTBP indicated that the

purified transferase exists as a dimer when bound to a detergent micelle or membrane vesicle (Cornell, 1989).

The anion-exchange-channel protein is responsible for the exchange of Cl^- and HCO_3^- ions across the erythrocyte membrane. The subunits of this protein also cross-link as a dimer (Staros & Kakkad, 1983; Jennings & Nicknish, 1985).

The present investigation aims to determine the quaternary structure of bovine PAS IV by chemical cross-linking with glutaraldehyde and DSS. Both glutaraldehyde and DSS are amine reactive bifunctional cross-linking agents. The reactive groups in these two cross-linking agents are an aldehyde and ester respectively. The aldehyde reacts with either primary amine functions of epsilon amine groups on lysine or available N-terminus amines to form an imide bond. The ester group reacts with the amine groups to form a stable amide bond. Since the reactive group is present on both ends of the cross-linker, a covalent linkage is formed between subunits that are associated together. The cross-linked protein products can be analyzed by electrophoresis. The cross-linking reaction thus serves to identify subunit interactions or quaternary structure.

2. EXPERIMENTAL PROCEDURES

Materials

The materials used in this study and their sources are listed in Table 1.

Source Company	Materials
Aldrich Chemical Co., Inc. (Milwaukee, WI)	N-Ethylmaleimide
Bio-Rad Laboratories (Richmond, CA)	bisacrylamide, alkaline phosphatase-conjugated goat-antirabbit IgG, Coomassie Brilliant Blue R-250, Triton X-100 and nitrocellulose membrane
Eastman-Kodak Co. (Rochester, NY)	o-Phenanthroline
Fisher Scientific (Fair Lawn, NJ)	Acrylamide
New England Nuclear (Boston, MA)	Endo F
Pierce Chemical Co. (Rockford, IL)	Disuccinimidyl suberate
Sigma Chemical Co. (St Louis, MO)	Glycine, SDS, Tris, BSA, nitro blue tetrazolium chloride, and SDS-PAGE molecular weight standards
Structure Probes Inc (West Chester, PA)	Glutaraldehyde
US Biochemical Corporation (Cleveland, OH)	BCIP

Table 1. The materials used in this study, and their sources.

Methods

Preparation of Membranes

Bovine MFGM was prepared by diluting fresh, unwashed cream with an equal volume of 0.1M citrate buffer, pH 7.0, containing 0.15 M NaCl. It was then stirred in a Waring blender at 30% speed at 4°C until butter formed. The aqueous buttermilk was filtered through four layers of cheesecloth and centrifuged for one hour at 100,000*g*. The membrane pellet was then suspended in 20mM Tris, containing 0.15 M NaCl (TBS) and recentrifuged to obtain the final membrane preparation.

Gel Electrophoresis

Proteins were separated by SDS-PAGE in 0.75 mm, 8.0% (w/v) acrylamide slab gels as described by Laemmli (1970). The molecular weight standards were as follows: myosin (205,000), β-galactosidase (116,000), phosphorylase-b (97,400), BSA (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000). The proteins were stained by Coomassie Blue.

Immunoblotting

Protein samples were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper, essentially as described by Towbin, et al. (1979). The current density used was 1 mA/cm^2. The nitrocellulose paper was then rinsed overnight with 2% (wt/vol) BSA/TBS. Affinity-purified rabbit antibodies to bovine PAS IV, diluted 1:200 in 2% (wt/vol) BSA/TBS, were then incubated with the paper for one hour and the nitrocellulose was sequentially treated as follows: 0.5 M NaCl/TBS, 15 min; 0.5% (vol/vol) Triton X-100/TBS, 15min; 0.2% (wt/vol) BSA/TBS, 15 min; alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1:1000 in 2% (wt/vol) BSA/TBS, 1 h; 0.5 M NaCl/TBS, 15 min;

0.5% (vol/vol) Triton X-100/TBS, 15min; TBS, 15 min; distilled water, 5 min. Preimmune serum was used in place of affinity-purified rabbit antibodies in control experiments. The control blots did not show any bands. The reactive protein bands were visualized using BCIP as the phosphatase substrate. 10 ml of substrate contained 9 ml barbitol buffer, 20 µl 2M $MgCl_2$, 50 µl BCIP (10 mg/ml in DMF), and 1 ml nitro blue tetrazolium chloride (1mg/ml in 50 µl DMF + 950 µl barbitol buffer).

Proteinase Digestion

Papain: Bovine MFGM was homogenized at a concentration of 1mg/ml in 5 mM cysteine, pH 6.2, containing 2 mM EDTA. The membrane preparation was then incubated with papain (1 unit/ml), for one hour at 37°C. After digestion, the sample was diluted 4X with 1% BSA/TBS, pH 7.4, and centrifuged at 17,000 rpm for 1 hour (4°C). The pellets obtained were then suspended in TBS and recentrifuged. The final pellet was homogenized in 40 mM Tris-HCl, pH 7.8, containing 50 mM $CaCl_2$. The protein concentration in the digest was determined by BCA assay and the digest was analyzed by SDS-PAGE.

Trypsin: 3.5 ml of the papain-digested membrane was treated with trypsin at 100 units/ml and incubated for 1 h at 37°C. The trypsin digest was then diluted 6X with 1% BSA/TBS, pH 7.4, and centrifuged for 30 min at 17,000 rpm (4°C). The pellet was rinsed and homogenized in TBS and recentrifuged for 30 min. The final pellet was homogenized in 2 ml of PBS (50mm, pH 7.0). The protein concentration in the digest was determined by BCA assay and the digest was analyzed by SDS-PAGE.

Endoglycosidase reactions

Bovine PAS IV (10 µg/tube) was combined with 20 µl 0.1 M sodium phosphate (pH 6.1), 0.6 µl β-mercaptoethanol, and 0.4 units of endo-β-N-acetylglucosaminidase (endo F) in a final volume of 30 µl and overlayed with mineral oil. The mixture was then incubated

at 37°C for 2–20 hours. In the 6 hour and 10 hour incubations, 0.6 µl 10% SDS and 3.0 µl 10% Triton X-100, were also present as detergents. In one 20 hour incubation, an additional 0.4 units of endo F was added after 10 hours. The reaction was terminated by diluting 20 µl of the reaction mixture with 20 µl of SDS-PAGE sample buffer and heating in a boiling water bath for 1 minute. The samples were analyzed by immunoblotting.

