LIGAND INDUCED CHANGES IN AMINO ACID ACCESSIBILITY OF P-GLYCOPROTEIN

by

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ABSTRACT

P-Glycoprotein (Pgp) is a 170 kDa plasma membrane protein belonging to the ATP-Binding Cassette (ABC) superfamily. The expression of this protein in tumor cells leads to the emergence of a multidrug resistance phenotype (MDR) which is thought to be responsible for the failure of chemotherapy in some forms of cancer. Pgp is an ATP-dependent drug efflux pump, using the energy of ATP hydrolysis to mediate drug transport. The mechanism of substrate recognition and transport, however, is poorly understood.

There is strong evidence that Pgp proceeds through various structural changes as it goes through its catalytic cycle. In the present study, non-selective fluorescent labeling with a residue-specific fluorescent modification agent was used to locate regions on Pgp that undergo conformational changes with substrate binding. A lysine-specific modification agent, DEACSE, was used to label Pgp and fluorescent peptide maps were generated by protease cleavage. The labeling profiles of Pgp in the presence of various substrates were compared to pinpoint regions that exhibited ligand-dependent changes in accessibility to DEACSE.

Several cleavage products show significant changes in fluorescence intensity with different substrates. Selected peptides were identified by MALDI-TOF mass analysis and N-terminal peptide sequencing. Two peptides with reduced labeling upon nucleotide binding were localized respectively to the amino (^{525}GAQLSGGQ**K**QR) and carboxyl (^{1170}GTQLSGGQ**K**QR) Walker C signature motif within the nucleotide binding domains of Pgp (modified lysine residue in bold and underlined). On the other hand, two peptides with increased labeling were observed in the predicted second cytoplasmic loop. Peptide ^{260}TVIAFGGQ**K**K appears to be the most

dynamic and experienced a significant increase in fluorescence while the second peptide, ^{284}LGI**K**K, encountered a more modest increase.

These findings indicate that the non-selective fluorescent modification technique developed in this study can be used to map in greater detail areas of conformational changes in Pgp. This technique should be applicable also to other protein systems, including a number of ABC proteins. Initial results from this study are consistent with the proposal by others that the Walker C signature sequence may be involved in coupling ATP binding and/or hydrolysis to substrate transport. In addition, they show that nucleotide binding can induce significant structural changes within the transmembrane domains located at a distance along the linear sequence of the protein from the nucleotide binding sites. These results provide evidence that supports a mechanism in which energy is transduced to effect a global conformational change associated with transport.

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LIST OF ABBREVIATIONS

ABC: ATP- binding cassette

AMPPNP: 5'-adenylylimido-diphosphate

ATP: adenosine 5'-triphosphate

CFTR: cystic fibrosis transmembrane conductance regulator

DEACSE: 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester

EDTA: ethylenediaminetetraacetic acid

HlyA: hemolysin A

HlyB: hemolysin B

MALDI-TOF-MS: matrix-assisted laser desorption ionization time-of-flight mass spectroscopy

MDR: multidrug resistance

MOPS: 3-(N-morpholino)propanesulfonic acid

NBD: nucleotide binding domain

Pgp: P-Glycoprotein

PMSF: phenylmethylsulfonyl fluoride

Rh123: rhodamine 123

RP-HPLC: reversed phase-high performance liquid chromatography

RT: room temperature

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

STE6: sterile 6

TMD: transmembrane domain

Tricine: N-tris(hydroxymethyl)methylglycine

Tris: tris(hydroxymethyl)aminomethane

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CHAPTER 1: INTRODUCTION

Introduction to ABC Transporters

Transmembrane transport is crucial to the survival of all living organisms. This process is required for many key cellular functions, from the import of nutrients to the export of cytotoxic endogenous or exogenous agents. Various membrane-associated proteins are responsible for these processes. One of the largest and most diverse family of these membrane proteins is the ATP-Binding Cassette (ABC) superfamily (for reviews see; Higgins, 1992; Fath and Kolter, 1993; Dean and Allikmets, 1995). This protein family is characterized by a highly conserved ATP-binding domain. Generally, these proteins utilize the energy derived from ATP hydrolysis to pump their substrates across the various membranes. This transport function requires the cooperation of multiple protein domains that can be encoded as multiple polypeptides or as a single protein.

A typical ABC protein contains 4 structural domains; 2 hydrophobic membrane-associated domains and 2 hydrophilic ATP binding domains (Figure 1). These proteins are of great interest due to their roles in many biological processes from bacteria to humans. In *E. coli*, the hemolytic toxin, Hemolysin A is transported by the Hemolysin B transport complex (Sheps *et al.*, 1996). In *S. cerevisiae*, the yeast a-mating factor is transported by the STE6 protein (Kuchler *et al.*, 1989). Lastly, in humans CFTR functions as a chloride specific channel (Riordan *et al.*, 1989). Interestingly, many of these proteins have been linked to clinical problems, including cystic fibrosis, caused by mutations of CFTR, and multidrug resistance in

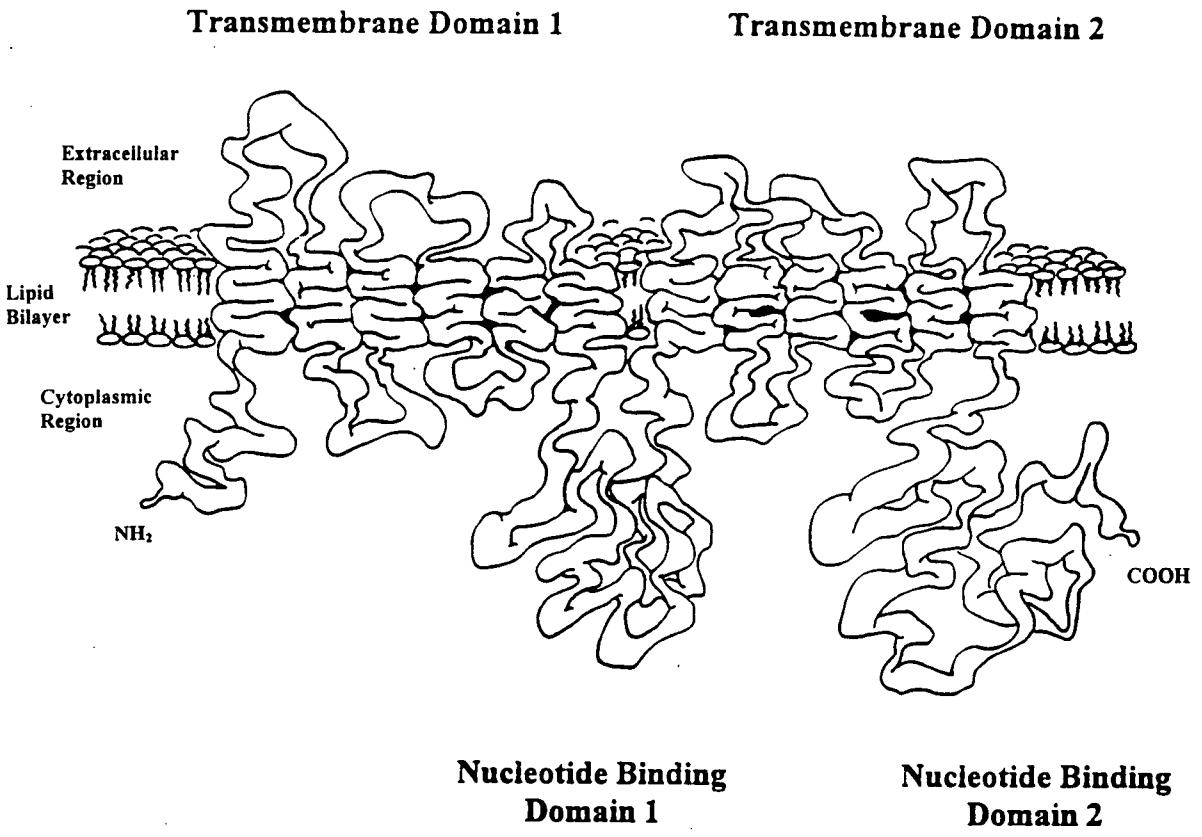


Figure 1: Putative structural organization of P-Glycoprotein.

cancer, caused by the over expression of Pgp. Most ABC proteins are very specific for a particular substrate (some examples are given above). However, P-Glycoprotein is of particular interest because of its ability to transport a wide variety of structurally dissimilar compounds and therefore give rise to multidrug resistance (MDR).

Introduction to P-Glycoprotein

P-Glycoprotein (Pgp) is a 170 kDa ABC plasma membrane protein containing 1276 amino acids (for reviews see; Gottesman and Pastan, 1993; Bosch and Croop, 1996). It consists of 2 transmembrane domains and 2 ATP-binding domains encoded on a single polypeptide. According to hydropathy predictions, Pgp is organized with each transmembrane domain consisting of six membrane spanning segments (Figure 1). This topology has been confirmed by several groups using epitope insertion (Kast *et al.*, 1995, 1996) and cysteine insertion (Loo and Clarke, 1995a). Several other groups, however, have discovered that Pgp may exist in other topologies (Bibi and Beja, 1994; Beja and Bibi, 1995; Zhang and Ling, 1991; Zhang *et al.*, 1993; Skach *et al.*, 1993; Skach and Lingappa, 1993, 1994). Zhang and Ling (1995) proposed that the transmembrane domains of Pgp are dynamic structures that can exist in different topologies regulated by specific cytoplasmic factors.

The overexpression of P-Glycoprotein in tumor cells leads to the emergence of the multidrug resistance (MDR) phenotype that is thought to be in part responsible for the failure of chemotherapy treatments for cancer (Riordan and Ling, 1985; Endicott and Ling, 1989; Ling, 1997). The mechanism by which Pgp confers MDR has been the focus of numerous studies

(Shapiro and Ling, 1995a; Sharom, 1997; Senior *et al.*, 1995). Purified and reconstituted Pgp alone has been demonstrated to act as an ATP-dependent drug efflux pump (Sharom *et al.*, 1993; Urbatsch *et al.*, 1994; Shapiro and Ling, 1995b).

Pgp couples the energy of ATP hydrolysis to transport of a wide variety of hydrophobic drugs out of the cell, thereby decreasing the intracellular drug concentration below cytotoxic levels. The most interesting characteristic of Pgp is its ability to interact with and/or transport an enormous range of structurally dissimilar substrates. Pgp has been demonstrated to interact with chemotherapy agents (Demmer *et al.*, 1997; Ferry *et al.*, 1995; Bruggemann *et al.*, 1992), and chemosensitizers, compounds able to reverse MDR (Demeule, *et al.*, 1997; Safa, 1988). Other studies indicate that Pgp can also interact with a number of steroids (Barnes *et al.*, 1996), lipids (Van Helvoort *et al.*, 1996), hydrophobic peptides (Sharma *et al.*, 1992; Sarkadi *et al.*, 1994) and ionophores (Eytan *et al.*, 1994).

According to the "hydrophobic vacuum cleaner" model (Gottesman, 1993), substrates are actively displaced, by Pgp, directly from the plasma membrane to the exterior of the cell. This has been confirmed experimentally (Shapiro *et al.*, 1997; Homolya *et al.*, 1993; De Graaf *et al.*, 1996). This mechanism helps to explain the broad substrate specificity of Pgp, since its hydrophobic substrates concentrate in the plasma membrane. As a result, substrates need not be recognized with high affinity. Indeed, Pgp has been found to possess relatively low affinities for some of its drug substrates (Liu and Sharom, 1996). Pgp has been shown to contain at least two sites for binding of transported substrates, with distinct but overlapping specificities (Shapiro and Ling, 1997b; Dey *et al.*, 1997; Ferry *et al.*, 1992; Tamai and Safa, 1991). Transport of substrates from either site appears to occur via the cytoplasmic leaflet of the plasma membrane to the

exterior of the cell (Shapiro and Ling, 1997a, 1998). With the recent biochemical and genetic information, the mechanism of ATP-dependent multidrug transport by P-Glycoprotein is just beginning to be appreciated.

Thesis Research

The ability to confer MDR by P-Glycoprotein requires the cooperation of all 4 protein domains; 2 hydrophobic membrane domains and 2 hydrophilic ATP binding domains. Loo and Clarke (1995b) has demonstrated that the different domains within Pgp interact. Furthermore, there is strong evidence that Pgp undergoes structural changes as it goes through its transport cycle. Using infrared spectroscopy to measure hydrogen/deuterium exchange, Sonveaux *et al.* (1996) discovered that Pgp undergoes tertiary conformational changes in the presence of different substrates. In the presence of MgATP there was an increased solvent accessibility to the protein surface. Conversely, in the presence of MgATP and the chemosensitizer verapamil, there was a decreased accessibility.

Fluorescence quenching experiments by Liu and Sharom (1996) indicated that there is "conformational communication" between the drug-binding site(s) and the ATP-binding site. Several groups have identified specific areas on the protein that may be essential for communication between domains. Based on information from limited proteolysis experiments, Wang *et al.* (1997) proposed that a large portion of the C-terminal transmembrane domain undergoes intramolecular motion upon nucleotide binding. Mechetner *et al.* (1997) demonstrated that the first extracellular loop changed in accessibility to an anti-Pgp antibody

upon nucleotide binding. In addition, Loo and Clarke (1997a) have demonstrated by oxidative cross-linking that transmembrane segments 6 and 12 undergo conformational changes during drug binding or ATP hydrolysis. Structural changes within the nucleotide binding domain of the hemolysin exporter, Hemolysin B (HlyB), a structurally homologous bacterial ABC protein (Gerlach *et al.*, 1986), was also detected by limited proteolysis (Koronakis *et al.*, 1993). However, it is of great interest to our laboratory to locate the areas on Pgp where these conformational changes are occurring with greater precision.

Protein modification agents are frequently used in biochemical and biophysical studies to obtain structural information (for review see Brinkley, 1992). A non-specific fluorescent labeling technique is proposed in this thesis to identify areas on P-Glycoprotein that undergo changes in solvent accessibility as a result of substrate binding and substrate-induced conformational changes. In order to pinpoint amino acid residues that alter in accessibility, a fluorescent residue-specific protein modification agent will be used to non-selectively label Pgp. Fluorescent tryptic peptides of the purified Pgp can be separated by reversed phase HPLC. Comparing the peptide maps would reveal peptides that exhibit changes in accessibility to the fluorescent label in the presence of substrates. These peptides can be identified by using a combination of matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) and N-terminal peptide sequencing.

Based on the hydrogen/deuterium exchange results of Sonveaux *et al.* (1996), a certain labeling profile would be expected for the control situation, with no substrate present, as shown by the illustration in Figure 2. MgATP should increase the accessibility of labeling overall, and the opposite should occur with MgATP and verapamil. On the other hand, residues involved in

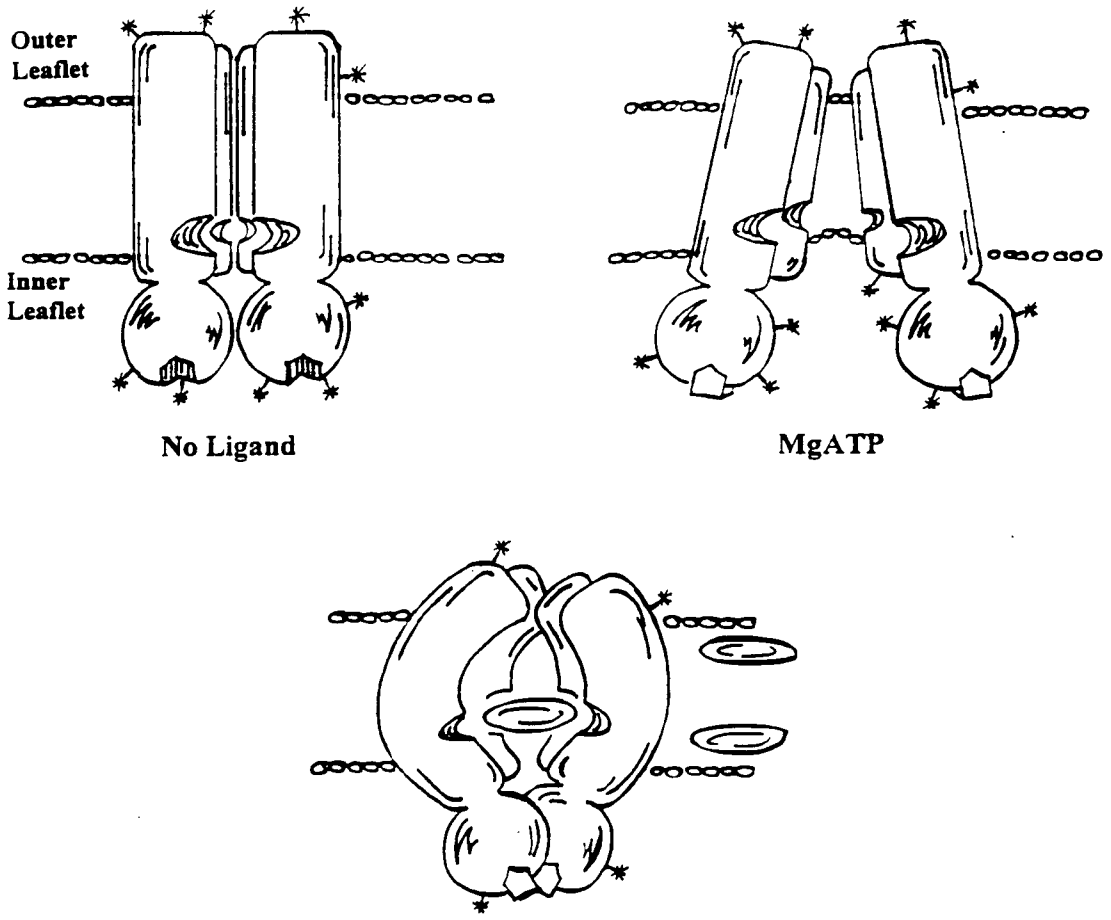


Figure 2: Illustration depicting possible fluorescent labeling results with the binding of different P-Glycoprotein substrates. *In this figure, (*) represent sites of fluorescent modification on Pgp in the presense of No Ligand, MgATP (□), and MgATP and verapamil (○).*

substrate binding should become less accessible in the present of the substrate. Furthermore, individual residues throughout the protein could show different behaviour than the average effect outlined by Sonveaux *et al.*

Structural information about Pgp has been obtained by photoaffinity labeling with photoactivatable drug analogues (Greenberger *et al.*, 1991; Greenberger, 1993; Bruggemann *et al.*, 1989; Borchers *et al.*, 1995) and ATP analogues (Sankaran *et al.*, 1997a, 1997b). Mutagenesis studies have also revealed a great deal of structural information (Loo and Clarke, 1995a, 1996, 1997b). The real advantage of the generalized labeling technique developed in this study is its ability to define more precisely regions on Pgp that undergo changes in conformation without imposing any bias onto the system.

