Kinetics of PAPS Translocase: Evidence for an Antiport Mechanism†

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ABSTRACT: In order to gain an understanding of the mechanisms involved in the transfer of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) from the cytosol where it is synthesized to the Golgi lumen where it serves as the universal sulfate donor for sulfate ester formation in higher organisms, we have undertaken a kinetic characterization of the PAPS translocase from rat liver Golgi. Analyzing the PAPS translocase activity in both intact Golgi vesicles and in a reconstituted liposome system, we have determined a number of physical and kinetic parameters. Strong competitive inhibition in *zero-trans* uptake experiments only with β-methylene PAPS and adenosine 3′,5′-bisphosphate (PAP) suggest the transporter is highly specific for the 3′-phosphate. The demonstration of *trans* acceleration as observed by stimulation of transport activity under exchange conditions suggests that the translocase is a carrier with distinct binding sites accessible from both faces of the membrane. The behavior of the PAPS translocase in the presence of equilibrium concentrations of PAP supports the function of an antiport mechanism. Thus the translocase is characterized by its kinetic properties as a specific transporter of PAPS which acts through an antiport mechanism with PAP as the returning ligand. This characterization of the transport activity has proved instrumental in the identification of a ∼230 kDa Golgi membrane protein as the PAPS translocase protein [Ozeran, J. D., Westley, J., & Schwartz, N. B. (1996) *Biochemistry 35*, 3695−3703 (accompanying paper)].

The transport of molecules of biological significance across the plasma membranes of a variety of cells has received considerable attention. Plasma membrane transporters have been described and characterized for a number of systems, including the nucleoside transporters in erythrocytes (Jarvis *et al.*, 1983) and intestinal cells (Huang *et al.*, 1993) and the erythrocyte choline transporter (Krupka & Devés, 1988). The erythrocyte ATP-driven sodium/potassium pump (Glynn & Karlish, 1990), the voltage-gated sodium/potassium channel of squid axons (Stuhmer *et al.*, 1989), and the sodium/calcium exchanger of cardiac muscle (Levitsky *et al.*, 1994) have also been well described. Among the metabolite transporters, neutral and cationic amino acid transporters of mammalian cells (Shotwell *et al.*, 1983; Closs *et al.*, 1993), sugar transporters in mammalian renal tubular cells (Silverman, 1976; Mullin *et al.*, 1986), rat ileal brush border membranes (Murer & Hopfer, 1974), and pancreatic β cells (Waeber *et al.*, 1994) have all been intensively studied. However, with the notable exception of mitochondrial transport proteins [for review see Palmieri *et al.*(1993)], there is a paucity of information concerning intracellular organelle metabolite transporters.

Sulfation is a critical modification in biological systems serving a wide variety of functions. 3′-Phosphoadenosine 5′-phosphosulfate (PAPS)[1] is the universal sulfate donor for sulfate ester formation in all higher organisms. Following synthesis of PAPS in the cytoplasm by the sequential action of ATP sulfurylase (ATP:sulfate adenylyl transferase, EC 2.7.7.4) and APS kinase (ATP:adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25) (Geller *et al.*, 1987; Lyle, 1993), PAPS must be transported into the Golgi lumen where sulfotransferases catalyze the formation of esters on most of the sulfated macromolecules (Dorfman, 1970; Vertel *et al.*, 1993).

As a highly charged, hydrophilic, unstable molecule, PAPS is unlikely to diffuse across the Golgi membrane in sufficient quantities to drive the sulfation of such a wide variety of molecules. Previous studies of PAPS transport, as well as nucleotide sugar transport, have been described only phenomenologically (Capasso & Hirschberg, 1984; Schwarz *et al.*, 1984; Milla *et al.*, 1992). In the current work, kinetic and mechanistic characterization of the PAPS translocase activity provides a better understanding of the function of these transporters of nucleotide metabolites. The PAPS translocase is a specific transporter of PAPS which acts though an antiport mechanism with adenosine 3′,5′-bisphosphate (PAP) as the returning ligand and is sensitive to inhibition by PAP and other structurally similar nucleotides. Utilizing this characterization, we have recently identified the Golgi membrane PAPS translocase which is responsible for the transfer of PAPS from the cytosol to the Golgi lumen as described in the accompanying manuscript (Ozeran *et al.*, 1996).