Cross-linking reactions

Solubilized bovine PAS IV, bovine MFGM, papain-digested MFGM, and MFGM digested with endo F for 20 hours were used for cross-linking. The reaction volume was 25 µl, and the cross-linking was carried out both in the presence and absence of β-mercaptoethanol. Glutaraldehyde was used only with the solubilized protein. The reactions were carried out at room temperature for 45 min with glutaraldehyde and 20 min with DSS. The reactions were quenched by addition of 10 M excess of glycine, pH 9.5. An equal volume of SDS-PAGE buffer containing 5% β-mercaptoethanol was added to the samples. The samples were boiled for one minute and then analyzed by SDS-PAGE or immunoblotting. The protein and cross-linker concentrations used for each reaction are indicated in Table 3.

Copper-Phenanthroline Oxidation of MFGM

Samples of MFGM containing 400 µg of protein were incubated for 10 min at 37°C in the presence or absence of the oxidation catalyst 1.5mM Cu(II) (1, 10-phenanthroline)$_3$. The reaction was terminated by the addition of an equal volume of SDS-PAGE sample buffer containing 5mM EDTA and 10mM N-ethylmaleimide, and boiling for one minute. EDTA was included to chelate Cu(II) and N-ethylmaleimide to block free sulfhydryl groups. The products were analyzed by immunoblotting.

Protein determinations

Protein concentrations were analyzed with the BCA reagent (Smith, et al., 1985); BSA was used as the standard.

Protein	μg protein in 25 μl reaction	Cross-linker concentration
Solubilized PAS IV	10 & 100	0.25 mM DSS; 8 mM glutaraldehyde
MFGM	5	1 mM DSS
Papain digested MFGM	10	1 & 3 mM DSS
Endo F digested MFGM	0.125	1 mM DSS

Table 3: Protein and cross-linker concentrations used in the cross-linking reactions.

3. RESULTS

Chemical cross-linking was used as a tool to determine the quaternary structure of bovine PAS IV. The experiments were performed with the solubilized and membrane-bound PAS IV.

Proteinase Digestion

PAS IV was not degraded when a preparation of bovine MFGM was digested with papain. Prior to papain digestion PAS IV accounted for only 2-5% of the MFGM proteins (Fig. 1, *lane 2*). As evident in the gel stained with Coomassie Blue, after digestion it constitutes about 75-80% of the of the MFGM proteins (Fig. 1, *lane 3*). Xanthine oxidase and butyrophilin, the major proteins of MFGM are degraded by papain. There is no significant difference in the extent of digestion between papain and papain+trypsin digests (Fig. 1, *lanes 3 & 4*). The amounts of protein present prior to and after proteinase digestion are indicated in Table 2.

Protein	mg	%
MFGM	20	100
Papain and Papain+Trypsin digests	0.6	3

Table 2: Protein concentrations in proteinase digests

Endoglycosidase Digestion.

A time course study of the deglycosylation of bovine PAS IV with endo F demonstrated a gradual decrease in the apparent M_r of PAS IV. The end point at

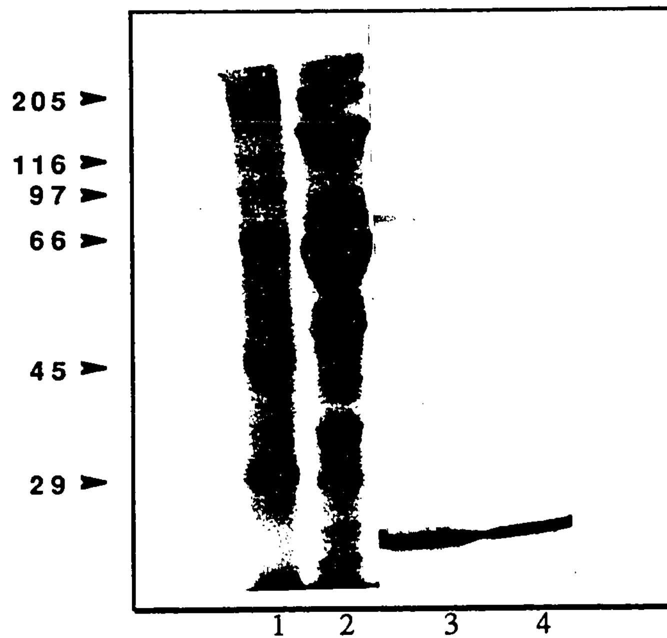


Figure 1. **Coomassie gel of Papain and Papain+Trypsin digestion of MFGM.**

Lane 1: Molecular weight markers
Lane 2: MFGM homogenate
Lane 3: Papain digested MFGM
Lane 4: Papain+trypsin digested MFGM

Lanes1&2 from one gel were combined with lanes 3&4 from another gel for this figure.

approximately 57kDa was reached with 10 hours of incubation (Fig. 2, *lane 5*). The end point was unaltered with 20 hours of incubation. Also, there was no change with 20 hour incubation done with twice the amount of enzyme (Fig. 3, lanes *4 & 5*). A total of seven bands could be identified (Fig. 2, *lanes 1-5*) and there were three bands at the end point (Fig. 2, *lane 5*; Fig. 3, *lanes 2, 4 & 5*). Six and ten hour control incubations in which endo F was absent did not change the apparent M_r of the substrates (Fig. 2, *Lane 1* and Fig.3, *Lane 1*).