Identifying regions of Pgp that undergo a change in solvent accessibility with the binding of different ligands would assist in understanding the mechanism of efflux by Pgp, thereby increasing our understanding of the mechanism by which cancer cells become multidrug resistant. This information would also be of great biochemical significance to the ABC transporter superfamily as a whole. In addition, the generalized labeling technique described in this thesis could be generally useful for studying conformational changes in other proteins.

CHAPTER 2: METHODS AND MATERIAL

Cell Culture

Drug-sensitive Chinese Hamster Ovary AuxB1 cells (Ling and Thomson, 1974) and the colchicine-selected drug-resistant CH^RB30 cells (Kartner *et al.*, 1985), derived from the AuxB1 cell line, were grown in α-Minimum Essential Medium (Stem Cell Technologies) containing 10% fetal bovine serum with 100 μg/ml of both streptomycin and penicillin (Canadian Life Technologies), under 5% CO_2 at 37 °C. B30 cells were maintained in 30 μg/ml colchicine. For plasma membrane isolation, cells were grown as monolayers in 1200 cm^2 roller bottles (Bellco Glass) and were harvested by physical shearing after incubation with citrate saline (15 mM sodium citrate, 134 mM KCl, pH 8) containing 0.5 mM EDTA (Shapiro and Ling, 1994). Each roller bottle yielded approximately $2x10^8$ cells. The cells were washed with citrate saline/EDTA and the cell pellets were stored at -80 °C.

Preparation of Plasma Membranes

Plasma membranes were isolated from both B30 and AuxB1 by discontinuous sucrose gradient centrifugation (Shapiro and Ling, 1994). Cell pellets containing 6 x 10^9 cells, were thawed and quickly suspended in cold buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM leupeptin, and 4 μg/ml

pepstatin A. All protease inhibitors were purchased from Sigma. The cell suspension was sonicated in a steel beaker set in ice for four 30 second bursts with a 30 second cooling period between bursts. The cell extract was pelleted in the ultracentrifuge (145,000 x $g_{max,}$ 4 °C, 1 h) and the supernatant containing soluble cell components was discarded. The membrane pellet was resuspended in a total of 42 ml of cold 16% (w/v) sucrose containing 5 mM Tris-HCl (pH 7.4) using a 40 ml glass Dounce homogenizer. This suspension was carefully layered on top of 31 ml of 31% (w/v) sucrose containing 5 mM Tris-HCl (pH 7.4), and centrifuged in a SW28 swinging bucket rotor (100,000 x g_{max}, 4 °C, 18 h). Plasma membrane vesicles collected from the 16/31 % sucrose interface, were divided into 1 ml aliquots, and stored at -80 °C until use.

Fluorescent Labeling of Plasma Membrane Vesicles

Fluorescent labeling experiments employed 300-500 µl of plasma membrane suspension that contained 120-200 µg of total protein, 15% of which was Pgp. Aliquots of plasma membranes were thawed and diluted in cold TNE buffer containing 50 mM Tricine-NaOH (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaN_3, 0.1 mM ouabain (Sigma). The optimum pH for maximum drug-stimulated ATPase activity was reported to be pH 7.4 (Al-Shawi and Senior, 1993). The inhibitor cocktail has been used by several groups (Sarkadi *et al.*, 1992, Al-Shawi and Senior, 1993) to eliminate activity from potential contaminating ATPases. Sodium azide was used to inhibit F_1 -F_0 -ATPase, ouabain to inhibit Na^+-K^+-ATPase and EGTA to inhibit calcium-dependent ATPase activity. The membranes were pelleted by centrifugation (100,000 x g, 4 °C, 45 min) to remove sucrose. The pellets were resuspended in a desired

volume of TNE buffer. The fluorescent labeling was carried out by incubating the plasma membrane suspension with the modification agent at 37 °C for 1-5 minutes with final probe concentration of 200-350 μM. All fluorescent labeling agents were obtained from Molecular Probes. The reaction was stopped by the addition of 15 mM ethanolamine.

Purification of Labeled P-Glycoprotein

DEAE-Cellulose Chromatography Purification of Labeled P-Glycoprotein - The labeling reaction samples were eluted through 3 mL spin columns by centrifugation (500 x g, 4 °C, 5 min). The spin columns were prepared with Sephadex G-50 (fine) (Sigma) pre-equilibrated with ME buffer containing 50 mM MOPS-HCl (pH 7.0) and 2 mM EDTA, and then prespun before use (500 x g, 4 °C, 5 min). The flow-through volumes were equalized with ME buffer. Zwittergent 3-12 (Calbiochem) was added to 6 mM and the membranes were vortexed vigorously and sonicated to ensure complete solubilization. A 200 μl anion exchange column was prepared with DEAE-cellulose (Whatman DE52) pre-equilibrated with MEZ buffer containing 50 mM MOPS-HCl (pH 7.0), 2 mM EDTA, and 6 mM Zwittergent 3-12, after the removal of fines. The labeled Pgp was purified from the solubilized membrane by a series of steps. First, the sample was eluted through the DEAE-cellulose column and the column was washed with equal volume of MEZ buffer. Shapiro and Ling (1994) found that, on a larger scale, elution with the MEZ buffer allowed for nearly quantitative yield of Pgp through the DEAE-cellulose column while most other membrane proteins were retained on the column. The flow

through and the wash were combined and concentrated with Centricon-100 concentrators (Amicon Inc.) by centrifugation (500 x g, 4 °C, 30 min). The retentate was also collected by centrifugation (500 x g, 4 °C, 10 min). Purified, fluorescently-labeled Pgp was precipitated overnight at -20 °C with 9 volumes of methanol. The protein was pelleted by centrifugation (16,000 x g, room temperature (RT), 15 min) and the supernatant was discarded. The pellet was resuspended in 50 mM NH_4HCO_3 (pH 7.8) for trypsin digestion.

Immunoprecipitation Purification of Labeled P-Glycoprotein - The samples from the labeling reaction were eluted through 3 ml spin columns by centrifugation (500 x g, 4 °C, 5 min). The columns were prepared with Sephadex G-50 (fine) pre-equilibrated with 10 mM Tris-HCl (pH 7.4), and then prespun before use (500 x g, 4 °C, 5 min). The flow-through was adjusted to 400 µl with 10 mM Tris-HCl. The membranes were solubilized by vigorous vortexing and sonication, using a bench top bath sonicator (Laboratory Supplies Inc.), after the addition of 100 µl of 5x buffer C [6% (v/v) Triton X-100, 1 M NaCl, 0.25 M Tris-HCl (pH 7.4), and 0.5% (v/v) SDS]. To each sample (equivalent to 120-200 µg of total protein) approximately 100 µl of C219 linked Protein A Sepharose CL-4B was added as a slurry in 1x buffer C. The affinity binding was allowed to proceed at 4 °C overnight, with gentle agitation on a nutator (Adams). The method of cross-linking C219 anti-Pgp monoclonal antibody (mAb) to Protein A Sepharose CL-4B (Pharmacia Biotech) is described in detail below. The immunoaffinity-Sepharose was washed twice with 1 ml of buffer D which contained 0.1% (v/v) Triton X-100, 0.03% (v/v) SDS, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4), twice with Tris buffered saline (TBS) containing 150 mM NaCl and 10 mM Tris-HCl (pH 7.4), and finally twice with MilliQ water (Millipore

Corp.). After careful removal of the MilliQ water, the labeled Pgp was eluted from the C219 - Sepharose with 400 µl of 0.1% (v/v)TFA in MilliQ water (pH 2), which was quickly neutralized with 100 µl of 0.1 M NH_4HCO_3 (pH 7.8) and subjected to trypsin digestion.

Preparation of C219-Protein A Sepharose CL-4B

The C219-Protein A Sepharose CL-4B were generated by cross-linking C219 anti-P-Glycoprotein monoclonal antibody to Protein A Sepharose CL-4B using a homobifunctional crosslinker dimethylpimelimidate (Sigma) as described by Harlow and Lane (1988). Protein A Sepharose CL-4B (approximately 0.450 gm) was allowed to swell in buffer D for 30 minutes at RT (wet bead volume was approximately 2 ml). The Sepharose was washed twice with 15 ml of buffer D, three times with 15 ml of TBS. C219 mAb was mixed with the slurry of Protein A Sepharose CL-4B at a concentration of 2 mg per millilitre of wet beads. This mixture was rocked gently at RT for 2 hours. For more complete binding, the slurry was rocked overnight at 4 ^{o}C. After incubation, the slurry was centrifuged (500 x g, RT, 2 min) and the supernatant was discarded. The C219 bound Sepharose was washed three times with 15 ml of 0.2 M sodium borate buffer (pH 9.0), resuspended in 15 ml of the same buffer and dimethylpimelimidate was added to a final concentration of 20 mM (75 mg). The reaction was allowed to proceed with gentle rocking for 30 minutes at RT. The cross-linking reaction was quenched by incubating the C219-Sepharose with 15 ml of 0.2 M ethanolamine (pH 8.0) for 2-4 hours at RT with gentle mixing. Finally, the C219-Sepharose was washed several times with TBS and stored in 15 ml of

TBS containing 0.02% (v/v) NaN_3. Sepharose was washed twice with 1x buffer C and then resuspended in an equal volume of 1x buffer C prior to use.

Selection of Fluorescent Probes

Different lysine-specific modification agents were tested for their sensitivity and stability. Aliquots of plasma membrane vesicles (120-200 μg of total protein) were labeled with the various fluorescent probes according to the procedure described above. The labeled Pgp was purified using both methods of purification. Purified Pgp was then digested overnight at 37 °C with 5-20 μg of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated Trypsin (Worthington Biochemical Corp.). The samples were frozen and evaporated to dryness using a SpeedVac (Savant Instruments Inc.). The tryptic peptides were solubilized in 20 μl MilliQ water and insoluble material was removed by centrifugation (16,000 x g, RT, 5 min). The Pgp tryptic peptides were separated using a Waters 600 HPLC (Waters Ltd.) with a 3.9 mm x 150 mm Nova-Pak C_{18} reversed phase column (4 μm particle size, 60 Å pore size). A linear gradient of 2-52% solvent B (0.1% (v/v) TFA in CH_3CN) in solvent A (0.1% (v/v) TFA in MilliQ water) at a flow rate of 1.2 ml/ min was used for the probe screening. Fluorescence was monitored with a Water 474 scanning fluorescence detector, with the gain on the detector set to 1000, and excitation and emission bandwidths set to 18 and 40 nm, respectively. Probes were selected by their ability to non-specifically label the protein and by the intensity of the fluorescence of the labeled tryptic peptides.

Effects of Various Substrates on DEACSE Labeling of P-Glycoprotein

7-Diethylaminocoumarin-3-carboxylic acid, succinimidyl ester or DEACSE (Molecular Probes), was selected based upon its sensitivity and stability. The labeling of B30 plasma membrane vesicles with DEACSE followed the procedure outlined above with minor modifications. Aliquots of plasma membranes (1 ml; approximately 400 μg total protein) were thawed and diluted in cold TNE2 buffer containing 50 mM Tricine-NaOH (pH 8.5), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaN_3, 0.1 mM ouabain. The membranes were pelleted by centrifugation (100,000 x g, 4 °C, 45 min). The pellets were resuspended in a desired volume of TNE2 buffer, the membrane vesicles were divided into 200 μl aliquots, and the aliquots were incubated with combinations of various Pgp substrates at 37 °C for 2 minutes. Pgp substrates (Sigma) included ATP, ADP, the non-hydrolyzable ATP analogue 5'-adenylylimido-diphosphate (AMPPNP), as well as various transported substrates such as verapamil, rhodamine 123, cyclosporin A, and vinblastine. DEACSE was added to a concentration of 250 μM and the suspension was incubated for an additional minute at 37 °C. The reaction was stopped by the addition of ethanolamine to a final concentration of 15 mM. The purification of differentially labeled Pgp was performed by immunoprecipitation with C219-Protein A Sepharose and the purified Pgp was digested with trypsin as described above. The fluorescently-labeled Pgp tryptic peptides were separated by RP-HPLC using a linear gradient of 19-33% solvent B in solvent A at a flow rate of 1.2 ml/ min. Fluorescence was detected with a Waters 474 scanning fluorescence detector. The excitation and emission wavelengths were 440 and 480 nm, respectively. The fluorescent profiles were compared between the different treatments to determine whether there

was an effect on non-specific fluorescent labeling in the presence of the various Pgp substrates. Differences in labeling were analyzed using the integrated area of individual peaks calculated using the Millennium Chromatography Manager software (Waters Ltd.). Quantitation of the Pgp band in polyacrylamide gels was performed using the densitometer equipped with ImageQuant software (Molecular Dynamics).

The integrated peak areas were compared between Pgp treated with different substrates and the control with no ligands, within each experiment, in order to calculate percent differences in DEACSE labeling. The percent difference in labeling between numerous experiments were pooled for statistical analysis. One-tailed t-tests of significance were used to evaluate the effect of substrates on DEACSE labeling of Pgp, with the null hypothesis being that the difference from the control was zero (Moore ad McCabe, 1993).

SDS-PAGE

Electrophoresis was performed according to Laemmli (1970). The P-Glycoprotein yield during optimization of the two purification procedures was monitored by 8% SDS-PAGE. For immunoprecipitation, the Pgp was eluted directly from the washed immunoaffinity Sepharose by the addition of 2x treatment buffer containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.005% bromphenol blue. For the labeling experiments done in the presence of various substrates, a portion of each sample (one third to one half) was removed for SDS-PAGE analysis, in order to ensure that all samples contained equal amounts of Pgp. The aliquots were dried by SpeedVac and 2x treatment buffer was added to solubilize the

lyophilized protein. The effectiveness of the trypsin digestion was evaluated using 12% SDS-PAGE. The digested sample was dried by SpeedVac and solubilized in 2x treatment buffer for electrophoresis.

Polyacrylamide gels containing DEACSE-labeled protein or peptides were first fixed with destaining solution I (50% methanol, 10% acetic acid), and then destaining solution II (7% acetic acid, 5% methanol). The gels were visualized by scanning in blue fluorescence mode using the Storm 860 phosphorimager/fluorimager (Molecular Dynamics). Afterwards, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma). All other gels were stained with Coomassie Brilliant Blue R-250 immediately after electrophoresis.

Effects of DEACSE Labeling on P-Glycoprotein Activity

Plasma membranes aliquots (1 ml, approximately 400 μg total protein) from both the drug resistant B30 cells (done in duplicate) and the parental drug sensitive AuxB1 cells were thawed and diluted in cold TNE2 buffer. The membranes were pelleted by centrifugation (100,000 x g, 4 °C, 45 min). The pellets were resuspended in 1 ml of TNE2 buffer and kept on ice for a brief period of time. The membranes were incubated at 37 °C for 2 minutes and 150 μl was removed from each sample and added directly to 2.5 μl of 615 mM cold ethanolamine (10 mM final concentration) to use as a control (t= 0 seconds). To the rest of the sample, DEACSE was added to a final concentration of 165 μM and the reaction was allowed to proceed at 37 °C. At 10, 30, 60, 120, and 300 second intervals, 150 μl of each sample was removed and placed directly into a cold solution of ethanolamine to a final concentration of 10 mM to quench the

labeling reaction. The samples were eluted through 3 ml Sephadex G-50 spin columns by centrifugation at 500 x g for 5 minutes at 4 °C. The columns were prepared as described above, but with Sephadex G-50 pre-equilibrated with TNE buffer. Each sample was diluted to 250 µl with TNE. The diluted samples served as a pool of membranes used to test Pgp activity.