MATERIALS AND METHODS

Materials. The radiolabeled compounds, [^{35}S]PAPS (>400 Ci/mmol), γ-[^{32}P]ATP (6000 Ci/mmol), UDP-[^{14}C]galactose (272.8 mCi/mmol), [^{3}H]H_2O (1 mCi/mL), [^{3}H]-2-deoxyglucose (8.0 mCi/mmol, 0.2 mCi/mL), and [^{3}H]methoxyinulin (230 mCi/gram, 0.2 mCi/mL) were purchased from New England Nuclear. Universol liquid scintillation fluid from ICN was used for scintillation counting. Ultrapure sucrose and dithiothreitol were purchased from Gibco BRL. Phos-

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[1] Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; APS, adenosine 5′-phosphosulfate; PAP, adenosine 3′,5′-bisphosphate.

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phatidylcholine, cholesterol, and stearylamine were purchased from Avanti Polar Lipids. ATP, ADP, AMP, IMP, adenosine-3′,5′-bisphosphate (PAP), HEPES, and bovine hemoglobin were obtained from Sigma. Silica and polyethyleneimine cellulose TLC plates with fluorescent indicator were from Merck. Ultrapure PAPS was purchased from Dr. Sanford Singer, University of Dayton, OH. β-Methylene-APS, β-methylene-PAPS, and 3′-[^{32}P]-β-methylene PAPS analogs were synthesized in our laboratory as described (Callahan *et al.*, 1989; Ng *et al.*, 1991; J. D. Ozeran, S. Lyle, K. Ng, J. Westley, and N. B. Schwartz, manuscript in preparation). SM-2 Bio-Beads adsorbent and Bio-Spin-30 chromatography columns were from Bio-Rad. Ultrapure "protein grade" NP-40 was from Calbiochem. Centricon 30 and Centriprep 30 concentrators, stirred ultrafiltration cells, and CM-30 ultrafiltration membranes were from Amicon. Reagent grade acetone was from Baker. All other chemicals were reagent grade. APS kinase from *Penicillium chrysogenum* was a generous gift of Dr. Irwin Segel, University of California, Davis, CA.

Methods. Golgi Preparation. Golgi-enriched membrane vesicles were prepared essentially by the method of Leelavathi *et al.* (1979) with modifications based on Geetha-Habib *et al.* (1984). Membranes layering between 21.6% and 40% sucrose, enriched 48-fold in galactosyltransferase activity, were used for further experiments.

Protein Assays. Protein determinations were made based on the method of Smith *et al.* (1985), using bovine serum albumin as the standard and Pierce's BCA reagent for color development. The Pierce standard or microtiter plate protocols were used for all samples.

PAPS Transport into Intact Golgi Vesicles. Transport of solutes into intact Golgi vesicles was assayed essentially as described by Perez and Hirschberg (1987). Membrane samples containing 1.0 mg of protein were incubated with [^{35}S]PAPS (2 μM for standard assays), [^{3}H]-2-deoxyglucose (0.2 μCi/mL) or [^{3}H]methoxyinulin (0.2 μCi/mL) in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM $MgCl_2$, and 1 mM DTT in a total volume of 1.0 mL. Following any preincubations of vesicles with inhibitors in 0.8 mL, assays were begun by the addition of 0.2 mL aliquots of Golgi vesicle suspensions containing approximately 0.5 mg of protein. Standard incubation was at 37 °C for 10 min. Reactions were stopped by placing the tubes on ice and immediately centrifuging at 95 000 rpm in a TLA-100.2 rotor for 15 min at 4 °C.

A 100-μL aliquot of supernatant was removed for scintillation counting to determine [S_m], the concentration of transported solute in the bulk reaction medium. Remaining supernatant was discarded, and the pellet surface was washed three times with ice-cold buffer over 60 s. Pellets were then resuspended in 0.5 mL of water and counted in a Packard β scintillation counter. [^{3}H]-2-Deoxyglucose served as a standard penetrator to provide a measurement of the total pellet volume. [^{3}H]Methoxyinulin served as a standard non-penetrator to measure bulk solvent trapped within the pellet. Vesicle volumes were typically 3−5 μL/mg of Golgi protein. Transport was calculated according to the definintions and formulae described by Perez and Hirschberg (1987).