Cross-Linking reactions

Cross-linking of Solubilized PAS IV: When β-mercaptoethanol was absent in the incubation mixture, bovine PAS IV cross-linked as a dimer with 0.25mM DSS (Fig. 4, *lane 6*). No change was observed in the amount of dimer when the concentration of protein was varied by a factor of 10 (Fig. 4, *lane 8*). With 8mM glutaraldehyde in the absence β-mercaptoethanol a small amount of the PAS IV cross-linked as a dimer. Higher molecular weight oligomers were also seen. As with DSS, the cross-linking pattern was not altered when the concentration of protein was varied by a factor of 10 (Fig. 4, *lanes 2 & 3*). When β-mercaptoethanol was present in the incubation mixture PAS IV migrated in the gel as a monomer (Fig. 4, *lanes 3,5,7 & 9*). 2μM and 20μM DSS were unable to cross-link bovine PAS IV as a dimer, in the absence of β-mercaptoethanol (Fig. 5, *lanes 3 & 5*).

Cross-linking of MFGM: When MFGM was cross-linked with 1mM DSS in the absence of β-mercaptoethanol no dimer was seen in the immunoblot (Fig. 6, *lane 3*). The amount of MFGM used for the incubation was such that it yielded 5 μg of PAS IV (calculated on the basis that PAS IV constitutes 2.5% of MFGM). A dimer was not observed even when the incubation time was increased--30, 45, 60, 120, and 180 minutes (Fig. 7, *lanes 2, 3, 4, 5 and 6*). Since there seemed to be no significant difference between

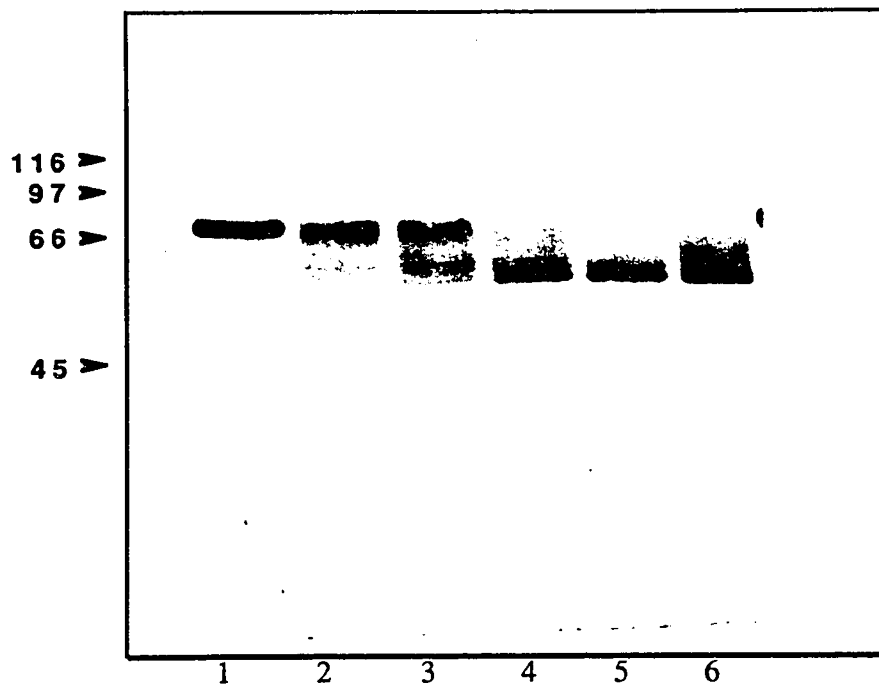


Figure 2. Immunoblot of endoglycosidase digestion of MFGM (2-10 hours).

Lane 1: 6 hour control without enzyme
Lane 2: 2 hour without detergents
Lane 3: 4 hour without detergents
Lane 4: 6 hour without detergents
Lane 5: 10 hour without detergents
Lane 6: 6 hour with detergents

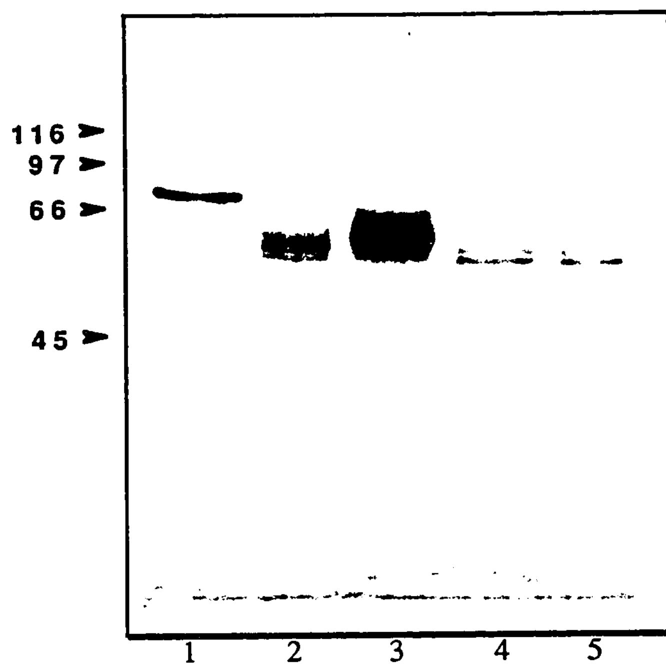


Figure 3. Immunoblot of endoglycosidase digestion of MFGM (10 & 20 hours).

Lane 1: 10 hour control without enzyme
Lane 2: 10 hour without detergents
Lane 3: 10 hour with detergents
Lane 4: 20 hour without detergents
Lane 5: 20 hour without detergents; twice the amount of enzyme