ATPase Assay - ATP hydrolysis mediated by Pgp was measured using 40 µl of the 250 µl of labeled membrane samples. The samples were diluted with assay buffer to a total volume of 200 µl. The final solution contained 15 mM Tricine-NaOH (pH 7.4), 20 mM NaCl, 0.2 mM EDTA-HCl (pH 7.4), 0.2 mM EGTA-NaOH (pH 7.4), 0.2 mM NaN_3, 0.02 mM ouabain, 3 mM $MgCl_2$, 1.5 mM ATP, and 2 mM dithiothreitol. The samples were incubated for 10 minutes at 37 °C. The reaction was stopped by the addition of 250 µl of ammonium molybdate colour reagent (6% SDS, 3% ascorbic acid, 0.5% ammonium molybdate). The release of inorganic phosphate was measured colormetrically at 850 nm according to Chifflet *et. al.* (1988), with potassium phosphate standards. The Pgp ATPase activity was calculated by subtracting the AuxB1 activity from the B30 activity, with correction for differences in the amount of protein.

Rhodamine 123 Transport Assay - Determination of rhodamine 123 transport activity of Pgp in B30 plasma membranes was preformed according to Shapiro and Ling (1997b). The rhodamine 123 transport measurements were made with a SLM-8100 spectrofluorometer (SLM-Aminco) equipped with double excitation monochromator. The cuvette holder was maintained at 37 °C for the duration of the transport experiment. The excitation and emission wavelengths were 497 nm and 526 nm, respectively. A 160 µl portion of the control or the labeled membrane vesicles

was diluted to 950 µl with TNE buffer. Rhodamine 123 was added to a final concentration of 1 µM. The solution was then transferred to a 4 mm x 10 mm quartz cuvette and incubated in the excitation light beam at 37 °C with magnetic stirring. The fluorescence was monitored for 5 minutes before the addition of 50 µl of concentrated ATP solution containing 100 mM Tricine-NaOH (pH 7.4), 60 mM $MgCl_2$, 30 mM ATP, and 40 mM dithiothreitol. The data collection proceeded for an additional 400 seconds. The fluorescence trace was normalized to a value of 1 immediately after the addition of the ATP solution, in order that the slope of the initial linear part of the fluorescence trace is the fraction of fluorescence lost per second. The slope of fluorescence decrease was use to determine the transport activity of Pgp in membrane vesicles labeled with DEACSE for various time intervals.

Protein Determination - The protein content of each sample was measured by the bicinchoninic acid (BCA) reaction (Sigma) supplemented with 0.5% SDS in order to solubilize membrane proteins. The BCA protein assay was selected for its compatibility with many detergents required to solubilize membrane proteins (Smith *et al.*, 1985). A 50 µl portion of each labeled membrane sample was solubilized with 0.5% SDS. The colour reagent was prepared by adding 1 part copper (II) sulfate pentahydrate 4% (w/v) to 50 parts bicinchoninic acid solution. To each sample, 1 ml of the colour reagent was added and the colour was allowed to developed for 30 minutes at 37 °C. The absorbance was measured at 562 nm with a Hitachi U-2000 spectrophotometer (ESBE Scientific), after the solution has been cooled to RT. The amount of protein in each sample was calculated against bovine serum albumin standards supplemented with 0.5% SDS and with the same buffer composition as the membrane samples.

Purification of DEACSE-Labeled Peptides and Determination of Labeling Stoichiometry

The immunoprecipitation purification of P-Glycoprotein was scaled up in order to purify sufficient amounts of peptides for sequencing and mass spectrometry. One 5-6 ml portion of plasma membranes corresponding to 10^9 cells was thawed and diluted with 40 ml of cold TNE2 buffer. The membranes were pelleted by centrifugation (100,000 x g, 4 °C, 45 min), and the membrane pellet were resuspended in 2 ml of TNE2 buffer. Prior to labeling, the membranes were incubated at 37 °C for 2 minutes. The fluorescent labeling was carried out by incubating the plasma membrane suspension with DEACSE to a final probe concentration of 100-250 μM and the reaction was allowed to proceed at 37 °C for 5 minutes. The reaction was stopped by the addition of ethanolamine to 12 mM and placement on ice. The membrane suspension was split and eluted through six 3 ml spin columns, by centrifugation (500 x g, 4 °C, 5 min). The columns were prepared with Sephadex G-50 pre-equilibrated with 10 mM Tris-HCl (pH 7.4), as described above. The flow-through was combined and adjusted to 4 ml with 10 mM Tris-HCl. The membranes were solubilized by vigorous vortexing and sonication after the addition of 1 ml of 5x buffer C.

The labeled Pgp was purified using 1 ml of C219-Protein A Sepharose CL-4B, added as a slurry in 1x buffer C. Binding was allowed to proceed at 4 °C overnight with gentle agitation. The C219-Sepharose was washed twice with 12 ml of buffer D, TBS, and MilliQ water. After careful removal of the MilliQ water, the labeled Pgp was eluted from the C219-Protein A Sepharose with 4 ml of 0.1% (v/v)TFA in MilliQ water, which was quickly neutralized with 1 ml of 0.1 M NH_4HCO_3. The DEACSE labeling stoichiometry was calculated from the amount of

DEACSE measured spectrophotometrically at 440 nm, and from the amount of purified Pgp measured by BCA protein assay supplemented with 0.5% SDS. The molecular weight of Pgp used for the stoichiometry calculation was 170 kDa.

The purified Pgp was digested with 50 μg of TPCK-treated trypsin at 37 °C overnight. The samples were frozen and evaporated to dryness using a SpeedVac. The pellet was redissolved in 100 μl of MilliQ water and insoluble material was removed by centrifugation (16,000 x g, RT, 5 min). The DEACSE-labeled peptides were separated by HPLC in two successive steps. The first purification involved HPLC separation using a linear gradient of 19-33% solvent B in solvent A, a change of 1% solvent B per 4 minutes, at a flow rate of 1.2 ml/ min. Due to the large amount of fluorescent material, the sensitivity of the HPLC was decreased by reducing the gain to 100 and the emission bandwidth to 18 nm. The peptides of interest were collected from the HPLC and dried using the SpeedVac. The labeled peptides were purified further by a second more shallow gradient that was specific for each individual peptide, with a change of 1% solvent B per 6 minutes. The peak of interest was again collected and dried.

Identification of DEACSE-Labeled Peptides

The peptides of interest were identified after the second purification step using a combination of matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) and amino-terminal peptide sequencing. The MALDI-MS and peptide sequencing were performed by the Nucleic Acid-Protein Service Unit (Biotechnology Laboratory, University of British Columbia).

For MALDI-TOF-MS, the lyophilized peptides were dissolved in 10 μl of solution containing 1 part CH_3CN and 2 parts 0.1% TFA (v/v) in water. Sample aliquots of 0.5 μl were mixed with an equal volume of a saturated solution of α-cyano-4-hydroxy cinnamic acid and dried onto a stainless steel target. The masses of the peptides were determined by a MALDI-TOF mass spectrometer (Ciphergen) in positive ion mode with the use of a nitrogen laser. External mass calibration was used.

The peptides were prepared for sequencing by adsorption onto a PVDF support of a Prosorb cartridge (PE Applied Biosystem). Prior to sequencing, methanolic Biobrene (PE Applied Biosystem) was applied to the PVDF-bound sample to fix small peptides to the membrane. N-terminal peptide sequencing was performed on a 476A automated protein sequencer (Perkin-Elmer Applied Biosystem) using standard gas phase Edman chemistry (Edman, 1950; Edman and Begg, 1967). The 476A sequencer was equipped with an on-line C_{18} reverse phase HPLC and 610A data analysis system.

CHAPTER 3: RESULTS AND DISCUSSION

Selection of Fluorescent Probes

The aim of this project was to evaluate the use of non-selective fluorescent labeling to identify regions on P-Glycoprotein that undergo changes in accessibility due to conformational changes. The lysine residue was a very attractive target for modification due to its relatively high abundance (82/1276 amino acids) and its global distribution throughout the Pgp molecule (Figure 3). Additionally, many fluorescent lysine-specific reagents are available commercially, a number of which were evaluated based on several criteria. First, the fluorescent probe should label non-selectively throughout the protein so that any areas that undergo ligand-dependent changes in solvent accessibility would be detected by the probe. Second, the probe signal needed to be sufficiently intense so that the labeled peptides may be detectable by the HPLC fluorescence detector. Third, the probe needed to be stable throughout the experimental and purification procedure, in order to maintain the fluorescent signal and the added mass due to the modification could be predicted for the identification of the labeled tryptic peptide of Pgp.

Numerous lysine-specific fluorescent modification reagents were screened by labeling equal aliquots of plasma membrane vesicle preparations. After purification of the labeled P-Glycoprotein with either purification procedure, the protein was enzymatically digested with trypsin. The labeled Pgp peptides were analyzed by HPLC to determined which labeling agents satisfied the above criteria. Figure 4 shows a comparison of the HPLC fluorescent peptide profiles resulting from four lysine modification reagents; fluorescamine, dansyl chloride,

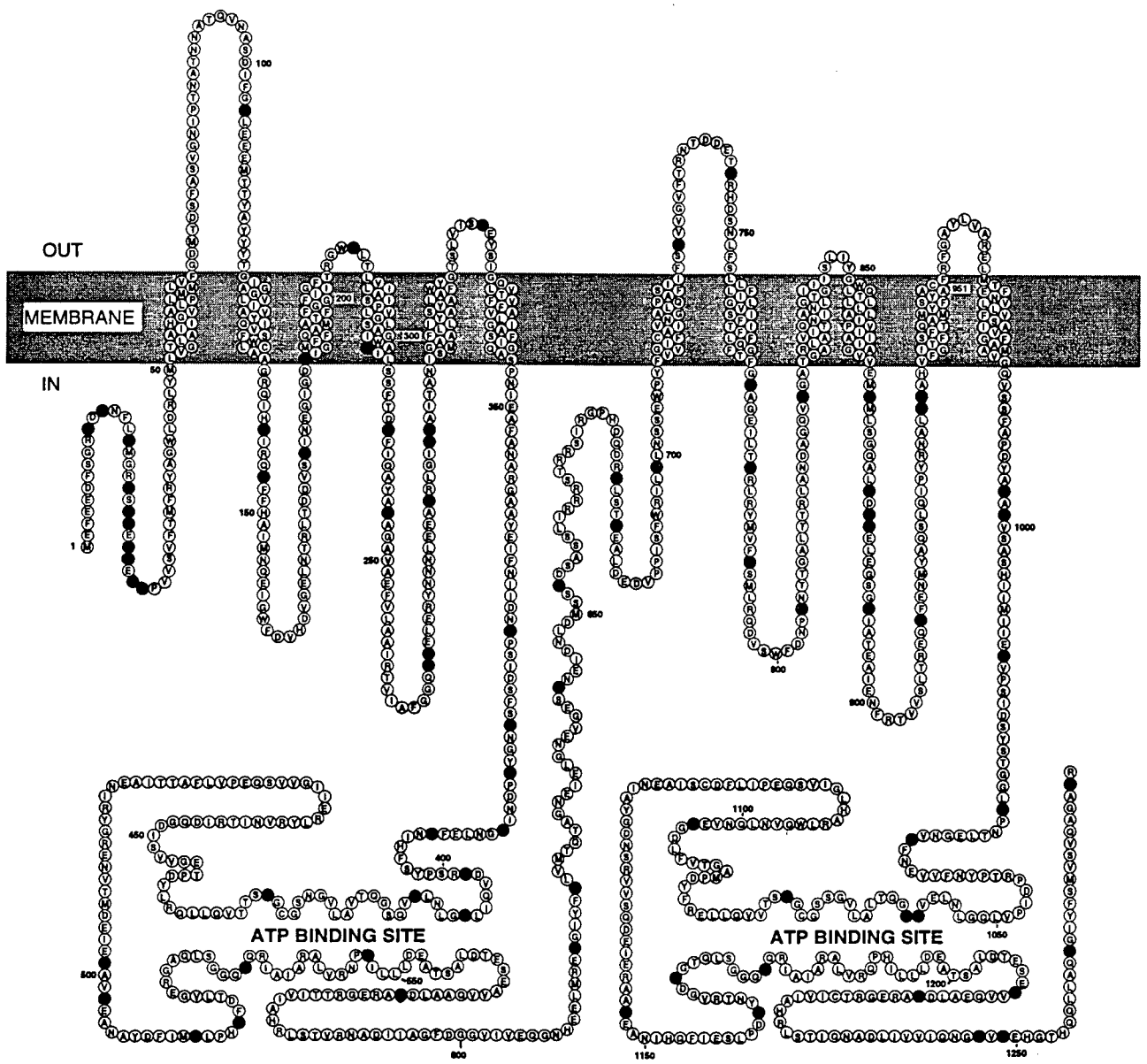


Figure 3: The location of lysine residues in the amino acid sequence of Chinese Hamster P-Glycoprotein-1. *Lysine residues are highlighted in black (●). This figure has been adapted from Shapiro et al., (1996).*

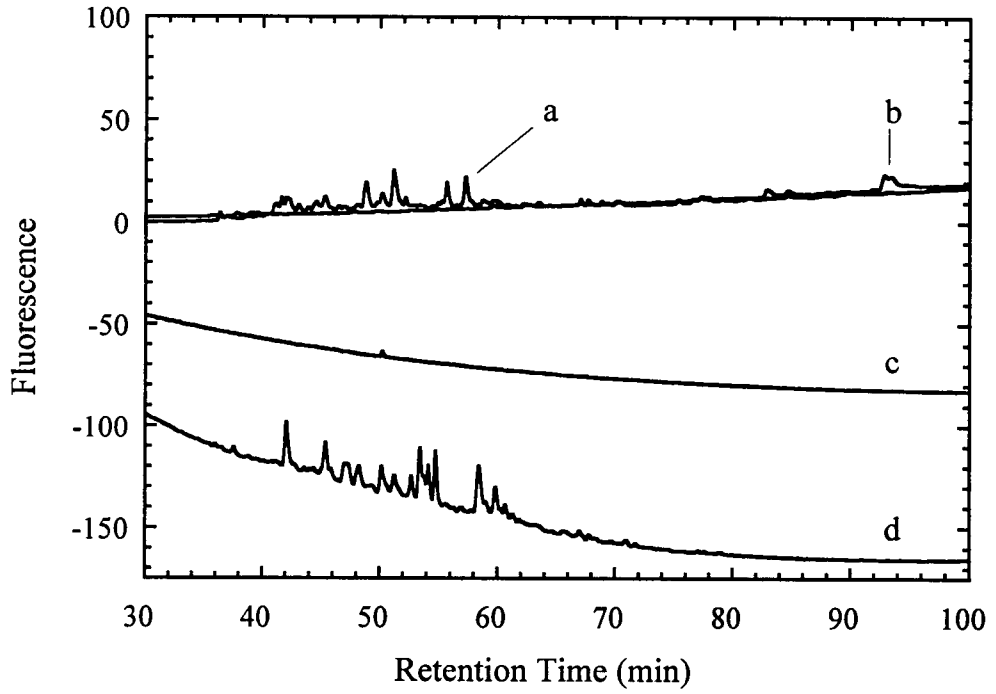


Figure 4: Chromatograms of trypsin digested Pgp labeled with various fluorescent probes. *Pgp was labeled with a)fluorescamine, b)dansyl chloride, c)lissamine rhodamine sulfonyl chloride, d)DEACSE.*

lissamine rhodamine sulfonyl chloride, 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester (DEACSE). Only fluorescamine and DEACSE were sufficiently intense to be detected by the HPLC fluorescence detector.

Fluorescamine and DEACSE (Figure 4), were initial candidates since both compounds have very intense fluorescence. Fluorescamine had an additional feature of being non-fluorescent until it undergoes reaction with primary amines, which takes place within milliseconds (De Bernardo *et al.*, 1974, Udenfriend *et al.*, 1972). DEACSE undergoes a rapid

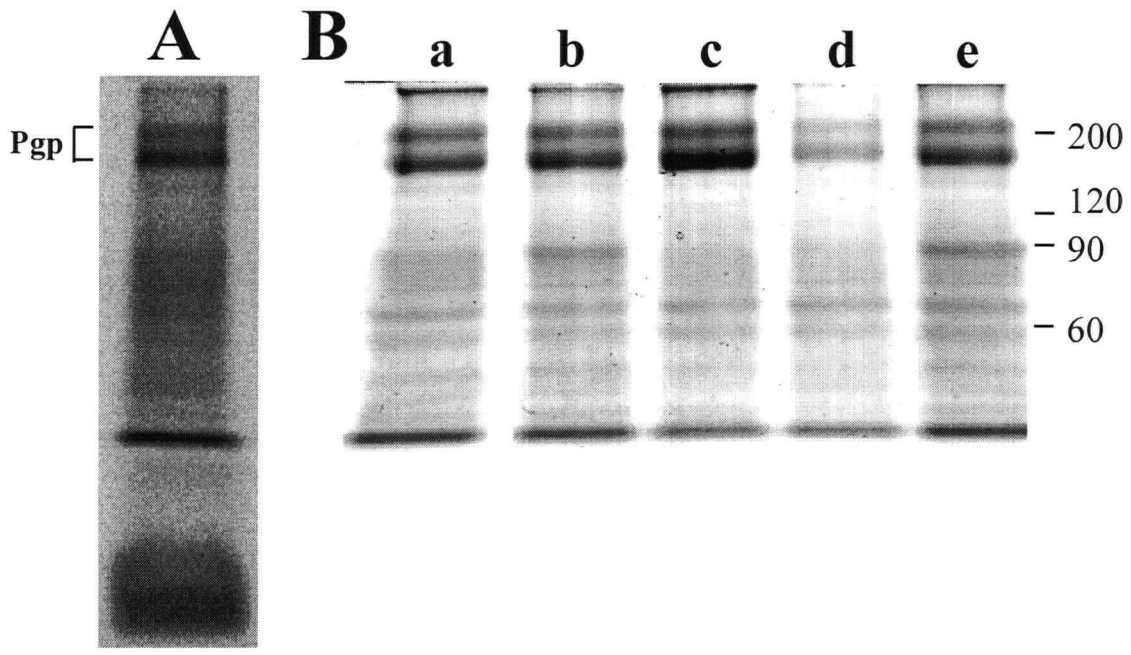


Figure 5: DEAE-cellulose purified Pgp labeled with different fluorescent probes. *Panel A - 8% SDS-PAGE of DEACSE-labeled Pgp was visualized on the Storm 860 in blue fluorescence prior to staining; Panel B - 8% SDS-PAGE of DEAE-cellulose purified Pgp a)unlabeled, and labeled with b)fluorescamine, c)lissamine rhodamine sulfonyl chloride, d) DEACSE, and e)dansyl chloride, stained with Coomassie Brilliant Blue. The position of Pgp is indicated.*

reaction with primary amines to form a stable adduct through an amide bond. In addition, DEACSE has spectroscopic characteristics that are compatible with the Storm 860 phosphorimager/fluorimager. In blue fluorescence mode (Figure 5, Panel A), the labeled protein and labeled peptides can be visualized in the polyacrylamide gels by the fluorimager.