Solubilization and Reconstitution of Golgi Membrane Proteins. Golgi membranes (5 mg of protein/15 mL) were solubilized with 0.5% NP-40 in solubilizing buffer on ice followed by centrifugation at 100 000*g* for 1 h to remove insoluble material. Solubilized proteins were concentrated to ∼10 mg/mL using either Centriprep-30 concentrators or a stirred ultrafiltration cell with a 30 000 MW cutoff membrane and used immediately or stored in 1-mL aliquots at −70 °C.

Detergent was removed from solubilized Golgi proteins (10 mg/mL) either by incubation with Bio-Rad SM-2 Bio-Beads (300 mg/mL of protein solution) for two sequential extractions or by passage through a 2-mL column of Pierce's Extracti-Gel D which had been pre-equilibrated with solubilizing buffer and "blocked" with a solution of 1 mg of hemoglobin/mL to prevent nonspecific protein binding (Milla *et al.*, 1992). Phosphatidylcholine liposomes were prepared by drying 200 mg of egg yolk phosphatidylcholine from 4 mL of chloroform under a stream of nitrogen and lyophilizing overnight. Lipids were resuspended in "PG" buffer [20 mM phosphate, pH 7.4, 1% (v/v) glycerol] to 30 mg/mL and sonicated under nitrogen until transparent. Detergent-depleted protein (∼1 mg) and liposomes were combined (∼1:10 w/w) in the absence or presence of defined concentrations of nucleotides and reconstituted by a freeze−thaw−sonication method which produced primarily small unilamellar vesicles (Deamer & Uster, 1983). In this method (Kasahara & Hinkle, 1976) the combined protein and liposome solutions were rapidly frozen in a dry-ice:acetone bath and thawed slowly at room temperature (∼15 min at 25 °C). Following five freeze−thaw cycles, solutions were sonicated briefly (∼5 s) using a microtip sonicator. Reconstituted Golgi protein samples were then assayed immediately for PAPS translocase activity.

PAPS Transport into Reconstituted Liposomes. PAPS transport in reconstituted vesicles was measured by a modification of the method of Zaruba *et al.*, (1988). Following a brief (1−2 s) sonication, reconstituted vesicles containing 0.25−1.0 mg of protein/mL were incubated with 2.0 μM [^{35}S]PAPS in buffer containing 20 mM phosphate, pH 7.4, 1 mM $MgCl_2$, and 1% (v/v) glycerol at 37 °C. Transport was terminated after 5 min by passing 100 μL of the reaction mixture through a Bio-Spin-30 column and collecting the void volume peak. Because of the size difference between the reconstituted vesicles and the [^{35}S]-PAPS, vesicles containing translocated PAPS eluted in the void volume while the smaller [^{35}S]PAPS was retained in the column.

Kinetic Data Analysis. All kinetic data were analyzed using the Microsoft QuickBASIC program "MacEnzkin" on an Apple Macintosh IIci computer. This program fits the initial velocity measurements as a function of substrate concentration to the best rectangular hyperbola by an iterative least-squares function.

RESULTS

Characterization of PAPS Transport in Intact Golgi Membranes. In order to establish the kinetic parameters of PAPS transport, a series of experiments was designed in which PAPS transport was assayed in the presence of a 200-fold range of substrate concentration. PAPS translocase activity is dependent upon PAPS concentration in the range 0.1−20.0 μM in a saturable fashion (Figure 1), is linear with time up to 20 min (Figure 1 inset), and equilibrates over

Table 1: Kinetic Constants of PAPS Translocase Activity

	K_m[a] (μM)	V_{max} (pmol/min/mg of protein)
PAPS translocase in intact Golgi membranes[b]	0.80 ± 0.01	10.86 ± 0.20
PAPS translocase in reconstituted vesicles	0.86 ± 0.05	21.59 ± 1.52
PAPS translocation of β-methylene PAPS by intact Golgi vesicles	15.4 ± 1.74	12.45 ± 0.16

[a] Values are derived from the nonlinear least-squares fits of the slopes and intercepts. [b] PAPS translocase activity was measured in intact Golgi vesicles and reconstituted vesicles with [^{35}S]PAPS in the range 0.1−200 μM as described in Materials and Methods. Translocation of β-methylene PAPS into intact Golgi vesicles was measured using 3′-[^{32}P]-β-methylene PAPS in the range 0.1−200 μM. Values for K_m and V_{max} were determined from nonlinear least-squares regression to the best-fit rectangular hyperbola using the MacEnzkin program on a Macintosh IIci.