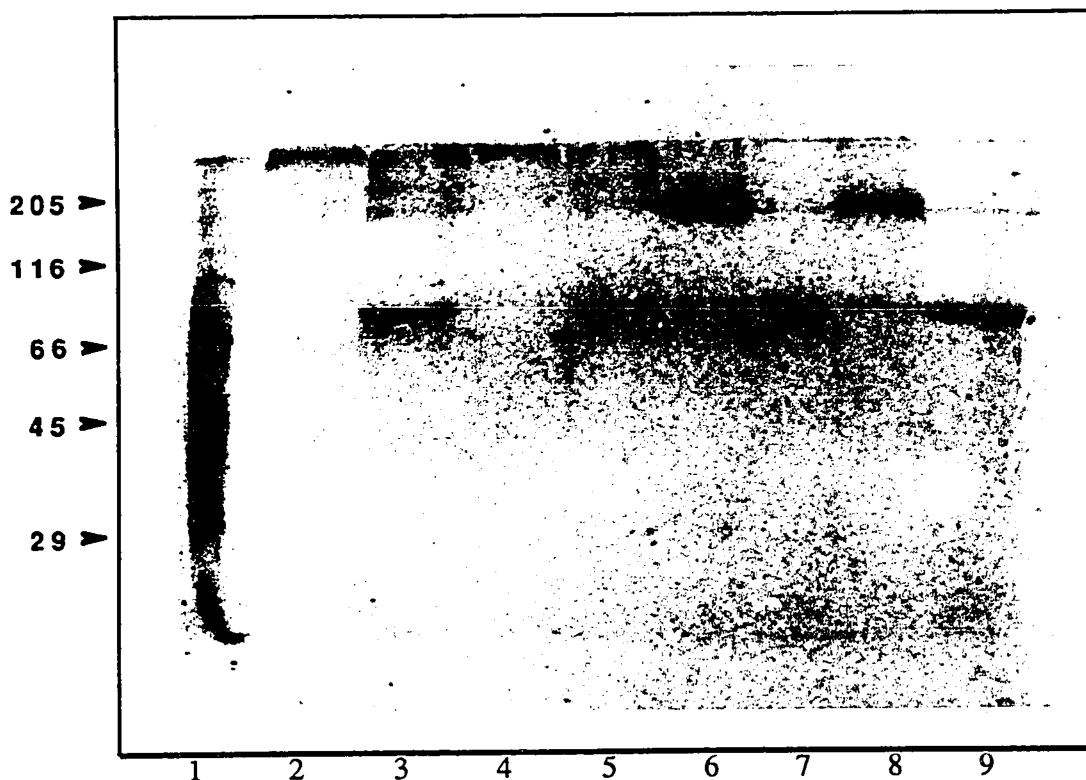


Figure 4. Coomassie gel of DSS and Glutaraldehyde cross-linking of solubilized PAS IV.

Lane 1: Molecular weight marker
Lane 2: 10μg PAS IV/25μl; - βME; 8mM glutaraldehyde
Lane 3: 10μg PAS IV/25μl; + βME; 8mM glutaraldehyde
Lane 4: 100μg PAS IV/25μl; - βME; 8mM glutaraldehyde
Lane 5: 100μg PAS IV/25μl; + βME; 8mM glutaraldehyde
Lane 6: 10μg PAS IV/25μl; - βME; 0.25mM DSS
Lane 7: 10μg PAS IV/25μl; + βME; 0.25mM DSS
Lane 8: 100μg PAS IV/25μl; - βME; 0.25mM DSS
Lane 9: 100μg PAS IV/25μl; + βME; 0.25mM DSS

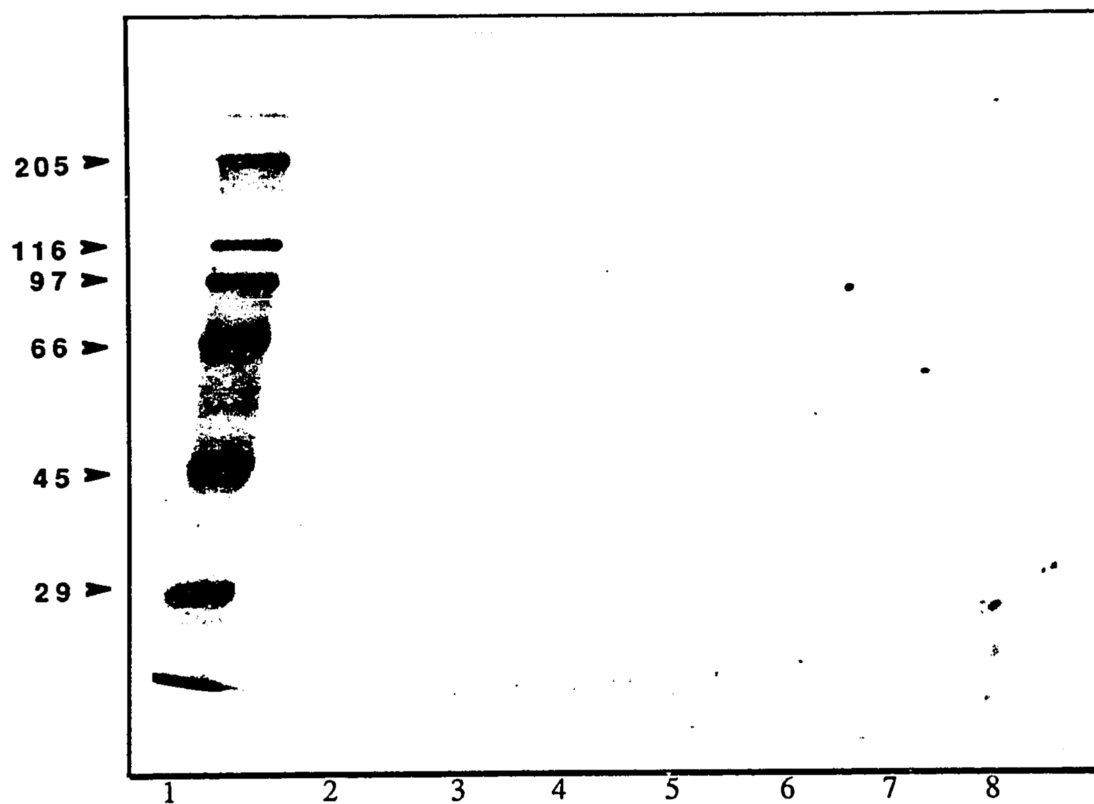


Figure 5. Coomassie gel showing cross-linking of PAS IV with different DSS concentrations.