Fluorescently labeled Pgp was initially purified by DEAE-cellulose anion-exchange chromatography which discriminates based on net charge of the protein. However, the yield of purified protein was very low and variable between the differently labeled samples, and the isolated Pgp was insufficiently pure (Figure 5, Panel B). Similar yields between dansyl chloride-labeled Pgp (lane e) and the unlabeled control may be due to the low reactivity of dansyl chloride toward Pgp leaving the labeled protein to possess a similar net charge to the unlabeled protein (Figure 4). DEACSE labeling (lane d) appeared to significantly reduce the yield of Pgp. This was probably due to effective reaction of DEACSE with Pgp (Figure 4), thereby eliminating the positive charges on the protein. The higher yield of lissamine rhodamine sulfonyl chloride-labeled Pgp, relative to the unlabeled control may, in part, be a result of the positive charge of this compound substituting for the modified lysine residue (lane c).

Due to the problems encountered with DEAE-cellulose chromatography, immunoprecipitation was later implemented. This purification procedure required a low pH elution to disrupt antibody-antigen binding in order to isolate the purified Pgp. Unfortunately, fluorescamine was found to be very pH sensitive (De Bernardo *et al.*, 1974) such that there was a significant loss of fluorescamine fluorescence with immunoaffinity purification. This particular characteristic of fluorescamine eliminated its candidacy and DEACSE was used for all subsequent labeling experiments.

DEAE-Cellulose Chromatography Purification of Labeled P-Glycoprotein

DEAE-cellulose chromatography was used by Shapiro and Ling (1994) as an initial purification step in their protocol to purifying functionally active P-Glycoprotein. The

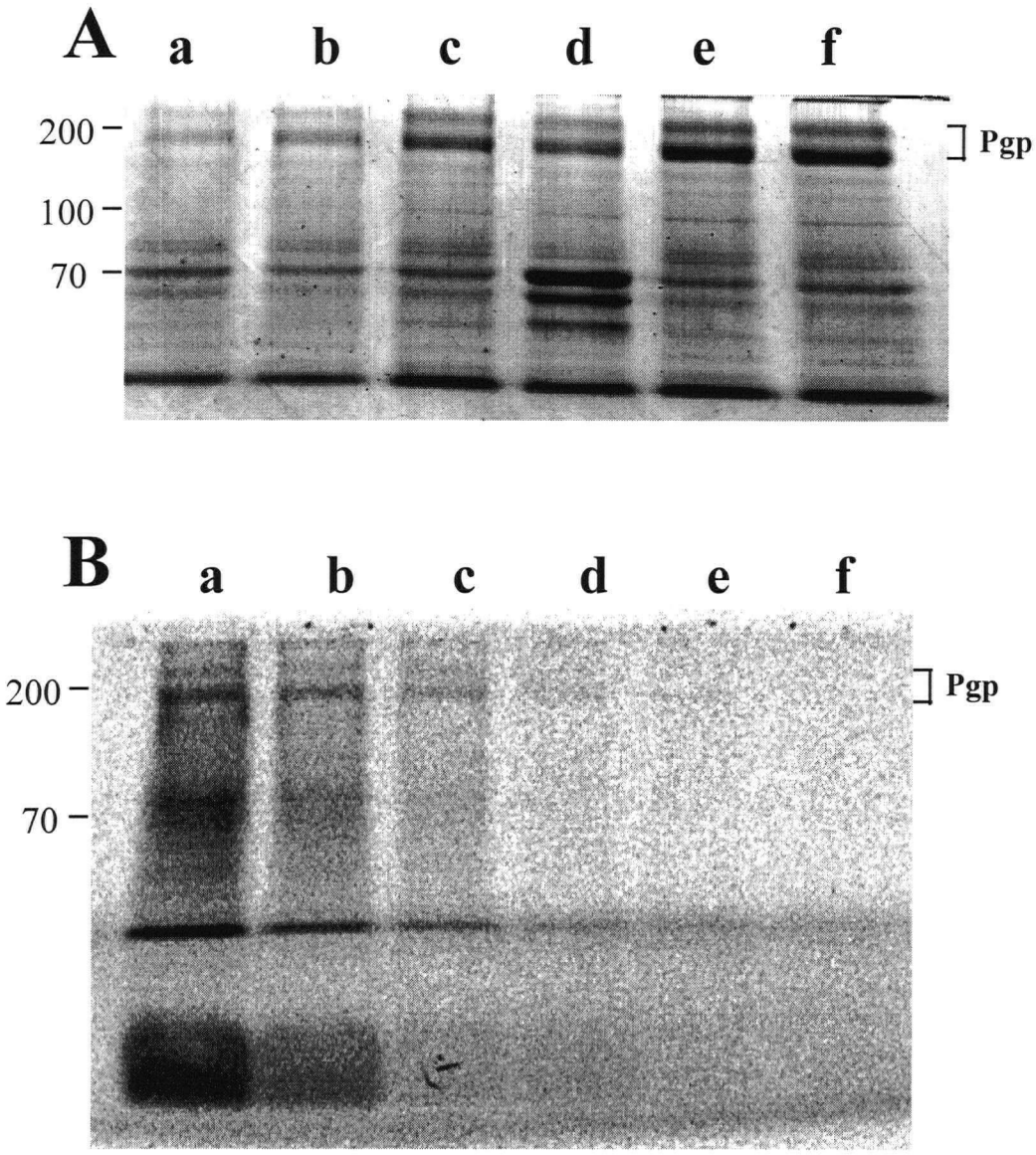


Figure 6: DEAE-cellulose purified Pgp labeled with different concentrations of DEACSE. *Panel A - 8% SDS-PAGE of purified Pgp labeled with various concentrations of DEACSE a)300 μM, b)100 μM, c)30 μM, d)10 μM, e)3 μM, f)unlabeled, stained with Coomassie Brilliant Blue; Panel B - 8% SDS-PAGE in Panel A scanned with Storm 860 in blue fluorescence prior to staining.*

conditions were optimized such that in the MEZ buffer (pH 7.0), near quantitative recovery of Pgp in the column flow-through was achieved, leaving other membrane proteins bound to the anion- exchange column. However, during the initial screening of fluorescent probes, this purification procedure was very problematic. The yield of labeled P-Glycoprotein was very low and very inconsistent between the differently labeled samples, all of which started with the same amount of plasma membrane vesicles (Figure 5, Panel B). This purification method involved anion-exchange chromatography with DEAE-cellulose, and therefore any perturbation in the net charge of a protein will effect its interaction with the ion-exchange column and ultimately alter its retention on the column. The modification of lysine residues would eliminate the positive charges on Pgp, and the protein would likely be retained on the anion-exchange solid phase. This clearly was the case with DEACSE, since labeling with an increasing amount of DEACSE actually decreased the yield of Pgp (Figure 6). Inconsistent yield also occurred when DEACSE was used to label equal quantities of membranes incubated with different Pgp substrates (Figure 7). The cause of this inconsistent yield may be due to differential labeling in the presence of the various substrates, as would be predicted. Differential labeling would result in species of labeled-Pgp with different net charges which would have different affinities for the ion-exchange column.

SDS-PAGE was used throughout the optimization of the DEAE-cellulose purification to monitor the yield of Pgp. P-Glycoprotein migrated as two distinct, diffused bands in the polyacrylamide gels in Figure 5, 6 and 7. This phenomenon has been observed by several group (Safa, 1988; Boscoboinik *et al.*, 1990), and has been attributed to heterogeneity of N-linked glycosylation (Greenberger *et al.*, 1987), as well as multiple forms of Pgp (Greenberger *et al.*, 1988). The polyacrylamide gels shown in Figure 5, 6 and 7 contain a large amount of

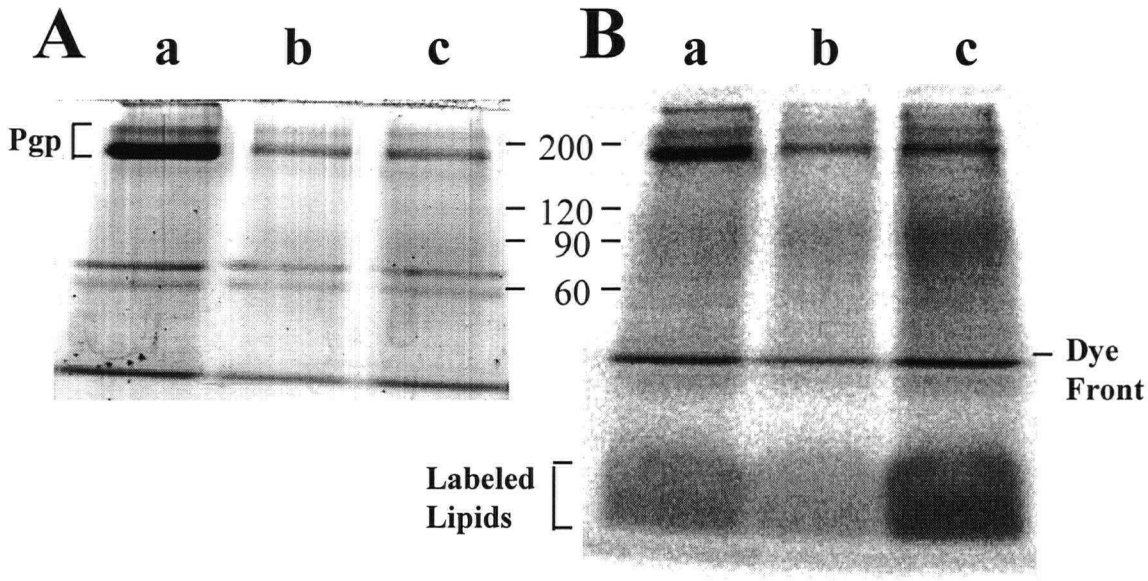


Figure 7: DEAE-cellulose purified Pgp labeled with DEACSE in the presence of different Pgp ligands. *Panel A - 8% SDS-PAGE of purified Pgp labeled with DEACSE in the presence of a)no ligand, b)3 mM ATP, 6 mM $MgCl_2$, c)3 mM ATP, 6 mM $MgCl_2$, 30 μM verapamil, stained with Coomassie Brilliant Blue; Panel B - 8% SDS-PAGE in Panel A scanned with Storm 860 in blue fluorescence prior to staining.*

contaminating labeled proteins other than Pgp. Also present, was a large amount of labeled lipids, most likely phosphatidylethanolamine, which ran slightly faster than the dye front (Figure 7, Panel B). The samples of labeled membranes were subjected to SDS-PAGE directly after Centricon 100 concentration. Many contaminating proteins are well below the 100 kDa molecular weight cut-off of the concentrators, thus the effectiveness of the concentrators may be a concern. It is the purpose of this study to examine changes in accessibility that occur within Pgp as it binds to its substrates. Therefore, the purity of the sample is very important and it was clear that the DEAE-cellulose purification procedure was inadequate for our purposes.

Another reason that DEAE-cellulose purification was inadequate for the purpose of this study was the extremely low yield of Pgp by this technique. The yield of purified Pgp from this procedure was too low to reveal any differential labeling with the presence of different substrates. If the purification yield from 300-500 μl of plasma membranes was 100%, then 15 μg of purified Pgp would be predicted to be isolated at the end of the purification. This is clearly not the yield obtained with DEAE-cellulose chromatography, based upon a qualitative comparison between the amount of Pgp isolated in Figure 7 versus Figure 11. It was also obvious the Pgp yield from DEAE-cellulose chromatography was drastically reduced by labeling the protein with DEACSE. Quantitation by densitometry of the Pgp band (Figure 6) revealed that labeling with 300 μM DEACSE (lane a) resulted in a 75% reduction in Pgp yield compared to the unlabeled control (lane f).

The methanol precipitation used to concentrate the labeled Pgp after DEAE-cellulose purification was also problematic. Using purified Pgp in detergent solution (Shapiro and Ling, 1994) the yield of protein from the methanol precipitation found to be only 20% of the original amount of Pgp with a number of protein concentrations (Figure 8). With the low amounts of P-Glycoprotein used, a carrier protein could facilitate the precipitation. However, the presence of the carrier protein would seriously compromise the purity of the labeled peptides that would be required for identification of these peptides. Other organic solvents were tested for their efficiency to precipitate Pgp, but the results were similar to methanol. Therefore, DEAE-cellulose purification and methanol precipitation resulted in poor and inconsistent yields of labeled Pgp, with a low degree of purity.

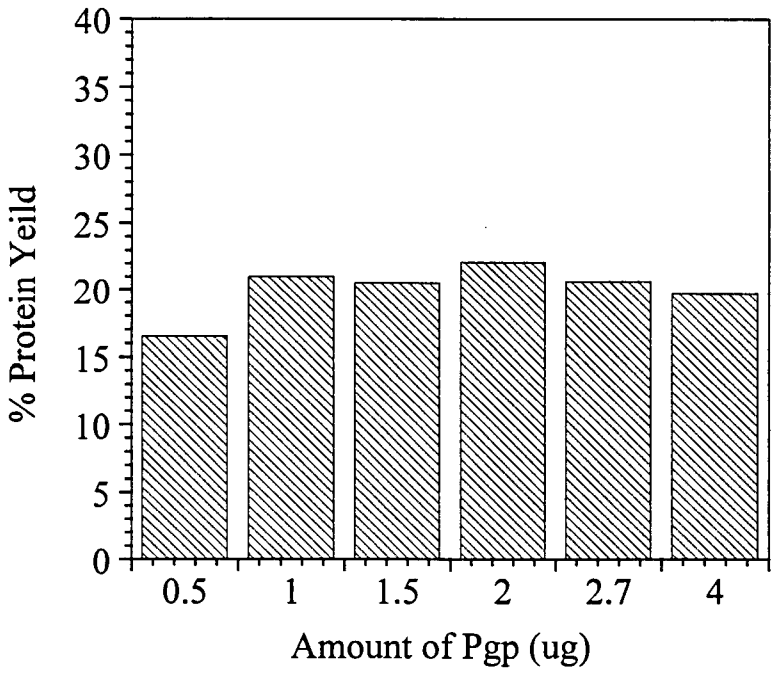


Figure 8: Yield of Pgp from methanol precipitation using various amounts of Pgp.

Immunoprecipitation Purification of Labeled P-Glycoprotein

Since the DEAE-cellulose purification method was inadequate, an immunoaffinity chromatography procedure was tested for use in the purification of labeled Pgp. The DEACSE label was conveniently monitored because of its spectrophotometric compatibility with the Storm 860 fluorimager. Immunoprecipitation was very effective in depleting solubilized B30 plasma membranes of Pgp. The Pgp isolated from the immunoaffinity matrix was estimated by densitometry to be approximately 70% pure, and void of any significant contamination (Figure 9).