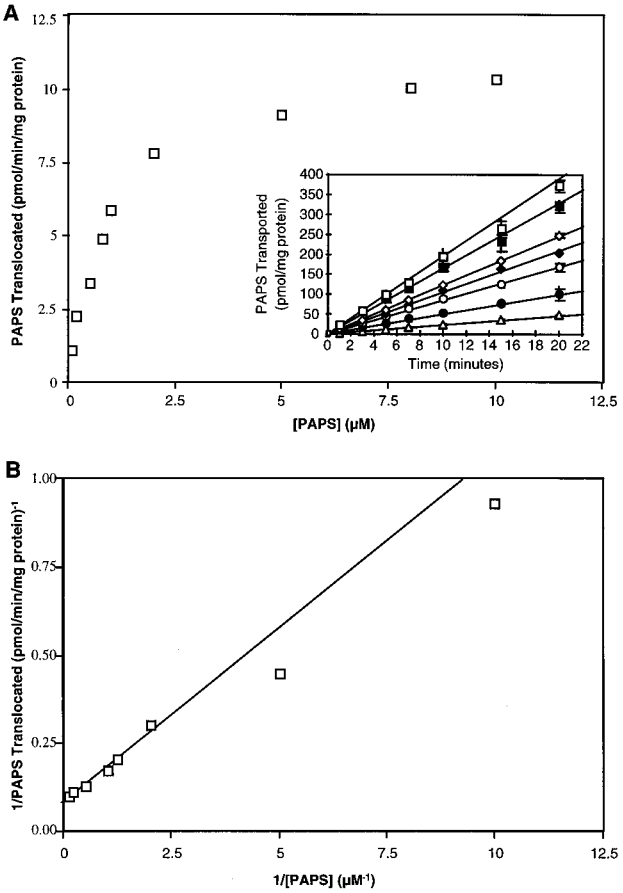

FIGURE 1: Dependence of PAPS translocase activity on PAPS concentration. Initial velocities of PAPS translocase activity were determined as a function of PAPS concentration. Golgi vesicles containing 1.0 mg of protein were assayed for uptake of [^{35}S]PAPS, and PAPS transport was calculated as described in Materials and Methods. Data are the means ± standard deviation for six determinations. (A) Hyperbolic plot of initial velocities of PAPS translocase over the range of PAPS concentrations from 0.01 to 10.0 μM. Inset: Initial velocities of PAPS translocase activity as a function of time. Assays of PAPS transport were terminated after 0, 1, 3, 5, 7, 10, 15, and 20 min and analyzed as described. [^{35}S]-PAPS concentrations were 0.1 (△), 0.2 (●), 0.5 (○), 1.0 (◆), 2.0 (◇), 5.0 (■), and 10 μM (□). Data are the means ± standard deviation for four determinations. (B) Initial velocity pattern in the form of a Lineweaver−Burke double-reciprocal plot. The line shown is modeled using the kinetic constants provided by nonlinear fitting of the entire data using the MacEnzkin program.

time (data not shown). Transport activity as a function of PAPS concentration in intact Golgi membranes yields an apparent $K_m = 0.83$ μM and an apparent $V_{max} = 10.86$ pmol of PAPS transported/min/mg of protein (Table 1). To investigate the possible relationships among the Golgi membrane PAPS translocase, the adenine nucleotide transporter of mitochondria (Vignais, 1976), and the erythrocyte nucleoside transporter (Jarvis, 1991), the inhibitors of mitochondrial adenine nucleotide transport (atractyloside, carboxyatractyloside, palmitoyl coenzyme A) and of erythrocyte anion transport [4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS)] were analyzed for their ability to inhibit PAPS transport. Under standard PAPS translocase assay conditions, these compounds inhibited activity only at very high concentrations (Table 2), suggesting that the PAPS translocase is not very closely related to these other transporters.

Table 2: Inhibition of PAPS Transport by Nucleotides and Inhibitors of Nucleotide and Anion Transporters[a]

compound	5 μM	50 μM
3′,5′-ADP	24	5
5′-ATP	96	57
5′-ADP	91	45
5′-AMP	93	52

compound	5 μM	10 μM	50 μM	100 μM
DIDS	ND[b]	ND	94.1	25.2
carboxyatractyloside	ND	89	78	67
atractyloside	87	79	65	ND
palmitoyl CoA	ND	94	86	75

[a] PAPS translocase activities are reported as percentage of control transport activity with no inhibitors present. [b] ND, not determined.