Lane 1: Molecular weight marker
Lane 2: PAS IV control
Lane 3: 2μM DSS; - βME
Lane 4: 2μM DSS; + βME
Lane 5: 20μM DSS; - βME
Lane 6: 20μM DSS; + βME
Lane 7: 200μM DSS; - βME
Lane 8: 200μM DSS; + βME

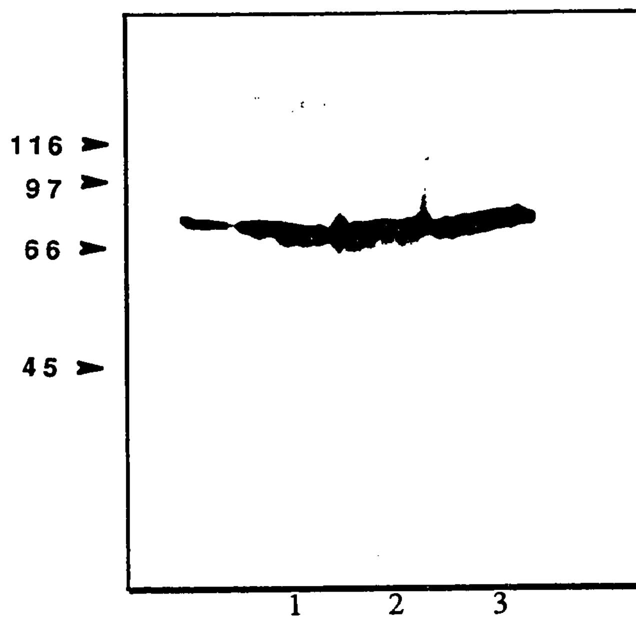


Figure 6. Immunoblot showing cross-linking of MFGM with 1 mM DSS.

Lane 1: MFGM control
Lane 2: MFGM; DSS; - βME
Lane 3: MFGM; DSS; + βME

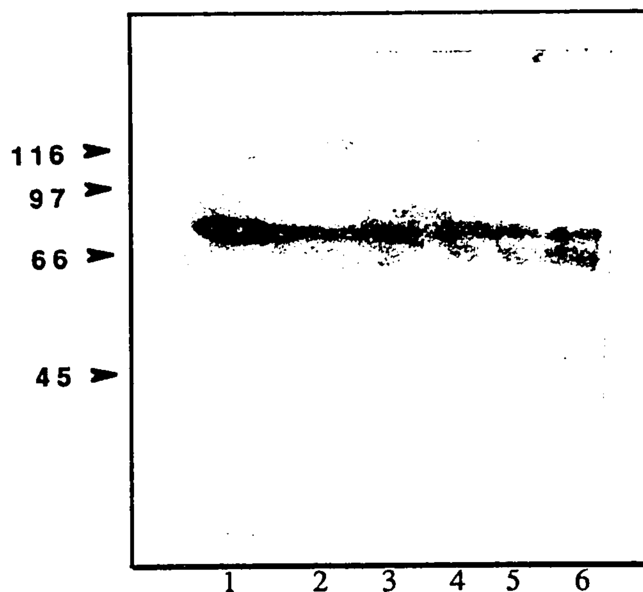


Figure 7. Immunoblot of MFGM cross-linking with 1mM DSS for various incubation periods.

Lane 1: 0 minute
Lane 2: 30 minutes
Lane 3: 45 minutes
Lane 4: 60 minutes
Lane 5: 120 minutes
Lane 6: 180 minutes

the various incubation periods and the bands were diffused in this blot, the 30 minute and 60 minute samples were re-run with the control. No dimers were seen (Fig. 8, *lane 2 and lane 3*).

Cross-linking of papain-digested MFGM: When the papain-digested MFGM was cross-linked with 1mM DSS, PAS IV was seen as a monomer in the presence and absence of β-mercaptoethanol (Fig. 9, *lane 4 and lane 5*). When the same samples were analyzed by immunoblotting, dimers were not observed. However, the control sample showed a number of smaller molecular weight bands (Fig. 10, *lane 1*). Such bands were not seen in the Coomassie blue stained control. An increase in DSS concentration (3 mM), also did not result in dimerization (Fig. 9, *lanes 6 & 7*). Lane 10 of Fig. 9 is solubilized PAS IV cross-linked with 1mM DSS in the absence of β-mercaptoethanol. It was included as a positive control.

Cross-linking of deglycosylated MFGM: The 20 hour deglycosylated MFGM when treated with 1mM DSS did not cross-link as a dimer both in the presence and absence of β-mercaptoethanol (Fig. 11, *lanes 2 & 3*).

Copper-Phenanthroline oxidation

Upon oxidation with Cu(II) (1, 10-phenanthroline)$_3$ as a catalyst, the membrane-bound PAS IV did not migrate as a disulfide cross-linked dimer (Fig. 12, *lane* 2). *Lane 1* of Fig. 12 is the control without the oxidation catalyst.

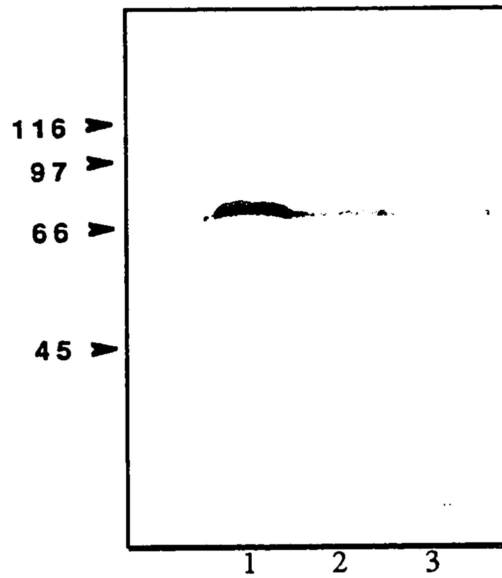


Figure 8. Immunoblot of MFGM cross-linking: 0, 30 and 60 minute incubations.