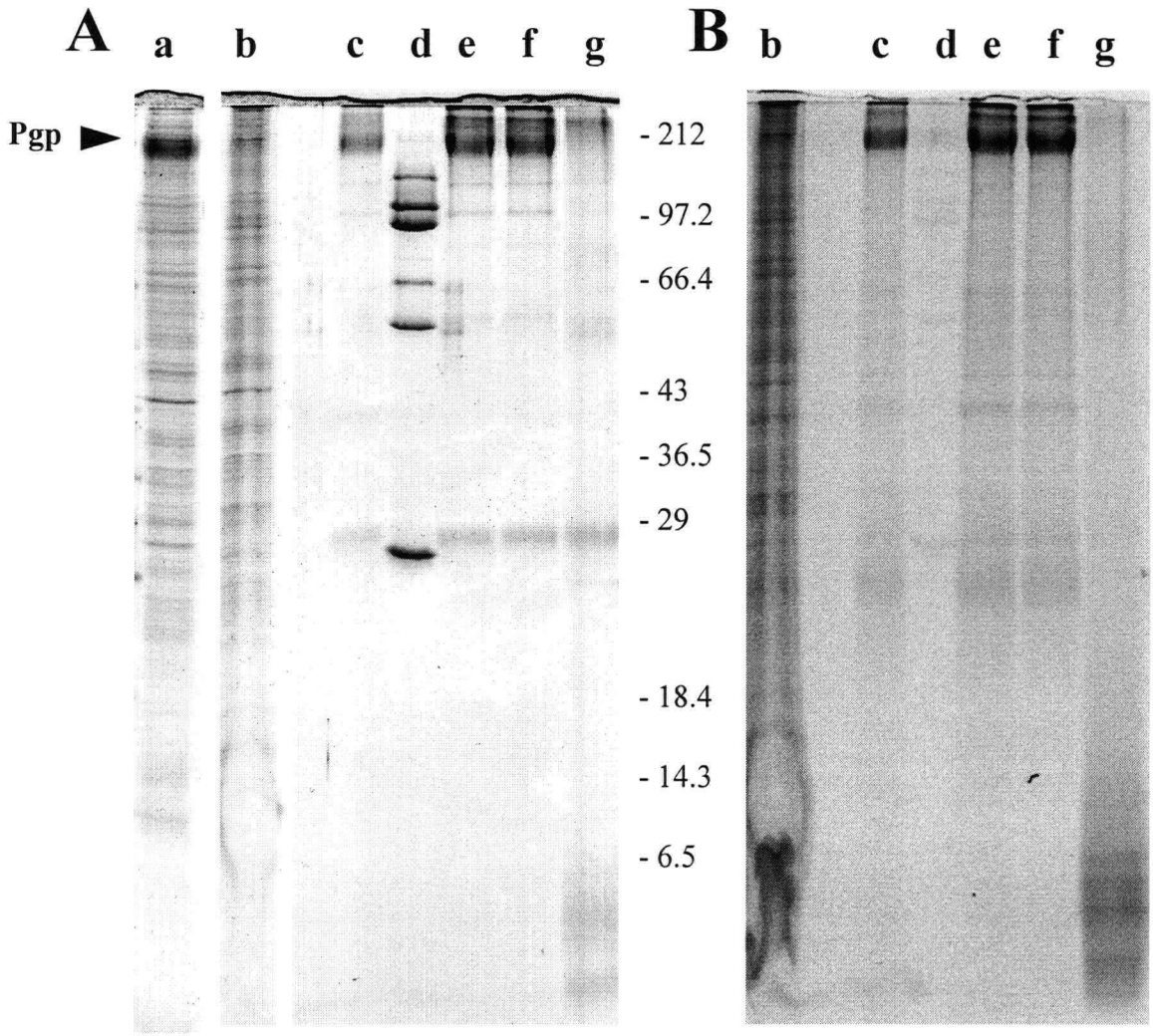


Figure 9: Immunoprecipitation purification and trypsin digestion of DEACSE-labeled Pgp. *Panel A - 12% SDS-PAGE of immuno-purified and trypsin digested DEACSE-labeled P-Glycoprotein stained with Coomassie Brilliant Blue; a)B30 membranes(unlabeled), b)immunoprecipitation supernatant, c)20 µl of immunoaffinity beads with labeled Pgp bound, d)5 µg C219, e) & f)5 µg TFA eluted labeled Pgp, g)approx. 8 µg labeled Pgp digested with trypsin. Samples were not boiled before electrophoresis.; Panel B - 12% SDS-PAGE in Panel A scanned with Storm 860 in blue fluorescence prior to staining.*

Figure 9 revealed that trypsin digestion of Pgp was very effective. Digestion of Pgp with TPCK-treated trypsin overnight at 37 °C, at a ratio of 1:3 (trypsin to Pgp) quantitatively reduced the Pgp to peptides under 7 kDa. In addition, there was a concern that trypsin activity may vary between samples because of the different substrates present. However, since Pgp ligands present during DEACSE labeling were removed by immunoprecipitation, they should have no effect on trypsin activity. Therefore, the immunoaffinity chromatography appear to be an effective purification procedure for isolating DEACSE-labeled Pgp, and was subsequently optimized for the purpose of this study.

Immunoaffinity chromatography with C494 anti-P-Glycoprotein monoclonal antibody has been previously used to purify functional Pgp (Shapiro and Ling, 1994). C494 mAb was bound to cyanogen bromide-activated Sepharose to generate an immunoaffinity matrix. This reaction would result in a physical linking of the antibody to the Sepharose in various orientations, some of which are unable to bind antigen. This would lead to a decreased capacity and efficiency of the immunoaffinity matrix. To eliminate this problem, Protein A-Sepharose was employed to correctly orientate the monoclonal antibody to ensure maximum antigen binding capacity. For our purification, the C219 mAb was used throughout (Georges *et al.*, 1990). The choice of C219 over C494 was arbitrary since immunoprecipitation using both antibodies gave high yield of labeled Pgp relative to DEAE-cellulose chromatography (data not shown). Maximum yield was achieved using a 3 fold excess of C219 over Pgp (Figure 10).

The elution of labeled Pgp from the immunoaffinity matrix can be accomplished by an incubation in a low pH buffer, which would disrupt the binding of Pgp to the C219 mAb. However, this would also disrupt the binding of the mAb to the Protein A-Sepharose, which

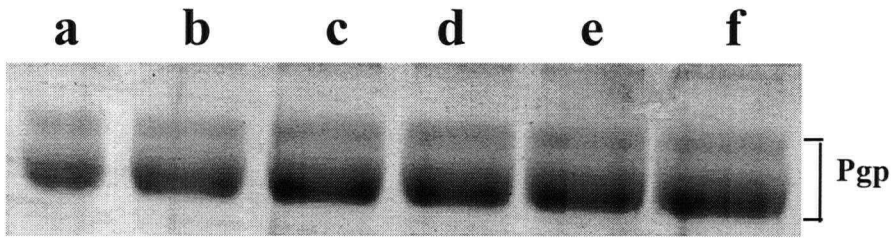


Figure 10: Purification of unlabeled Pgp with various amounts of C219 monoclonal antibody. *Unlabeled Pgp from 140 μl of B30 plasma membranes, containing approximately 9 μg of Pgp, was purified using various amounts of C219 anti-P-Glycoprotein monoclonal antibody; a)10 μg, b)20 μg, c)30 μg, d)50 μg, e)70 μg, f)100 μg.*

would compromise the purity of the labeled Pgp. To solve this problem the C219 mAb was physically coupled to the Protein A-Sepharose by chemically cross-linking using dimethylpimelimidate. Although it was possible that the cross-linking reagent may modify lysine residues that are essential for antigen binding, however, a reduction in the capacity of the beads following cross-linking was not noticed. Antibody-Protein A-Sepharose has been shown to be very stable through a wide pH range (Schneider *et al.*, 1982) and very effective for purifying membrane proteins (Peltz *et al.*, 1987).

There was concern that DEACSE may modify lysine residues in close proximity to the C219 epitope of Pgp and hinder antibody binding. Comparing the yields of unlabeled Pgp and Pgp labeled with increasing concentrations of DEACSE, no significant differences was found

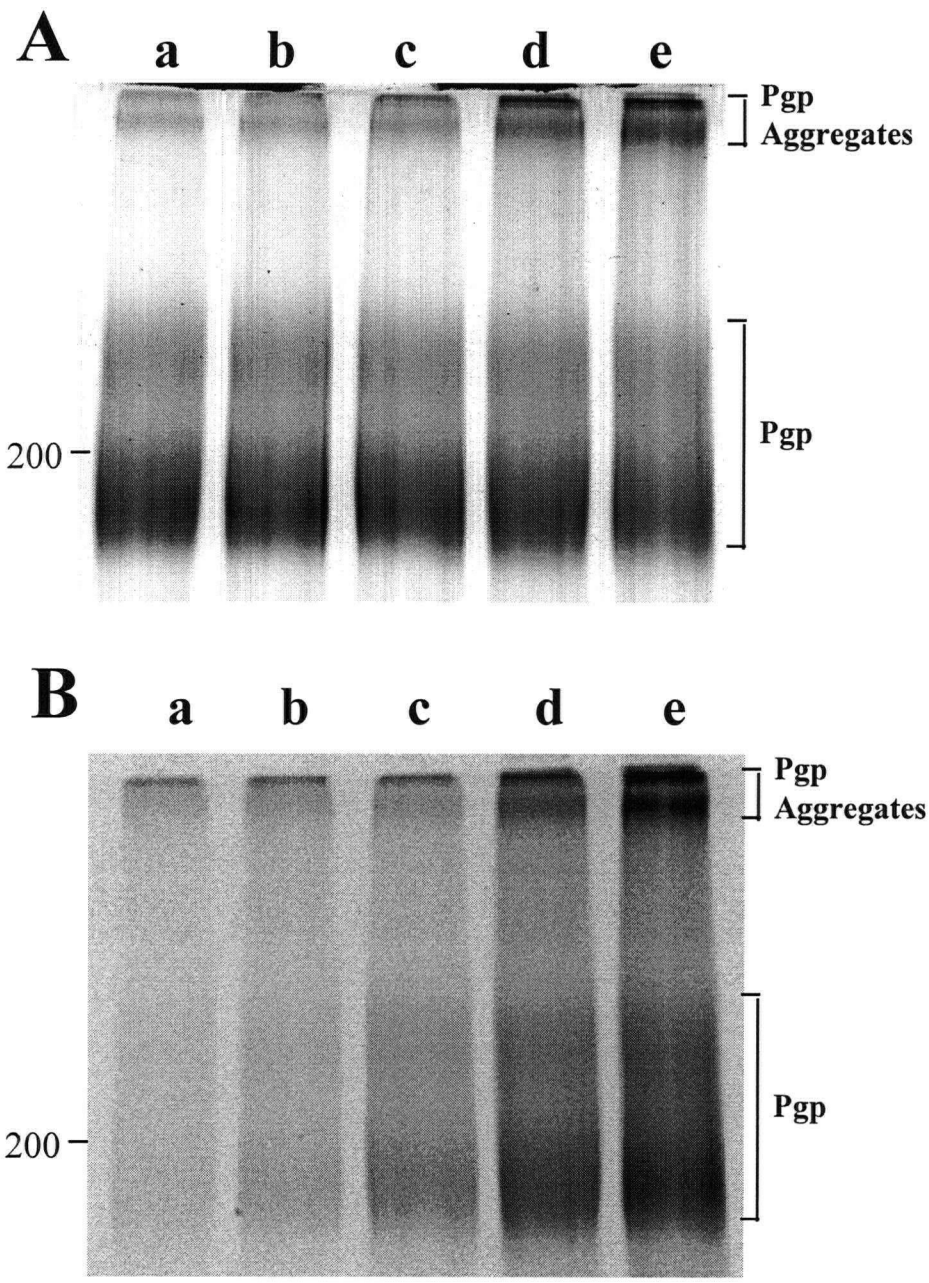


Figure 11: Immunoprecipitation purified Pgp labeled with different concentrations of DEACSE. *Panel A - 8% SDS-PAGE of purified Pgp, a)unlabeled, labeled with; b)2 μM, c)25 μM, d)125 μM, e)250 μM of DEACSE, stained with Coomassie Brilliant Blue; Panel B - 8% SDS-PAGE in Panel A scanned with Storm 860 in blue fluorescence prior to staining.*

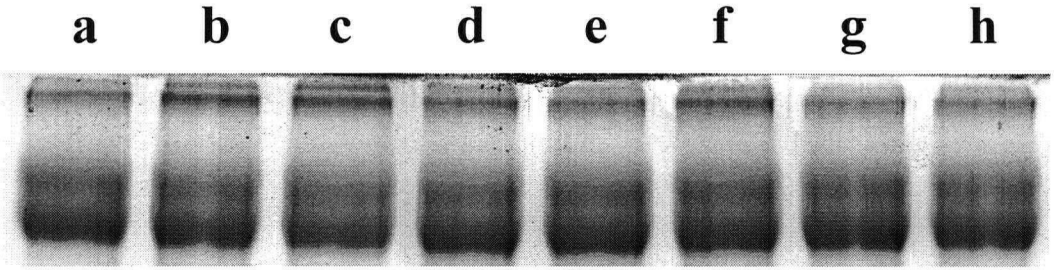
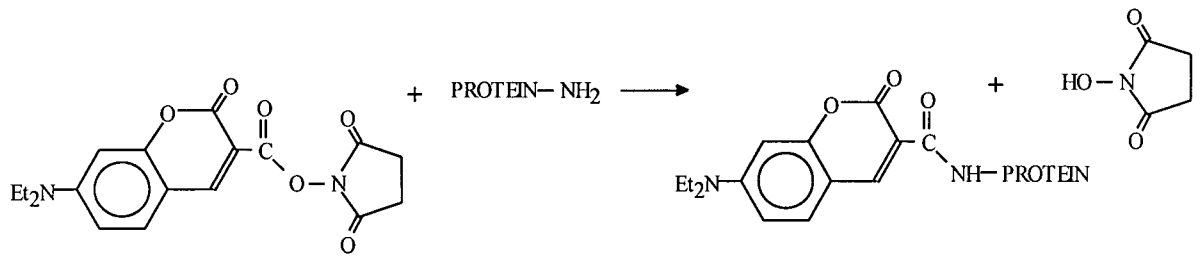


Figure 12: Immunoprecipitation purified Pgp labeled in the presence of different Pgp substrates. *8% SDS-PAGE of purified Pgp labeled with DEACSE in the presence of; a)no ligand, b)3 mM AMPPNP, 6 mM $MgCl_2$, c)11 μM verapamil, d)3 mM ATP, 6 mM $MgCl_2$, 11 μM verapamil, e)2 μM cyclosporin A, f)3 mM ATP, 6 mM $MgCl_2$, 2 μM cyclosporin A, g)4 μM vinblastine, h)3 mM ATP, 6 mM $MgCl_2$, 4 μM vinblastine, stained with Coomassie Brilliant Blue.*

between the amount of Pgp recovered (Figure 11). However, a slight increase in higher molecular weight Pgp aggregates was observed with higher concentrations of DEACSE. Moreover, there was no significant differences in the yield of labeled Pgp between samples labeled in the presence of various Pgp substrates and inhibitors (Figure12). It appeared that immunoprecipitation of Pgp was an effective method for purifying differentially labeled Pgp. The yield of label Pgp was significant relative to the DEAE-cellulose purification method and the yield was consistent between samples. Therefore immunoprecipitation was used for all subsequent labeling experiments.

Effect of Substrates on DEACSE Labeling of P-Glycoprotein

Non-specific fluorescent labeling of P-Glycoprotein with DEACSE was used to localize areas of the protein that undergo changes in accessibility due to ligand binding and ligand-induced conformational changes (Sonveaux *et al.*, 1996; Wang *et al.*, 1997; Liu and Sharom, 1996; Mechetner *et al.*, 1997). The reaction of DEACSE with primary amino groups on proteins is shown in Figure 13. Coumarin succinimidyl ester compounds are highly fluorescent and form very stable amide adducts with primary amines (Khalfan *et al.*, 1986). Changes in accessibility accompanying substrate binding and conformational changes would be indicated by a change in the DEACSE fluorescent labeling profile of the protein. Differentially labeled peptides can then subsequently be purified and identified.



DEACSE: MWt = 358
λ_{ex} = 440 nm
λ_{em} = 480 nm
ε = 56 000 M^{-1} cm^{-1}

Adduct Mass = 243

Figure 13: Reaction scheme of DEACSE with primary amino groups on proteins.

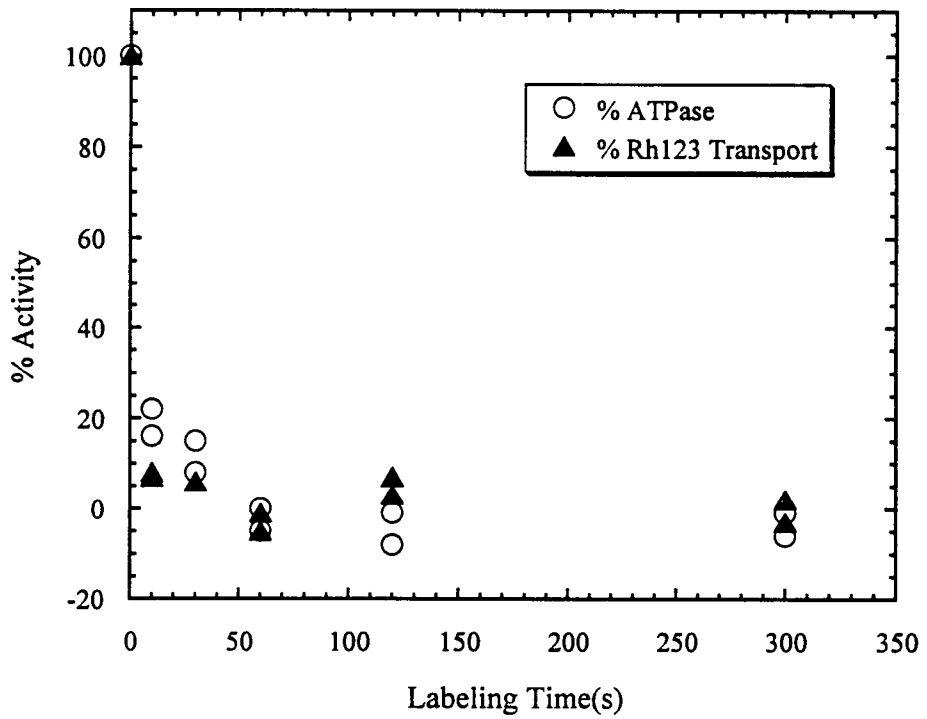


Figure 14: Effect of DEACSE labeling on ATPase and rhodamine 123 transport activity of Pgp.