To study the structural requirements of ligands for PAPS binding and transport, a series of experiments was designed in which PAPS transport was assayed in the presence of several adenosine nucleotide compounds. 3′-β-Methylene PAPS, PAP, ATP, ADP, AMP, and GTP were tested for their ability to inhibit PAPS transport activity (Figures 2−4). Since the intercepts of the primary plots are scattered around a single value, while the slopes vary linearly with respect to the inhibitor concentrations, the observed behavior corresponds to competitive inhibition. This competitive nature and the relative affinities of the PAPS translocase for each of the inhibitors suggest that PAPS binding is selective for adenine nucleotides containing 3′-phosphate groups, although specificity is limited with regard to 5′ substitutions on the substrate. Additionally, transport of β-methylene PAPS was assayed using 3′-[^{32}P]-β-methylene PAPS (Figure 5). The apparent K_m and V_{max} for β-methylene PAPS transport were 15.4 μM and 12.45 pmol/min/mg of protein, respectively (Table 1). These results suggest that transport of the analog, β-methylene PAPS, differs from transport of the natural substrate, PAPS, only in the affinity of the translocase for β-methylene PAPS as a substrate. It would appear, therefore, that the β-methylene substitution does not interfere with translocation *per se*.

Characterization of Reconstituted PAPS Transport. In order to purify and characterize the isolated translocase, a standardized reconstitution system was neccessary. The translocase activity was solubilized and reconstituted as described in the accompanying manuscript (Ozeran *et al.*,

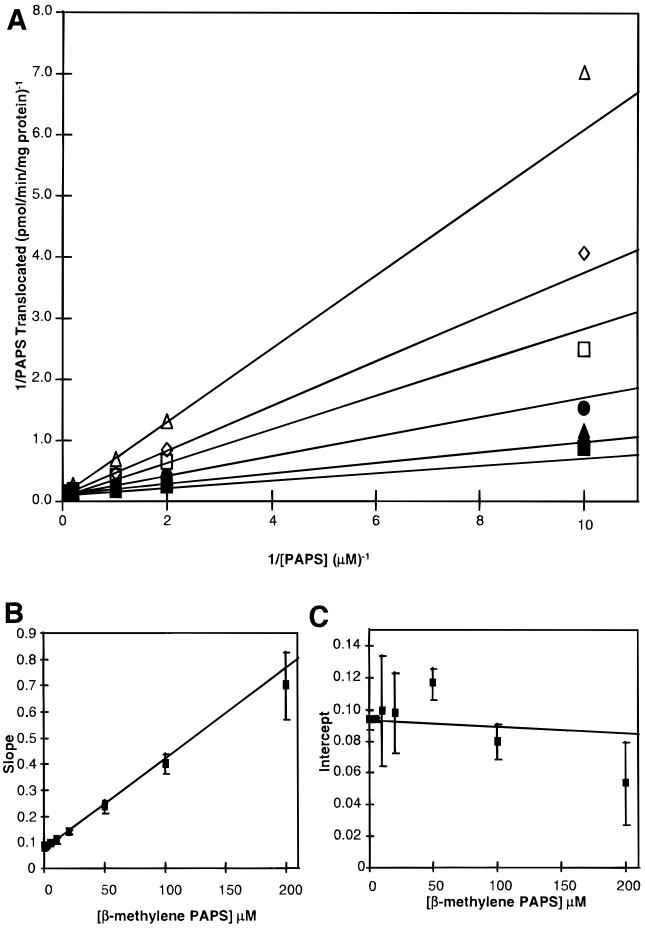

FIGURE 2: Inhibition of PAPS translocase by β-methylene PAPS. Golgi vesicles containing 1.0 mg of protein were assayed for uptake of [^{35}S]PAPS in the presence or absence of β-methylene PAPS at concentrations from 10 to 200 μM, and PAPS transport was calculated as described in Materials and Methods. Data are the means ± standard deviation for six determinations. (A) Lineweaver−Burke double-reciprocal plot of the inhibition pattern for PAPS translocase in the presence of the following concentrations of β-methylene PAPS: (■), uninhibited; (▲), 10 μM; (●), 20 μM; (□), 50 μM; (◇), 100 μM; (△), 200 μM. (B) The secondary plot of primary slopes versus β-methylene PAPS concentration. (C) The secondary plot of primary intercepts versus β-methylene PAPS concentration.