Lane 1: 0 minute
Lane 2: 30 minutes
Lane 3: 60 minutes

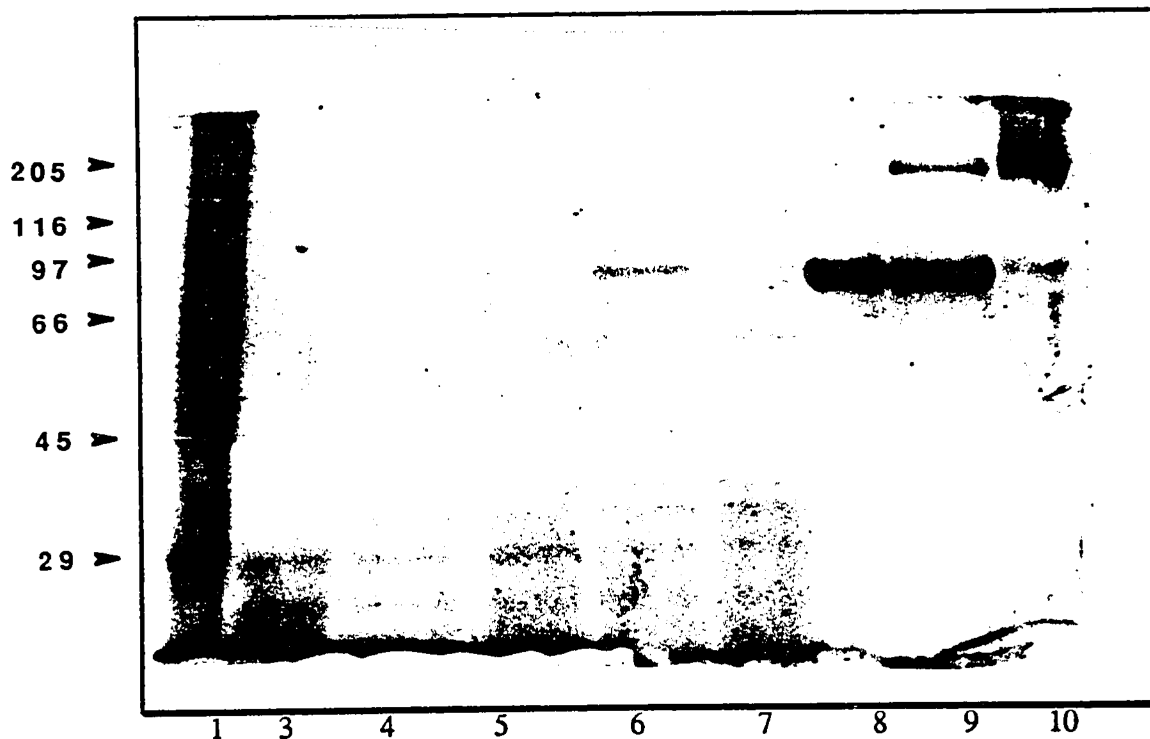


Figure 9. Coomassie gel showing cross-linking of papain digested MFGM.

Lane 1: Molecular weight marker
Lane 2: MFGM homogenate (not included)
Lane 3: Papain digested MFGM control
Lane 4: Papain digested MFGM;1 mM DSS; + β ME
Lane 5: Papain digested MFGM;1 mM DSS; - β ME
Lane 6: Papain digested MFGM;3 mM DSS; + β ME
Lane 7: Papain digested MFGM;3 mM DSS; - β ME
Lane 8: Solubilized PAS IV control
Lane 9: Solubilized PAS IV; 1 mM DSS; + β ME
Lane 10: Solubilized PAS IV; 1 mM DSS; - β ME

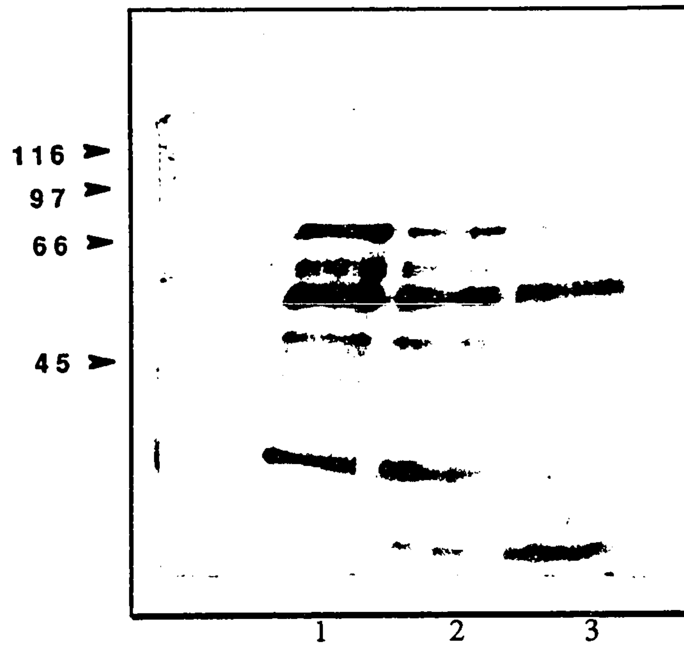


Figure 10. Immunoblot of papain digested MFGM cross-linking.

Lane 1: Papain digested MFGM control
Lane 2: Papain digested MFGM;1 mM DSS; + β ME
Lane 3: Papain digested MFGM;1 mM DSS; - β ME

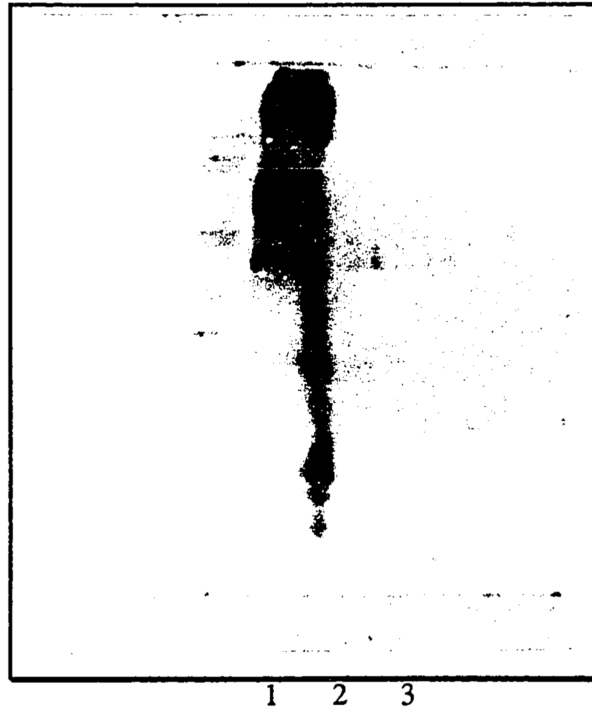


Figure 11. Immunoblot showing cross-linking of endo F digested MFGM.