It is important to consider the effect of DEACSE labeling on the activity of P-Glycoprotein. The ATPase and rhodamine 123 transport activities of Pgp as a function of DEACSE labeling was monitored (Figure 14). Both activities were rapidly inhibited upon labeling with DEACSE, indicating that lysine residues essential for function were modified. Azzaria *et al.* (1989) demonstrated mutation of the lysine residue in the highly conserved Walker A motif (GCGKST) of either amino or carboxyl nucleotide binding domains (NBDs) abolished Pgp's ability to confer resistance, whereas, a lysine mutation just outside the Walker A motif was tolerated. In addition, the two NBDs have been found to behave cooperatively in catalysis

(Urbatsch *et al.*, 1995a, 1995b; Loo and Clarke, 1994b, 1995c), suggesting that inhibition of only one NBD would be sufficient for inactivation of the whole protein.

The DEACSE labeling reaction was demonstrated to be very rapid with the majority of labeling occurring within the first 3 minutes (data not shown). Labeling of Pgp with DEACSE in the presence of different ligands was preformed quickly (1 minute) and at high pH (pH 8.5). This pH does not alter Pgp activity (Al-Shawi and Senior, 1993) but enhances the labeling reaction by favoring uncharged lysine species. The relatively rapid rate of reaction would potentially label the most abundant conformer of Pgp present with each substrate. In addition, a non hydrolyzable analogue of ATP, AMPPNP, was employed in an effort to enhance the stability of certain conformers.

A criteria for the selection of a suitable probe was the modification of numerous sites within the protein. This would optimize the likelihood of locating regions within Pgp that undergo a conformational change. DEACSE was able to label a number of lysine residues, shown by the HPLC chromatogram of DEACSE-labeled peptides (Figure 15). The stoichiometry of DEACSE labeling was calculated to be, approximately 5 moles of DEACSE per mole of Pgp. The chromatogram revealed a definite pattern of labeling and the varying intensities of the peaks implied that different lysine residues have different accessibility to DEACSE, as expected for the protein in its native state in B30 plasma membrane vesicles. Certain peaks on the chromatogram were not well resolved. As a consequence, any differential labeling of these peaks may be masked by overlapping components. To improve resolution, a shallower linear gradient of 20-31% solvent B in solvent A (1% solvent B per 6 minutes) was subsequently used.

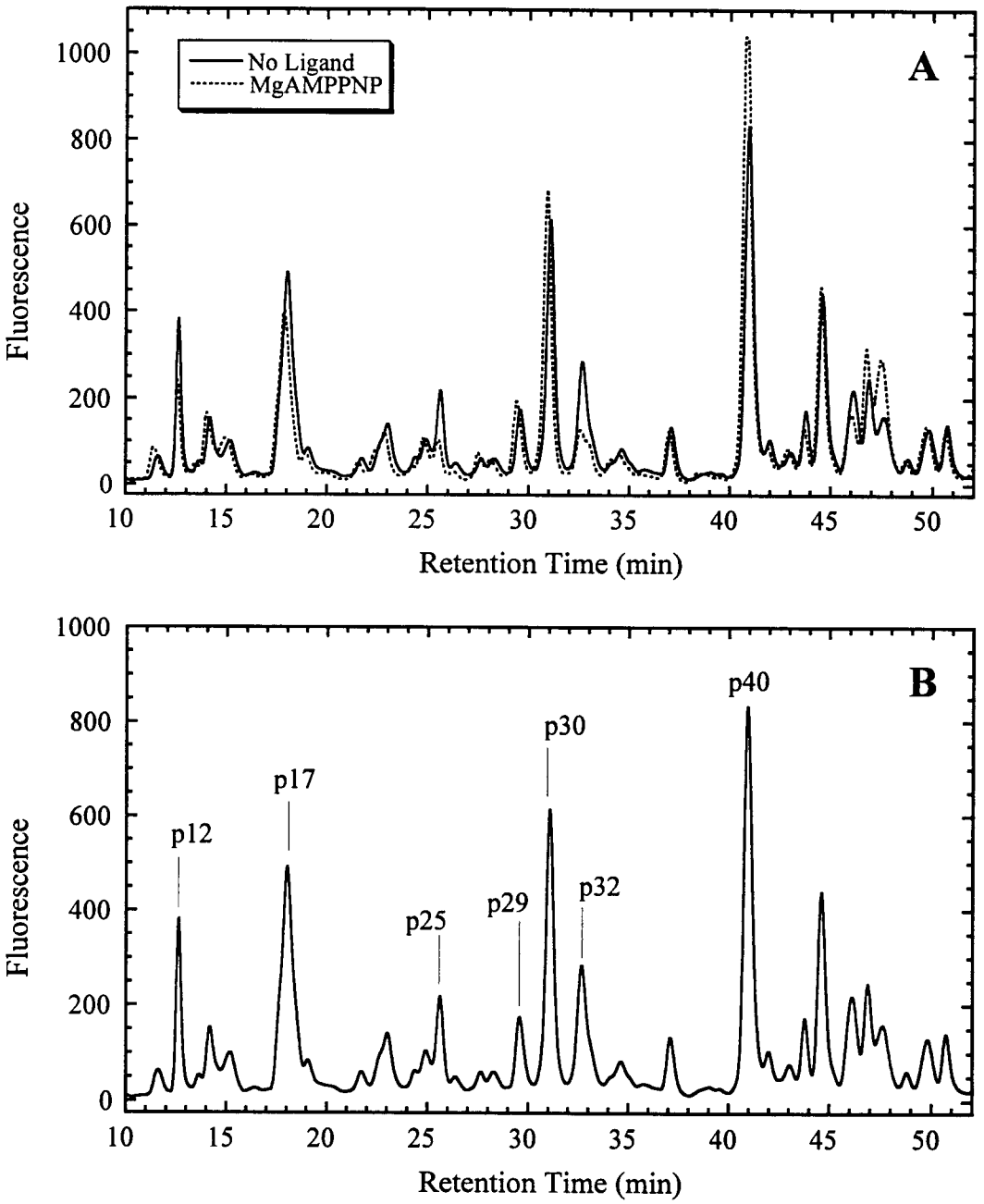
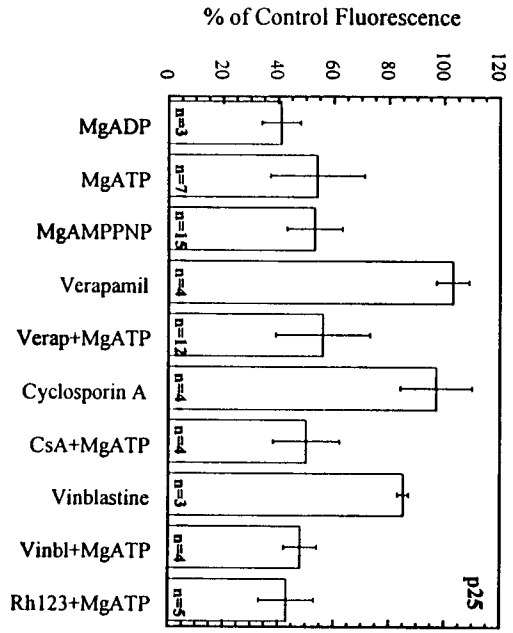


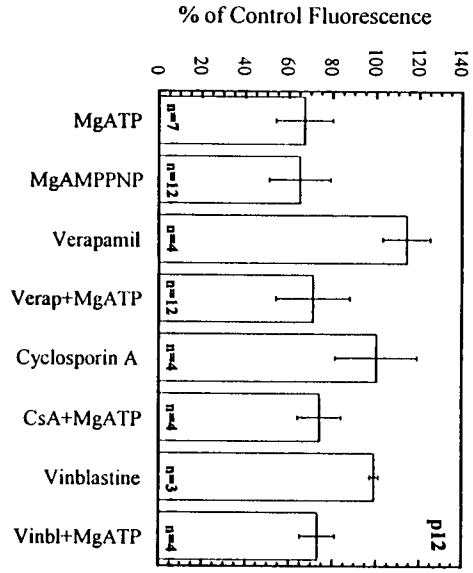
Figure 15: Chromatogram of DEACSE-labeled tryptic peptides of Pgp. *Chromatogram A shows the DEACSE-labeled peptide profile of Pgp labeled in the absence of ligand or in the presence of MgAMPPNP. Chromatogram B identify peptides that appear to undergo consistent changes in labeling in the presence of different substrates.*

When DEACSE labeling was performed in the presence of ATP or ATP analogues and/or various drugs and chemosensitizers, several peptides showed consistent and significant changes in fluorescence intensity, whereas others are unaffected (Figure 15). Certain peaks, such as p30, exhibited relatively constant fluorescence intensity over a broad range of substrates (Figure 16). In this case, the relatively high fluorescence intensity of this peptide reveals that this particular lysine residue is located on an exposed region of the protein. However, changes in accessibility of this peptide upon substrate binding was not detected by this technique. On the other hand, peptides p12, p17 and p25, experienced a substantial reduction in labeling of 35% (n=12, s=14%, P_{p12}«0.0005), 22% (n=15, s=9%, P_{p17}«0.0005), and 47% (n=15, s=10%, P_{p25}«0.0005) respectively, relative to the control, in the presence of MgAMPPNP (Figure 16). This decreased accessibility may be due to a conformational change in the presence of nucleotides, or to blockage of the labeled residues by the nucleotide. Furthermore, there was a statistically significant enhancement of DEACSE labeling of peptide p40 in the presence of MgAMPPNP (20%, n=14, s=17%, P_{p40}<0.0005) and peptide p29 in the presence of MgATP and verapamil (53%, n=11, s=35%, P_{p29}<0.0005). These effects are indicative of ligand-dependent conformational changes within the protein that increases the accessibility of a lysine residue in these particular peptides.

Identification of DEACSE-labeled Peptides

The location of the peptides that experienced ligand-dependent changes in accessibility to DEACSE within the amino acid sequence of Pgp requires conclusive identification of these


% of Control Fluorescence
0
20
40
60
80
100
120
MgADP
MgATP
MgAMPPNP
Verapamil
Verap+MgATP
Cyclosporin A
CsA+MgATP
Vinblastine
Vinbl+MgATP
Rh123+MgATP
n=3
n=7
n=15
n=4
n=12
n=4
n=4
n=3
n=4
n=5
p25


% of Control Fluorescence
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40
60
80
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120
140
MgATP
MgAMPPNP
Verapamil
Verap+MgATP
Cyclosporin A
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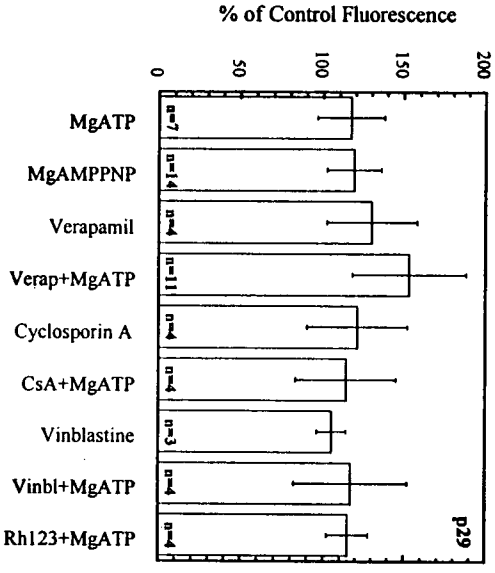
n=12
n=4
n=4
n=3
n=4
p12


% of Control Fluorescence
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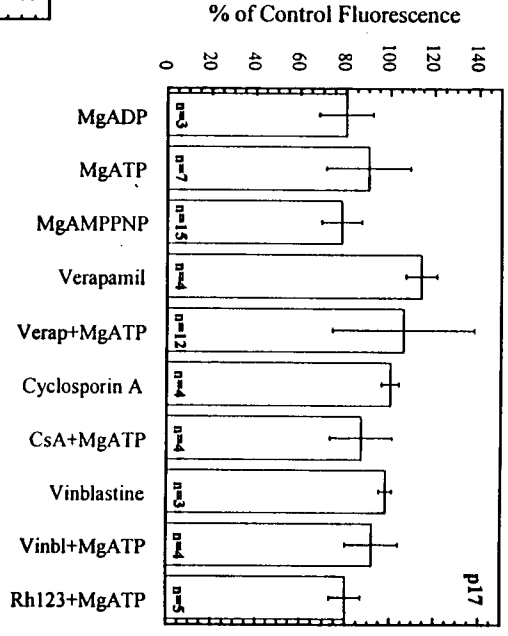
100
150
200
MgATP
MgAMPPNP
Verapamil
Verap+MgATP
Cyclosporin A
CsA+MgATP
Vinblastine
Vinbl+MgATP
Rh123+MgATP
n=7
n=14
n=4
n=11
n=4
n=4
n=3
n=4
n=4
p29


% of Control Fluorescence
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20
40
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120
140
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MgATP
MgAMPPNP
Verapamil
Verap+MgATP
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Vinblastine
Vinbl+MgATP
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n=3
n=4
n=5
p17

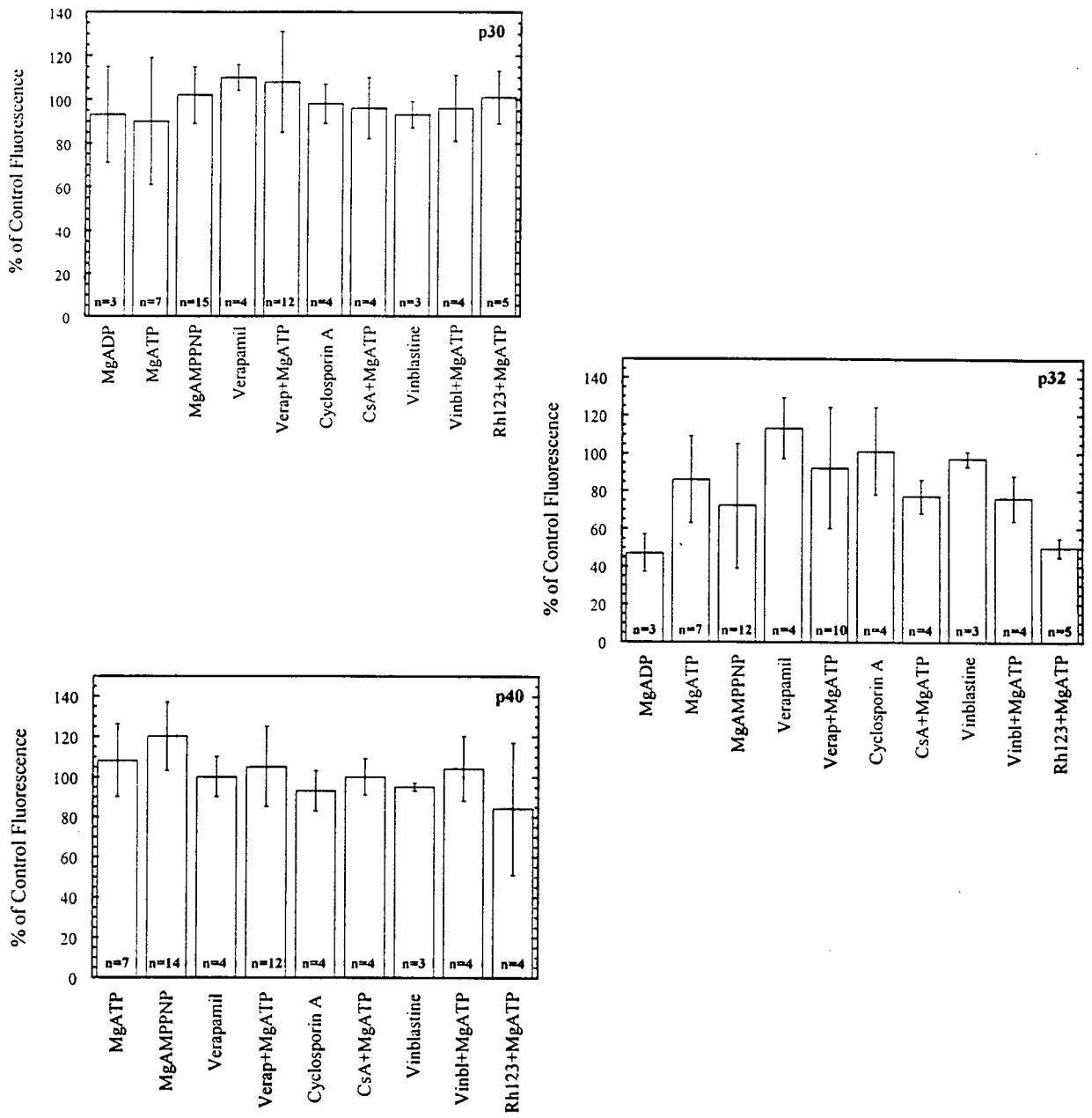

Figure 16: DEACSE differential fluorescent labeling results for several Pgp peptides.

peptides. N-terminal sequencing and MALDI-TOF mass spectroscopy were used in conjunction for identification of these peptides. MALDI-TOF mass spectroscopy is a very sensitive and accurate technique for determining the mass of peptides and proteins (Beavis ad Chait, 1990; Hillenkamp and Karas, 1990; Hillenkamp *et al.*, 1991; Cotter, 1992), and is able to detect subpicomole amount of total sample (Chait *et al.*, 1993). N-terminal Edman sequencing, though less sensitive than MALDI-TOF-MS (Wang *et al.*, 1993), resolves ambiguity in identification based on mass alone.