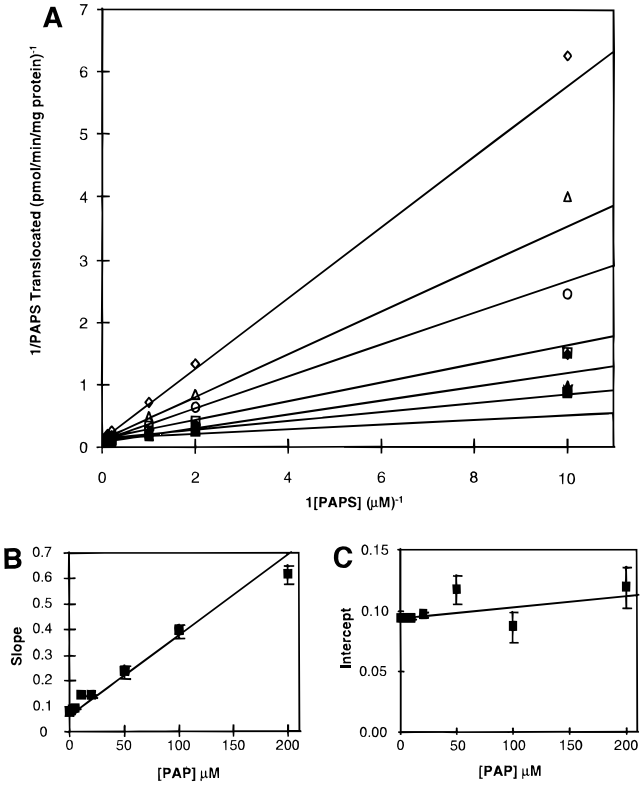

FIGURE 3: Inhibition of PAPS translocase by adenosine 3′,5′-bisphosphate. Initial velocities of PAPS translocase activity were determined as a function of PAPS concentration in the presence and absence of adenosine 3′,5′-bisphosphate. Golgi vesicles containing 1.0 mg of protein were assayed for uptake of [^{35}S]PAPS as described in the presence or absence of PAP at concentrations from 5 to 200 μM, and PAPS transport was calculated as described. Data are the means ± standard deviation for six determinations. (A) Lineweaver−Burke double-reciprocal plot of the inhibition pattern for PAPS translocase in the presence of the following concentrations of PAP: (■), uninhibited; (▲), 5 μM; (◆), 10 μM; (□), 20 μM; (○), 50 μM; (△), 100 μM; (◇), 200 μM. (B) The secondary plot of primary slopes versus PAP concentration. (C) The secondary plot of primary intercepts versus PAP concentration.

1996). In order to confirm that the activity measured in the reconstituted assay corresponded to that of the intact Golgi membranes, a series of experiments was designed to characterize the reconstituted PAPS translocase as a function of time, protein concentration and substrate concentration. The PAPS translocase activity of reconstituted Golgi protein was assayed under standard assay conditions, using samples of reconstituted vesicles containing 0.1−5.0 mg of protein. For substrate dependence, [^{35}S]PAPS concentration was varied in the range 0.1−10.0 μM and incubated for 5 min at 37 °C, and samples were processed as described. [^{35}S]PAPS which had been taken up by liposomes eluted in the void volume. Because of the typically smaller size of liposomes versus Golgi vesicles (Racker, 1985), zero-time kinetics might be expected to not yield accurate results; therefore dependence on time of PAPS translocase in reconstituted liposomes was critical to the use of this assay as a tool for characterization. For example, if the uptake of [^{35}S]PAPS was not linear with time throughout the experimental range, it could be due to the smaller vesicles "filling-up" more rapidly and reaching a point where [^{35}S]PAPS is transported out of liposomes as rapidly or even more rapidly than it is transported into the liposomes. However, as shown in Figure 6, although PAPS translocase activity in reconstituted liposomes did begin to visibly decrease after ∼12 min, the period of the standard reconstituted assay (0−5 min) was entirely within the linear phase of uptake.

The linear dependence on protein concentration exhibited by the reconstituted PAPS translocase (Figure 7) further supports the validity of the reconstituted assay by indicating that, under these conditions, PAPS translocase is measurable with a high degree of accuracy and reproducibility considering the difficulty in solubilizing and reinserting the translocase into the artificial bilayer. Similarly, the normal form of the dependence on substrate concentration exhibited by the reconstituted translocase (Figure 8) supports the conclusion that the activity being measured in the assay is, in fact, the same as that measured in intact Golgi vesicles. The apparent K_m for the reconstituted translocase was 0.86 μM, and V_{max} was 21.6 pmol/min/mg of protein (Table 1).