Lane 1: PAS IV; 1 mM DSS; - β ME
Lane 2: endo F digested MFGM; 1 mM DSS; + β ME
Lane 3: endo F digested MFGM; 1 mM DSS; - β ME

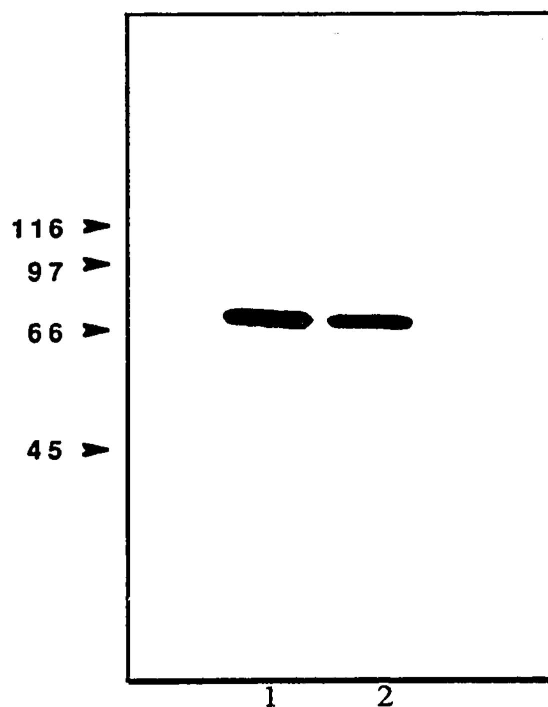


Figure 12. Immunoblot of copper-phenanthroline oxidation of MFGM.

Lane 1: MFGM control without oxidation catalyst
Lane 2: MFGM in the presence of copper-phenanthroline catalyst

4. DISCUSSION

In the absence of β-mercaptoethanol, solubilized PAS IV cross-linked as a dimer with DSS and glutaraldehyde. In addition, cross-linking with glutaraldehyde yielded higher molecular weight oligomers. In the presence of β-mercaptoethanol, PAS IV did not cross-link; instead, it migrated as a monomer. This difference can be explained as follows: β-mercaptoethanol reduces the disulfide linkages between the subunits to form monomers. These monomeric subunits are not close enough for the cross-linker to react. In the absence of β-mercaptoethanol, the subunits are linked together via disulfide linkages. Since the subunits are close together, the cross-linker forms a covalent linkage between them. β-Mercaptoethanol was present in the SDS-PAGE buffer and the samples were boiled in this buffer after the cross-linking reaction. This shows that β-mercaptoethanol does not have the capacity to break the covalent linkage once it has formed. There was no change in the amount of dimer formed when the protein concentration was increased by a factor of 10. This indicates that no random intermolecular collisions are participating in the cross-linking reactions. With solubilized Na^+K^+-ATPase, a similar result has been reported with different protein concentrations (Craig, 1988).

Having obtained a cross-linked dimer with the solubilized protein, the protocol was extended to the native membrane-bound form of the protein. However, PAS IV did not cross-link as a dimer when it was bound to the membrane. The absence of the dimer in the membrane-bound form could be attributed to two different reasons:

1. The cross-linking reagent might be sufficiently reactive to cross-link all the neighboring proteins in the membrane. In this situation, the cross-linked product will be very large and therefore will not enter the SDS gel. It is also possible that the neighboring proteins interfere with the cross-linking reaction.

2. PAS IV is a glycoprotein and the N-linked oligosaccharides on the protein surface might interfere with the cross-linking reaction.

To eliminate the first possibility, the MFGM was digested with papain and with papain+trypsin. It is known that PAS IV is resistant to proteinases when bound to the membrane (Greenwalt & Mather, 1985). PAS IV was the major protein present after digestion and most of the other proteins were degraded. The amount of PAS IV recovered after digestion was 3% which is in the range of 2.5–5% reported earlier (Greenwalt & Mather, 1985). The presence of smaller molecular weight fragments in the immunoblot indicated that PAS IV was not as "resistant" to proteinases as is evident in the Coomassie stained gel. There was no significant difference between the papain digested and papain+trypsin digested samples—the papain digestion alone is sufficient to degrade most proteins. Therefore, the papain digested sample was used for the cross-linking reaction. Since PAS IV was the major constituent of this digest, it was thought that the interference due to other membrane proteins would be considerably decreased. However, even with the use of the papain digested MFGM, PAS IV did not cross-link as a dimer. It migrated as a monomer and there was no aggregation, indicating that it was not cross-linked to other proteins.

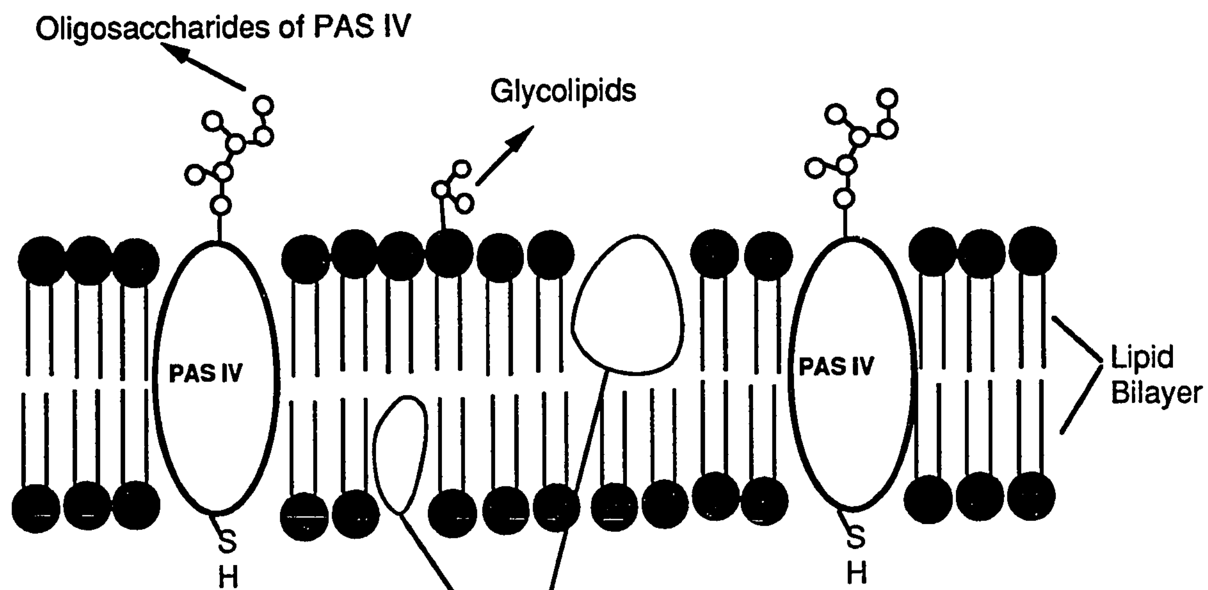
For ruling out the possible interference of the oligosaccharides, the MFGM was deglycosylated using endo F. A time course study of the deglycosylation of PAS IV with endo F identified seven bands. This would correspond to the presence of six N-linked oligosaccharides in bovine PAS IV. The time course study thus serves as an effective method to quantify the number of oligosaccharides present in PAS IV. The maximum possible deglycosylation was attained at 10 hours and three bands were seen (2 oligosaccharides). The extent of deglycosylation was unchanged with 20 hours of incubation, and even with 20 hours of incubation with twice the amount of enzyme. It is