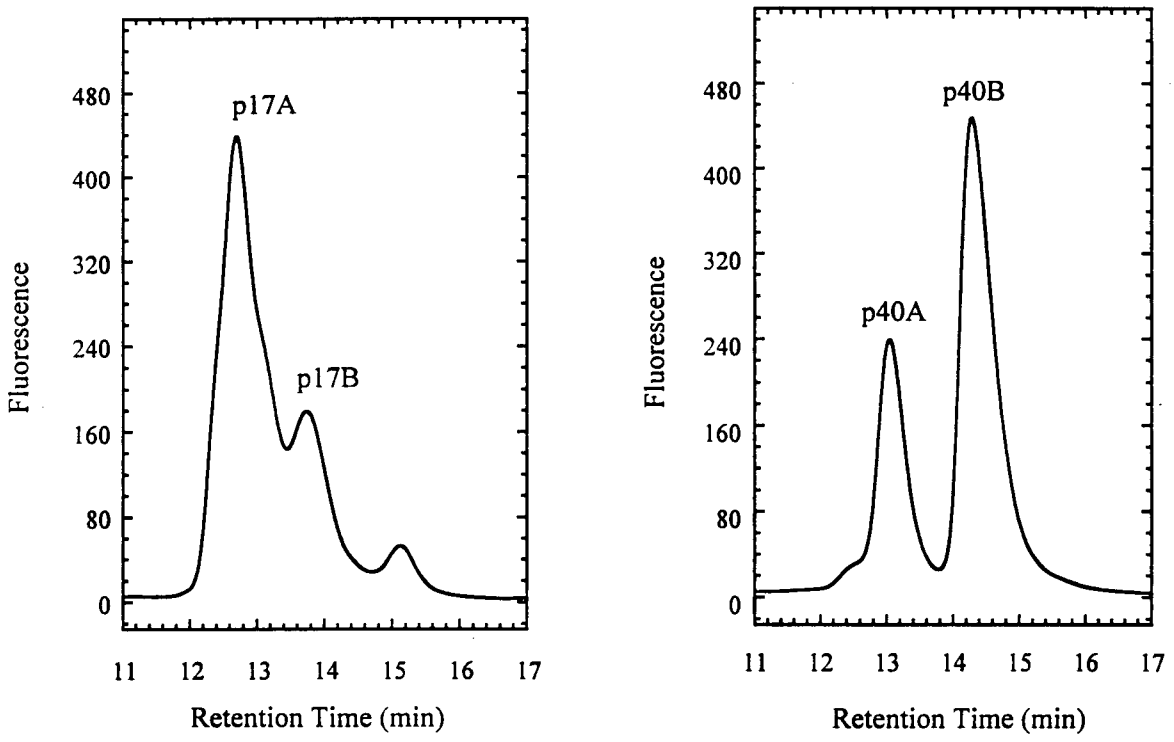

Figure 17: Resolution of multiple species under peaks p17 and p40.

Multiple aliquots of purified peptides were prepared and pooled for identification purposes. Three peptides (p17, p30, and p40) having large fluorescence signals were selected for identification. A high degree of purity was required in order to obtain definitive identification. Therefore, the peptides were subjected to a second round of purification on the HPLC using a more shallow gradient specific for each individual peptide in order to increase peak resolution. Peptides p17 and p40 were resolved into multiple components (Figure 17). A total of 5 peptides (p17A/B, p30, p40A/B) were subjected for MALDI-TOF-MS and N-terminal sequencing analysis. Peptides p17A, p30, p40A and p40B were unambiguously identified. The mass spectrums are shown in Figure 18 and the sequencing and mass data are summarized in Table 1.

Peak p17A was composed of two near-identical peptides originating from either the amino (^{525}GAQLSGGQ**K**QR; labeled residue in bold and underlined) and carboxyl (^{1170}GTQLSGGQ**K**QR) nucleotide binding domains and enveloped the Walker C signature motif (LSGGQKQR) that is highly conserved among the ABC proteins (Figure 19). The Walker C motif resides between the Walker A and Walker B sequences which are themselves conserved over a broad class of ATP binding proteins, which encompasses the ABC proteins (Walker *et al.*, 1982). The predicted secondary structures of the NBDs of several ABC proteins (including Pgp) based upon the structure of adenylate kinase revealed that 2 specific loop regions are only present with the ABC proteins (Hyde *et al.*, 1990). The two loops, named loop 2 and loop 3, were proposed to be located on the surface of the proteins, with loop 3 containing the Walker C signature motif. This is consistent with the relatively strong labeling of peptide p17A by DEACSE. Since non-ABC proteins that possess only the Walker A and Walker B motifs are still able to bind and hydrolyze ATP, it is apparent that the conserved Walker C region, in ABC

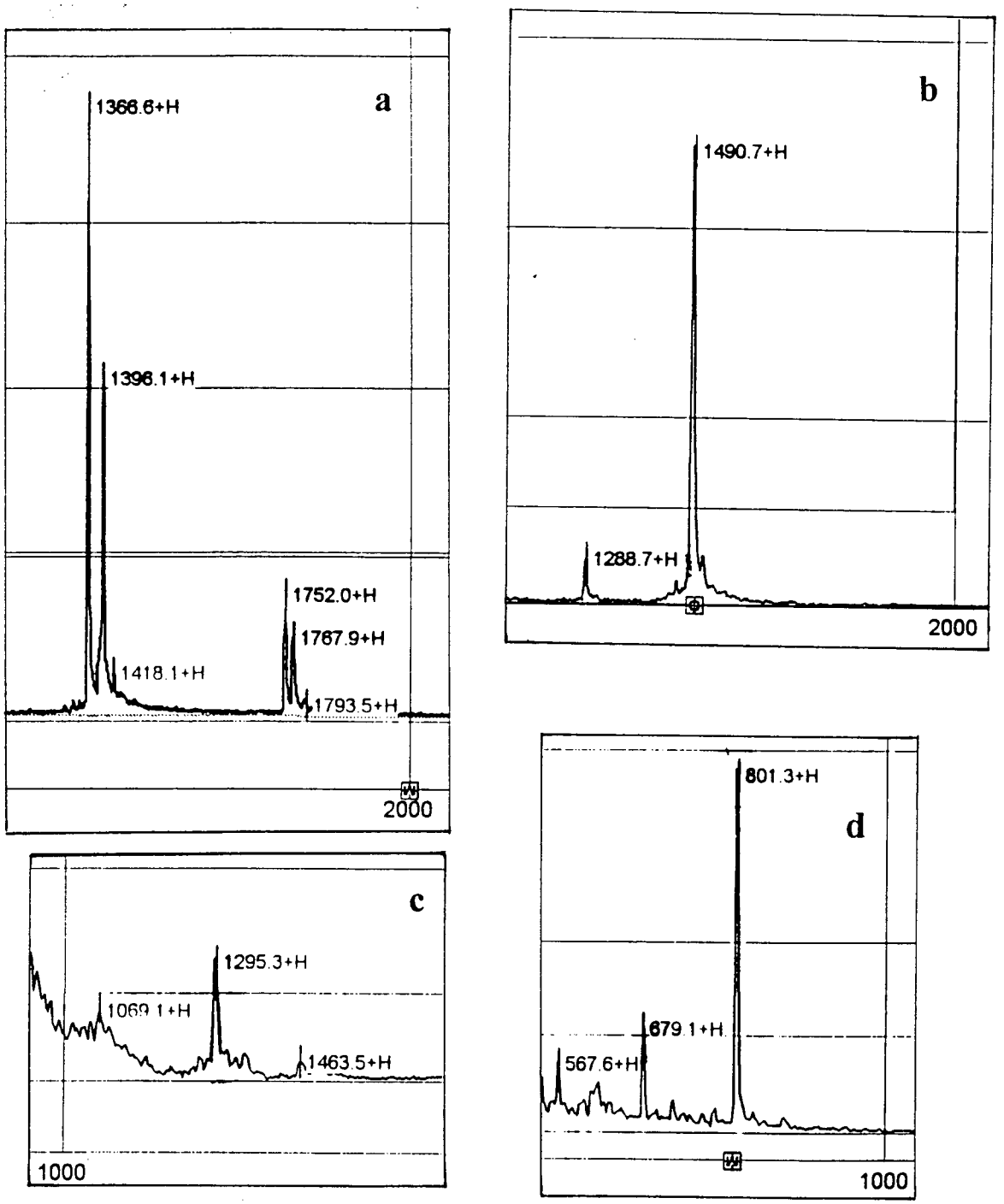

Figure 18: MALDI-TOF mass spectrum of several DEACSE-labeled peptides. *Measured mass for peptides; a)p17A, b)p30, c)p40A, d)p40B are given.*

Peptide	Peptide Sequence	Theoretical Mass	Measured Mass	% Error
p17A	525-GAQLSGGQ**K**QR	1374.5	1366.6	0.6
	1170-GTQLSGGQ**K**QR	1403.5	1396.1	0.6
p30	274-YNNNLEEA**K**R	1495.6	1490.7	0.3
p40A	260-TVIAFGGQ**K**K	1293.5	1295.3	0.1
p40B	284-LGI**K**K	803.0	801.3	0.2

Bold and underlined letters indicate DEACSE-labeled residues.

Table 1: MALDI-TOF-MS and N-terminal sequencing results for several DEACSE-labeled peptides.

proteins, may be essential for an alternative, ABC-specific function. The authors speculated that these loops may be responsible for coupling of ATP hydrolysis to the transport event by ATP-dependent conformational changes. The NBDs have been found to be in close proximity to the membrane surface (Liu and Sharom, 1998) which would facilitate potential energy transduction between the NBDs and the TMDs. Interestingly, computer modeling experiments with the N-terminal NBD of CFTR, a structurally homologous ABC protein (Riordan *et al.*, 1989), based upon the large domain of aspartate aminotransferase predicted that the Walker C motif may interact with another part of CFTR and functions to modulate ATPase activity (Hoedemaeker *et al.*, 1998). Mutagenesis studies on the N-terminal Walker C motif of Pgp has also revealed that several residues in this region were important for Pgp stability, while others were essential for both stability and drug-stimulated ATPase activity. Surprisingly, mutation of other amino acids,

including the DEACSE modified lysine residue, had little effect on stability or ATPase activity (Bakos *et al.*, 1997).

The ligand dependence of peptide p17 labeling with DEACSE (Figure 16) is consistent with an alternate role other than ATP binding and hydrolysis for the Walker C motif. Labeling of this peptide was only slightly reduced in the presence of nucleotides (22%, n=15, s=9%, $P_{p17:MgAMPPNP}$«0.0005), but slightly enhanced in the presence of verapamil (14%, n=4, s=7%, $P_{p17:verap}$=0.015). In contrast, labeling of peptides p12 and p25 drastically diminished in the presence of nucleotides, 35% (n=12, s=14%, P_{p12}«0.0005), and 47% (n=15, s=10%, P_{p25}«0.0005) respectively, suggesting that p12 and p25 may play a more active role in ATP binding. A number of groups have reported conformational changes in the nucleotide binding domain with the binding of various substrates. Liu and Sharom (1996) observed quenching of the NBD bound MIANS probe in the presence of drug substrates, which they interpreted as a conformational change in the NBD with drug binding. Koronakis *et al.* (1993) reported that the nucleotide binding domain of hemolysin exporter HlyB, a closely related member of the ABC superfamily of proteins (Gerlach *et al.*, 1986), undergo a conformational change in the presence of MgATP that protects the HlyB cytoplasmic domain from proteinase K digestion.

Peptides p30, p40A and p40B all belonged to the second intracellular loop of the N-terminal half of Pgp, between putative transmembrane helices 4 and 5 (Figure 19). The DEACSE labeling data (Figure 16) indicated that the accessibility of peptide p30 (^{274}YNNNLEEA**K**R) did not change significantly in the presence of substrates. The unresolved peptide p40 experienced a modest but statistically significant increase in accessibility to the label with MgAMPPNP (20%, n=14, s=17%, P_{p40}<0.0005), and to a lesser extent MgATP (Figure 16).

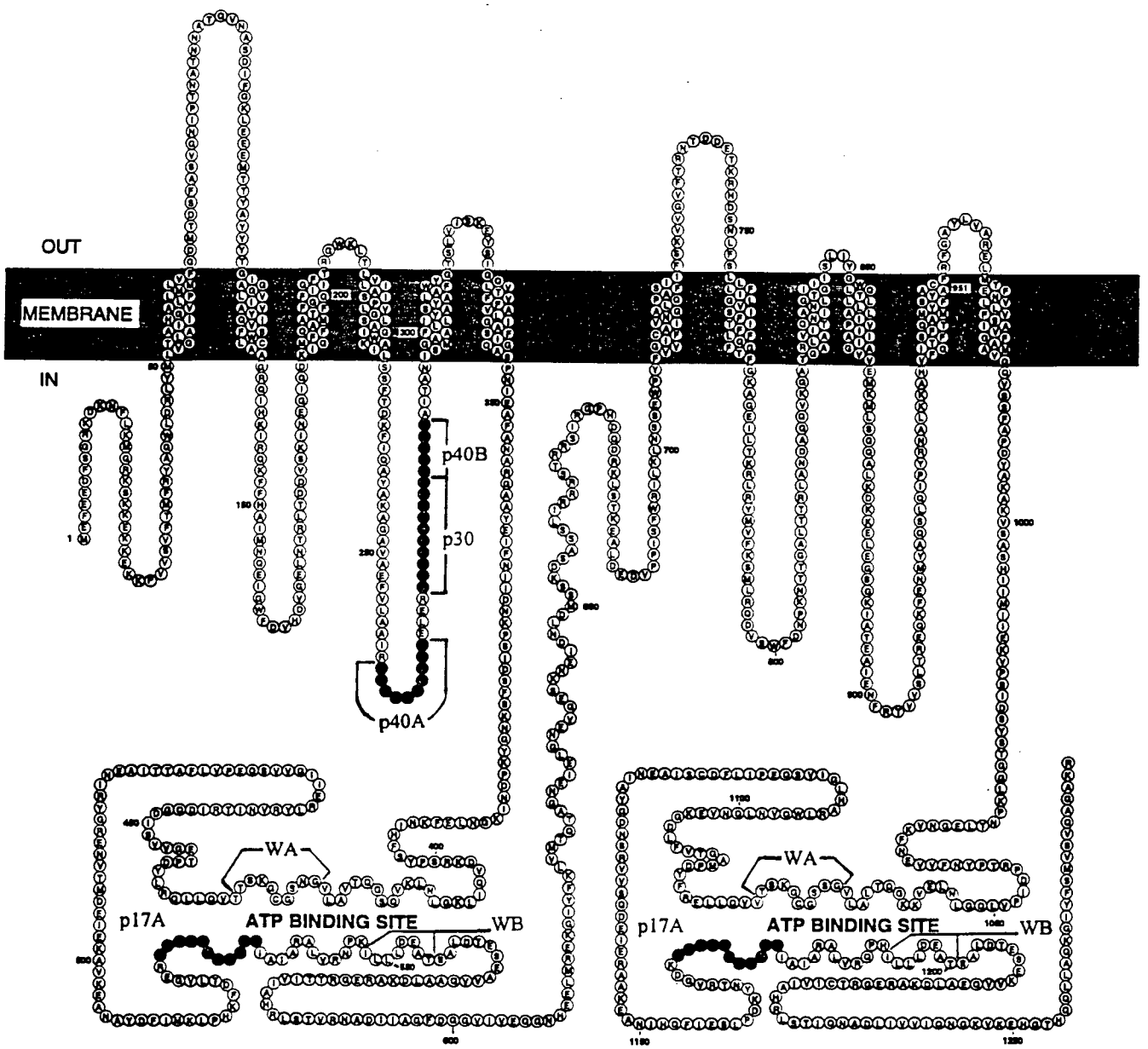

Figure 19: The location of DEACSE-labeled peptides p17A, p30, p40A and p40B within the amino acid sequence of Pgp. *The location of the Walker A (WA) and the Walker B (WB) conserved motifs within the nucleotide binding domains of Pgp are also indicated. This figure has been adapted from Shapiro et al., (1996).*

However, the differential labeling measured with p40 was an average of two peptides, p40A (^{260}TVIAFGGQ**K**K) and p40B (^{284}LGI**K**K), which were resolved by chromatography with a second gradient (Figure 17). Recent results have indicated that peptide p40A undergoes a more pronounced increase in labeling in the presence of MgAMPPNP than peptide p40B. Peptide p40A increased approximately 45%, whereas the increased in labeling was more modest for peptide p40B, approximately 16% (Figure 20). The high fluorescence intensities of peptides p30 and p40 indicated that the putative cytoplasmic loop that contain these peptides is readily accessible to the modification agent. The differential labeling of peptides within this loop indicated it likely plays a very dynamic role in the catalytic cycle of Pgp and that the different segments of the loop may take on different roles in the catalytic mechanism. It appears that with ATP binding, this intracellular loop undergoes substantial molecular motion that resulted in increased accessibility of certain segments (p40A and p40B) while the accessibility of other segments (p30) remain unchanged.