The reconstituted PAPS translocase activity was also measured in the presence of the two strong competitive inhibitors of transport, β-methylene PAPS (Figure 9) and PAP (Figure 10) at inhibitor concentrations ranging from 5 to 100 μM. The kinetic constants determined by these assays

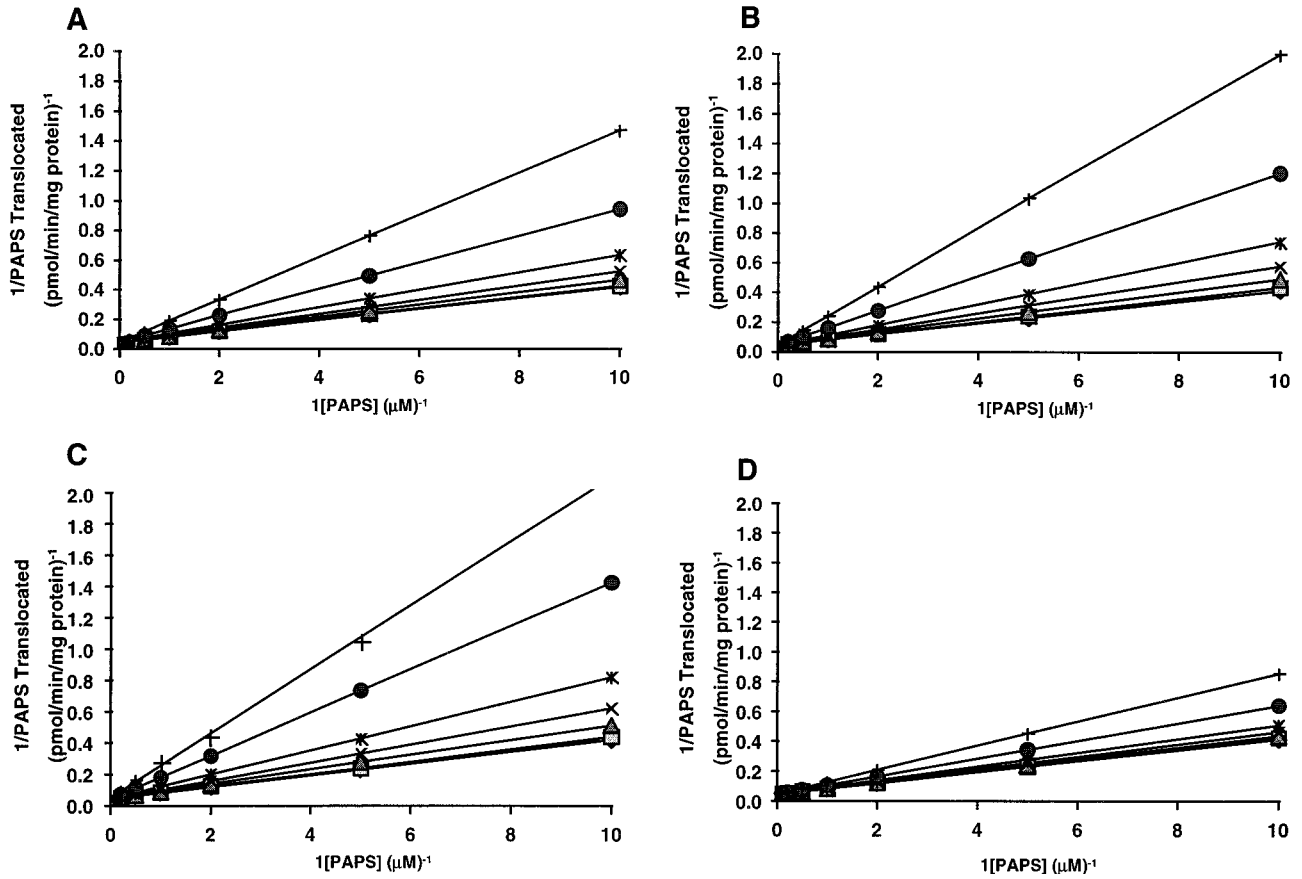

FIGURE 4: Inhibition of PAPS translocase by purine nucleotides. Lineweaver−Burke double-reciprocal plot of the inhibition pattern for PAPS translocase in the presence of the following concentrations (all in μM) of (A) ATP: (◆), uninhibited; (□), 1; (△), 5; (×), 10; (●), 50; (+), 100. (B) ADP: (◆), uninhibited; (□), 1; (△), 5; (×), 10; (●), 50; (+), 100. (C) AMP: (◆), uninhibited; (□), 1; (△), 5; (×), 10; (●), 50; (+), 100. (D) GTP: (◆), uninhibited; (□), 1; (△), 5; (×), 10; (●), 50; (+), 100.