probable that the orientation of PAS IV within the membrane prevents the enzyme from acting on these two oligosaccharides. The 20 hour deglycosylated MFGM did not yield dimers upon cross-linking.

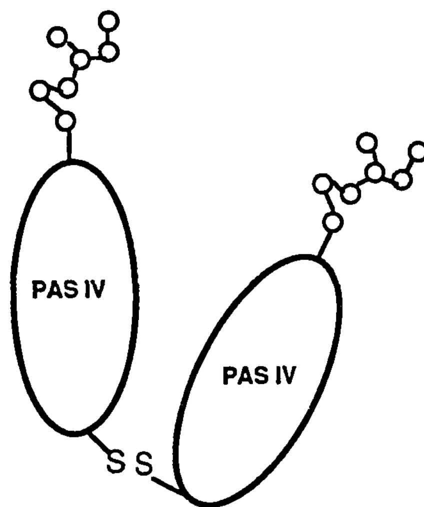
Copper-phenanthroline complex has the ability to catalyze the air oxidation of sulfhydryl groups in a protein to form disulfide bonds (Kobashi, 1968). Oxidation with the copper-phenanthroline complex has been used to study the subunit interactions of proteins (Askari et al., 1980; Falke & Koshland, 1987). Therefore, copper-phenanthroline oxidation was used as yet another cross-linking method. MFGM was oxidized by this method and the samples were analyzed on a non-reducing gel. PAS IV migrated as a monomer indicating that it was not cross-linked to another subunit via disulfide linkages.

Differences in the cross-linking patterns of solubilized and membrane-bound forms of the same protein have been reported by other workers. When $Na^{+}K^{+}$-ATPase was solubilized with a nonionic detergent octaethylene glycol dodecyl ether and cross-linked with glutaraldehyde an α,β-dimer was formed. However, when membrane-bound enzyme was cross-linked with glutaraldehyde none of the covalent products was capable of entering the SDS gel (Craig, 1988). On the other hand the membrane-bound enzyme formed an α,α-dimer with copper-phenanthroline complex (Huang et.al, 1988). Purified cytidylyltransferase when bound to a detergent micelle or membrane vesicle existed as a dimer on cross-linking with glutaraldehyde and DSP. In the absence of a membrane or micelle, the dimers self-aggregated in a reversible manner (Cornell, 1989).

Membrane bound PAS IV did not form dimers upon cross-linking. Neither was aggregation observed, indicating that it was not cross-linked to other proteins in the membrane. It migrated as a monomer even after the cross-linking reaction. A possible explanation for this observation is illustrated in Fig. 13. When bound to the membrane, the



Membrane-bound PAS IV



Solubilized PAS IV

Figure 13. Schematic representation of membrane-bound and solubilized PAS IV.

monomers of PAS IV are separated from each other by other proteins and lipids in the membrane. Since they are not close to each other, the cross-linker is unable to form a linkage between any two monomers. Solubilization with the detergent Triton-X 114 removes the membrane layer. During the solubilization procedure, the membrane preparation is stirred, centrifuged, and dialyzed. In any of these processes, two monomers of PAS IV are occasionally brought close together. Air-oxidation of the free sulfhydryl groups on the monomers results in the formation of a disulfide bond. The two monomers now exist as a dimer since they are linked together by a disulfide bond. Also, in the solubilized form the orientation of PAS IV may be such that it facilitates the formation of a disulfide-linked dimer. Such an orientation may not have been possible in the membrane-bound form. The probability that other forces such as hydrophobic interactions allow the formation of dimers is very low, since a difference in cross-linking pattern was observed both in the presence and absence of β-mercaptoethanol. A difference would not have been observed if the subunits were held together by other forces. β-mercaptoethanol reduces the disulfide linkages between the subunits to form monomers and in its presence PAS IV migrated as a monomer. In the absence of β-mercaptoethanol the subunits are still linked together via a disulfide bond. Solubilized PAS IV was therefore seen as a dimer upon cross-linking.

5. CONCLUSIONS

Solubilized bovine PAS IV exists as a dimer as indicated by cross-linking with DSS and glutaraldehyde. The cross-linked product is not a result of random collisions between various subunits. This is evident from the fact that an increase in PAS IV concentration did not alter the amount of dimer formed. PAS IV did not cross-link as a dimer when present as the native membrane-bound form. It did not cross-link even after the removal of:

(i) the neighboring proteins in the membrane; and

(ii) the N-linked oligosaccharides.

Either of these factors could possibly interfere with the cross-linking reaction. Also, oxidation of MFGM did not yield a dimer. It is probable that the solubilization procedure alters the orientation of PAS IV such that it exists as a dimer.

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