The increased labeling of peptides p40A and p40B in the presence of nucleotide correlates well with the hydrogen/deuterium exchange results from Sonveaux *et al.* (1996). Using infrared spectroscopy, the authors found that, in the presence of MgATP a significant portion of Pgp became more accessible to the solvent for exchange. They estimated that approximately 76 amino acids of the slowly exchanging residues became more exposed to the solvent. Coincidentally, this is approximately the size of the cytoplasmic loop of interest. However, we would expect that the increased hydrogen/deuterium exchange would involve other areas of the protein as well as this loop. Several groups have identified other areas within the transmembrane domains that experienced some type of molecular motion with the binding of

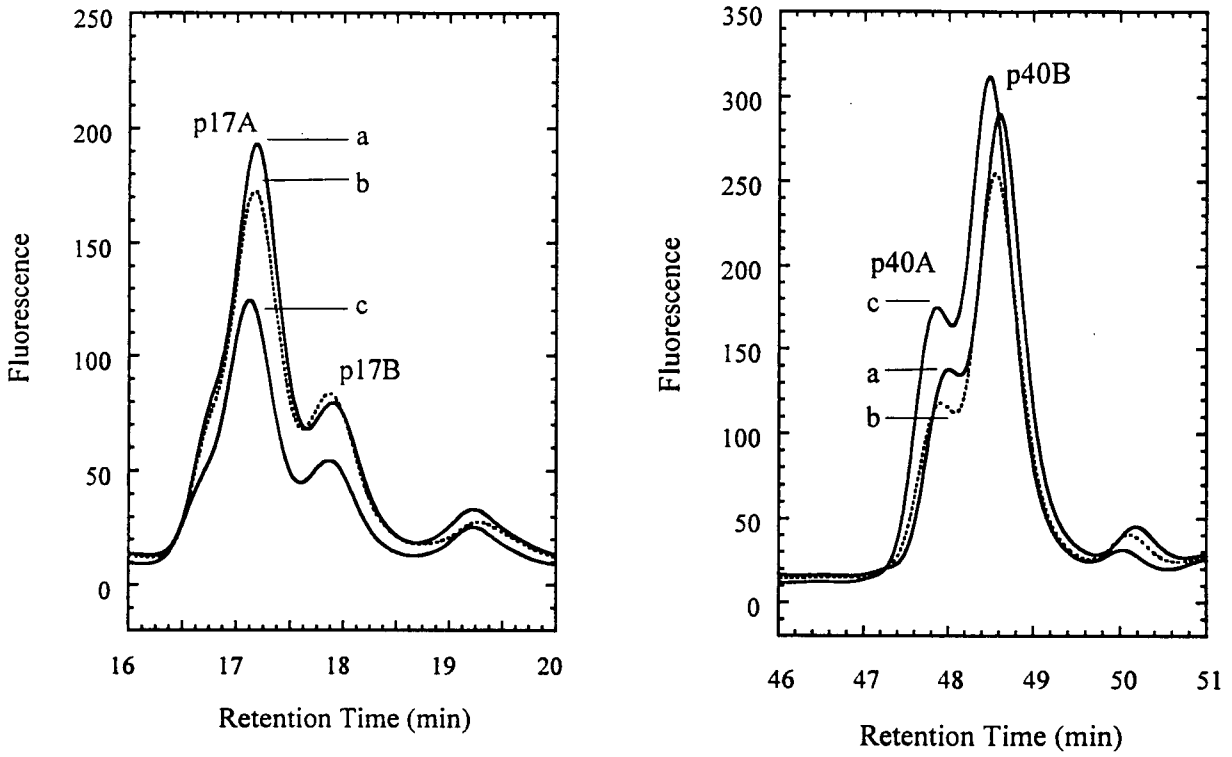

Figure 20: Change in DEACSE labeling of several peptides with different substrates. *Differential labeling of peptides p17A, p40A and p40B in the presence of a)no substrates, b)rhodamine 123, and c)MgAMPPNP.*

nucleotide substrates. These regions have been specifically mapped to the first extracellular loop (Mechetner *et al.*, 1997) and to a large region containing putative transmembrane helices 7 through 10 within the carboxyl-terminal transmembrane domain (Wang *et al.*, 1997). In addition, Loo and Clarke (1997a) reported that transmembrane helices 6 and 12 undergo conformational changes during drug binding or ATP hydrolysis. The hydrogen/deuterium exchange experiment also observed a decrease in solvent accessibility of a significant portion of Pgp with MgATP and verapamil. Interestingly, the opposite effect was observed with peptide

p29, where an increased accessibility to DEACSE was detected in the presence MgATP and verapamil (Figure 16). However, due to the relatively low abundance of this peptide, identification was not possible at this time.

The transmembrane domains of P-Glycoprotein have been shown to play an essential role in substrate binding and transport. Areas within the TMDs have been demonstrated by photoaffinity labeling experiments with photoactivatable drug analogues (Greenberger *et al.*, 1991; Greenberger, 1993; Bruggemann *et al.*, 1989; Borchers *et al.*, 1995) to interact with transported substrates. Specific membrane spanning sequences have been shown, by mutagenesis studies, to be important in drug interactions. Mutagenesis within putative transmembrane helices altered drug resistance profiles (Loo and Clarke, 1993a, 1993b; Taguchi *et al.*, 1997; Hanna *et al.*, 1996) and reduced photolabeling of Pgp by iodoarylazidoprazosin and azidopine (Kajiji *et al.*, 1993). In addition, transport of substrates by Pgp has been shown, by fluorescence studies, to occur from the cytoplasmic leaflet (Shapiro and Ling, 1997a, 1998), which would likely involve the use of the cytoplasmic loops between the transmembrane segments. These regions have also been shown to play a crucial role in Pgp function. A natural Pgp mutant with a point mutation in the first cytoplasmic loop is preferentially resistant to colchicine (Choi *et al.*, 1988). Photoaffinity labeling experiments (Safa *et al.*, 1990) and kinetic studies (Rao, 1995) showed that this mutation affected the interaction of Pgp with specific drugs. Currier *et al.* (1992) demonstrated that residues in the first cytoplasmic loop play an essential role in function and relative drug specificity by swapping specific amino acids in the first cytoplasmic loop of Class-I Pgp with corresponding amino acids of Class-III Pgp, a Pgp isoform that does not confer MDR (Ruetz and Gros, 1994; Smit *et al.*, 1994).

The second cytoplasmic loop has also been shown to be essential for Pgp folding and function. Mutagenesis studies by Loo and Clarke (1994a) established that glycine residues in this loop was essential for Pgp folding. Several glycine to valine mutants in this region resulted in proteins unable to confer resistance. The mutant proteins, with apparent mass of 150 kDa rather than 170 kDa, were believed to be misfolded and were retained in the endoplasmic reticulum. Other glycine mutations in this putative cytoplasmic loop as well as cytoplasmic loop 1 and 3 altered the drug resistance profile of the transfected cells. Furthermore, another group demonstrated that this particular loop was specifically photolabeled by [^{125}I]iodomycin, a daunomycin derivative (Demmer *et al.*, 1997).

It is apparent that the second cytoplasmic loop plays a key role in proper protein folding and drug binding. In the labeling experiments this loop was found to undergo an increased solvent accessibility as a result of MgATP/MgAMPPNP binding. From the iodomycin photolabeling results (Demmer *et al.*, 1997), we would expect to see a decrease in DEACSE labeling in the presence of daunomycin. Labeling experiments indicated that the proposed effect does not occur. However, a slight decrease in labeling of approximately 10% was observed with peptide p40A and p40B, in the presence of rhodamine 123 (Figure 20).

Various groups have found that Pgp may contain two or more drug binding sites (Shapiro and Ling, 1997b; Dey *et al.*, 1997; Ferry *et al.*, 1992; Tamai and Safa, 1991). By fluorescence studies, Shapiro and Ling (1997b) have proposed that Pgp contains two cooperative substrate binding sites with different specificities, one site specific for Hoechst 33342 (H site) and the other specific for rhodamine 123 (R site). Anthracyclines, including daunomycin, were found to inhibit rhodamine 123 transport while stimulating Hoechst 33342 transport. The authors

proposed that anthracyclines interact with the R site. This finding may in part explain the results obtained with rhodamine 123.

Furthermore, Pgp was found to possess relatively low affinities for its drug substrates (Liu and Sharom, 1996). Pgp may be able to compensate for the low affinity of its substrates through active transport directly from the lipid bilayer (Shapiro *et al.*, 1997), where the concentration of hydrophobic substrates are high. The lack of effect of various drug substrates on DEACSE labeling may be, in part, attributed to the low affinity of drug binding. In order to be confident of the modest effect of rhodamine 123, the labeling will have to be repeated to obtain statistically significance.

CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

In this thesis, a generalized fluorescent labeling technique was used to locate regions of Pgp that undergo a change in solvent accessibility as a result of ligand binding or ligand-dependent conformational changes. A lysine-specific fluorescent probe (DEACSE) provided high sensitivity of detection, chemical stability and the necessary global labeling of the protein. Immunoprecipitation with C219 monoclonal antibody covalently linked to Protein-A Sepharose provided a near quantitative yield of highly purified Pgp from resistant B30 plasma membrane vesicles.

Pgp was labeled with DEACSE in the presence of a number of ligands, including drug substrates, chemosensitizers, and adenine nucleotides. Several Pgp peptides that exhibited changes in the accessibility of lysine residues to DEACSE in the presence of different substrates were conclusively identified by a combination of MALDI-TOF mass spectroscopy and N-terminal peptide sequencing. Peak p17A exhibited decreased accessibility to DEACSE in the presence of nucleotide substrates. This fluorescent peak consisted of 2 near-identical peptides (^{525}GAQLSGGQ**K**QR and ^{1170}GTQLSGGQ**K**QR) that mapped to the nucleotide binding domains and contained the Walker C signature motif conserved amongst ABC proteins. The nucleotide dependence labeling of these two peptides is consistent with the structural changes found within the nucleotide binding domain of a homologous bacterial ABC protein (Hemolysin B) in the presence of MgATP (Koronakis *et al.*, 1993). Three additional peptides were identified and mapped to the second cytoplasmic loop of Pgp, between putative transmembrane helices 4 and 5. Interestingly, each of the 3 peptides in this short cytoplasmic loop of approximately 60

residues respond differently to the presence of ligands, suggesting the involvement of parts of this loop in ligand binding and/or ligand-induced conformational changes.

Two peptides p40A (^{260}TVIAFGGQ**K**K) and p40B (^{284}LGI**K**K) within this cytoplasmic loop exhibited significant increased labeling with nucleotide substrates. Increased accessibility of Pgp in the presence of nucleotide substrates has been documented by several groups (Sonveaux *et al.*, 1996; Wang *et al.*, 1997; Loo and Clarke, 1997a). Most of the reported structural changes have been localized to numerous regions within the transmembrane domains of Pgp, indicating that these domains are likely very dynamic. Furthermore, several possible topologies for Pgp have been reported (Kast *et al.*, 1995, 1996; Bibi and Beja, 1994; Beja and Bibi 1995; Zhang and Ling, 1991; Zhang *et al.*, 1993; Skach *et al.*, 1993; Skach and Lingappa, 1993, 1994). Although different topologies have not so far been correlated with ligand binding, these miscellaneous results indicate that Pgp has a highly mobile structure.

The second cytoplasmic loop has been shown to be involved in daunomycin binding (Demmer *et al.*, 1997). Yet in the presence of several different transport substrates, including daunomycin, no changes in DEACSE labeling were observed. Recent preliminary experiments, however, indicated a slight decrease of approximately 10% in the DEACSE labeling of p40A and p40B in the presence of rhodamine 123. Perhaps the reason for such a modest effect of the various drug substrates on DEACSE labeling is because of the low affinity of drug binding, as reported by Liu and Sharom (1996). In order to be certain that this effect is statistically significant, this experiment would need to be repeated.

The DEACSE labeling approach targeted lysine residues, thus the search for ligand-dependent conformational changes was restricted to the relatively hydrophilic regions within

Pgp, although several lysine residues are located in close proximity to, or within, putative transmembrane helices. In addition, the acetonitrile gradient used in this study was ideal in resolving relatively hydrophilic peptide mixtures (Hanakam *et al.*, 1995) and therefore highly hydrophobic peptides derived from the membrane spanning domains of Pgp are not expected to be resolved. The transmembrane domains (TMDs) of Pgp, however, are known to be involved in drug binding and transport. This has been shown by fluorescent studies (Shapiro *et al.*, 1997), photoaffinity labeling experiments (Greenberger *et al.*, 1991; Greenberger, 1993; Bruggemann *et al.*, 1989; Borchers *et al.*, 1995), as well as mutagenesis studies (Loo and Clarke, 1993a, 1993b; Taguchi *et al.*, 1997; Hanna *et al.*, 1996), as previously discussed. It has been established directly (Shapiro *et al.*, 1997) and indirectly (Homolya *et al.*, 1993; De Graaf *et al.*, 1996) that Pgp transports its substrates from the plasma membrane. To better study these hydrophobic regions, a number of different solvent systems (Moll and Thompson, 1994; Lew and London, 1997) could be used for future DEACSE labeling studies to complement the work in this thesis.

The identification of peptides by N-terminal sequencing was limited by the relatively low sensitivity of the technique, given the small amounts of labeled peptides available. Due to this lack of sensitivity, only 5 peptides were conclusively identified. Recent advances in mass spectrometry using ladder generating chemistry, which parallels that of DNA sequencing chemistry, may allow for more sensitive protein sequencing (Chait *et al.*, 1993; Wang *et al.*, 1993). Such an improvement would enable us to identify and map the less abundant DEACSE-labeled peptides of interests, such as p12, p25, and p29. In addition, other chemically-reactive residues, such as glutamate and aspartate, could be modified in the future to confirm labeling results from this study and/or to look for additional accessibility changes of Pgp.

The Walker C signature motifs of ABC proteins have been predicted to be involved in coupling nucleotide binding and/or hydrolysis to substrate transport (Hyde *et al.*, 1990; Hoedemaeker *et al.*, 1998). Non-specific DEACSE labeling has identified specific lysine residues within both the NBDs and the TMDs of Pgp that experienced changes in accessibility associated with nucleotide binding and therefore may participate in the transduction of energy from ATP hydrolysis to drug transport. Using resonance energy transfer, the distance between the NBDs and the membrane surface was calculated to be relatively short (Liu and Sharom, 1998), facilitating possible energy coupling between the NBDs and the TMDs. A similar approach could be used to investigate possible communication between the Walker C motifs with various areas of the transmembrane domains. Site-directed mutagenesis could be used to introduce specific residues for modification into the amino acid sequence corresponding to peptides p17A and p40A, allowing the incorporation of appropriate fluorophores. The distance between these two regions could then be calculated using fluorescence energy transfer in the presence and in the absence of nucleotide substrates. This information would be useful for formulating a model of the interactions between the Walker C motifs and the second cytoplasmic loop.

An advantage of the non-specific fluorescent labeling technique used in this thesis is its ability to locate regions on Pgp that exhibit ligand-induced changes in accessibility as a result of possible structural changes. Additional labeling studies with a number of different protein modification agents could provide an inventory of these peptides that could be used to interpret the complex mechanism of Pgp. Recently, Shapiro and Ling (1997b) reported that Pgp contains two distinct drug binding sites that interact cooperatively. The binding of substrate to one site

stimulated Pgp-mediated transport from the other site. The cooperation of these two sites may occur through additional ligand-induced conformational changes which could be investigated by non-specific fluorescent labeling. Furthermore, this method could be used to identify parts of Pgp involved in substrate binding at either of the proposed sites with the use of photoaffinity substrate analogues specific for each site. Thus a structural map of ligand binding sites and areas of ligand-dependent conformational changes in Pgp could be compiled.

The non-selective fluorescent labeling and purification techniques described in this thesis could be a potentially useful tool to specifically locate areas that undergo changes in accessibility as a result of conformational changes in other protein systems, including other ABC proteins such as Hemolysin B (HlyB). HlyB is a bacterial inner membrane ABC protein consisting of only 1 NBD and 1 TMD (for review see Sheps *et al.*, 1996). The hemolysin transport complex in *E. coli* is thought to exist as 2 molecules of HlyB, functioning as a homodimer, along with 2 molecules of Hemolysin D (HlyD) and a bacterial outer membrane protein TolC. This complex functions as an exporter of Hemolysin A (HlyA) across both membranes of *E.coli*. Koronakis *et al.* (1993) reported that the nucleotide binding domains of hemolysin exporter HlyB undergoes an ATP-dependent conformational change that protects the HlyB cytoplasmic domain from proteinase K digestion. The technique described in this thesis could potentially pinpoint specific areas of Hly B that experience altered accessibility in the presence of nucleotide and could reveal additional mechanistic information about the HlyB transport complex.

Another ABC transporter of great interest is the cystic fibrosis transmembrane conductance regulator, CFTR. Various naturally occurring mutations in this gene results in the emergence of cystic fibrosis (for review see Gadsby *et al.*, 1995). Like Pgp, CFTR is predicted

to consist of 2 repeated units in tandem, each containing a membrane spanning domain with 6 putative transmembrane helices, and a cytoplasmic nucleotide binding domain. However, these two units are separated by a unique R domain that is thought to be responsible for the regulation of chloride channel activity of CFTR (Riordan *et al.*, 1989). Gunderson and Kopito (1995) has demonstrated that CFTR exists in 3 different conductance states. The transition between the different conductance states was believed to be mediated through nucleotide-induced structural changes. In addition, phosphorylation of a number of sites in the R domain by cAMP-dependent kinases has been found to be essential for channel opening (Rich *et al.*, 1991; Hwang *et al.*, 1994). Rich *et al.* (1991) proposed that phosphorylation of the R domain causes conformational changes resulting in the release of this domain from a position that blocks the chloride channel. Due to the abundance of lysine residues in CFTR, lysine specific modification could prove to be very informative. Generalized fluorescent labeling would be ideal for specifically locating areas on CFTR that undergo these proposed structural changes. This information would contribute towards a better understanding of the complex mechanism that couples the different domains of this protein, as well as other ABC proteins, to ATP hydrolysis and substrate transport.

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