Table 3: Kinetic Constants for PAPS Translocase Inhibition

Nucleotide	K_I (μM)[b]	K_m[PAPS][c]	V_{max}
	(A) Intact Golgi Vesicles[a]		
β-methylene PAPS	7.62 ± 0.04	0.784 ± 0.012	10.82 ± 0.22
adenosine 3′,5′-diphosphate	5.38 ± 0.04	0.801 ± 0.033	10.34 ± 1.11
AMP	18.90 ± 0.20	0.797 ± 0.015	10.86 ± 0.27
ADP	23.51 ± 0.16	0.798 ± 0.015	10.95 ± 0.31
ATP	35.35 ± 0.17	0.799 ± 0.010	10.91 ± 0.20
GTP	84.66 ± 0.35	0.798 ± 0.003	10.83 ± 0.05
	(B) Reconstituted Liposomes[d]		
β-methylene PAPS	7.94 ± 0.12	0.798 ± 0.056	21.37 ± 0.79
adenosine 3′,5′-diphosphate	6.36 ± 0.57	0.821 ± 0.028	21.46 ± 0.70

[a] PAPS translocase activity was measured in intact Golgi vesicles using [^{35}S]PAPS concentrations in the range 0.1−200 μM. Concentrations of the inhibitors ATP, ADP, AMP, PAP, and β-methylene PAPS were in the range 0.1−200 μM. [b] Values for K_I are derived from the linear least-squares fits of the slopes and intercepts of the primary plot versus inhibitor concentration. [c] Values for K_m and V_{max} are derived from the nonlinear least-squares fits of the slopes and intercepts of the uninhibited translocase. [d] PAPS translocase activity was measured in reconstituted liposomes using [^{35}S]PAPS concentrations in the range 0.1−200 μM. Concentrations of the inhibitors β-methylene PAPS and PAP were in the range 0.1−200 μM.

are nearly identical to those of intact Golgi translocase (Table 3), indicating the similarity between the PAPS translocase activity in intact Golgi and in reconstituted liposomes. Thus, these results validate the use of this assay to monitor PAPS translocase activity during further studies.

Equilibrium Analyses of PAPS Translocase. In order to characterize the PAPS translocase activity more fully and to study the effects of substrates and inhibitors at the *trans* face of the membrane, it was necessary to design several experiments to analyze the equilibrium exchange behavior of the PAPS translocase. By forming reconstituted PAPS translocase liposomes in the presence of fixed concentrations of PAPS or PAP, we were able to record measurements for the initial rates of PAPS transport under "equilibrium exchange" conditions for PAPS and "*zero-trans*" conditions for PAPS in the presence of equilibrium concentrations of PAP. For PAPS equilibrium exchange measurements, soluble Golgi proteins (~1 mg) were reconstituted in the presence of unlabeled PAPS at concentrations ranging from 1 to 200 μM.

Examination of PAPS translocase activity as a function of equilibrium concentration of PAPS (Figure 11) showed hyperbolic kinetics with an apparent K_m = 7.89 μM and V_{max} = 87.81 pmol/min/mg of protein. This represents an approximately 5-fold stimulation in the overall activity versus intact Golgi vesicles and a 4-fold stimulation over PAPS translocase in reconstituted vesicles. In addition, a slight increase in the translocase's efficiency (K_m/V_{max}) was noted.

To determine the effect of a competitive inhibitor (PAP) in equilibrium versus PAPS, liposomes were formed in the presence of 1−200 μM PAP. Assays of PAPS translocase activity were initiated by the addition of [^{35}S]PAPS to concentrations of 0.1−10.0 μM. Following 5 min of incubation at 37 °C, samples were treated as described previously.

A striking result was seen in the measurement of PAPS uptake into vesicles preloaded with PAP (Figure 12). Rather

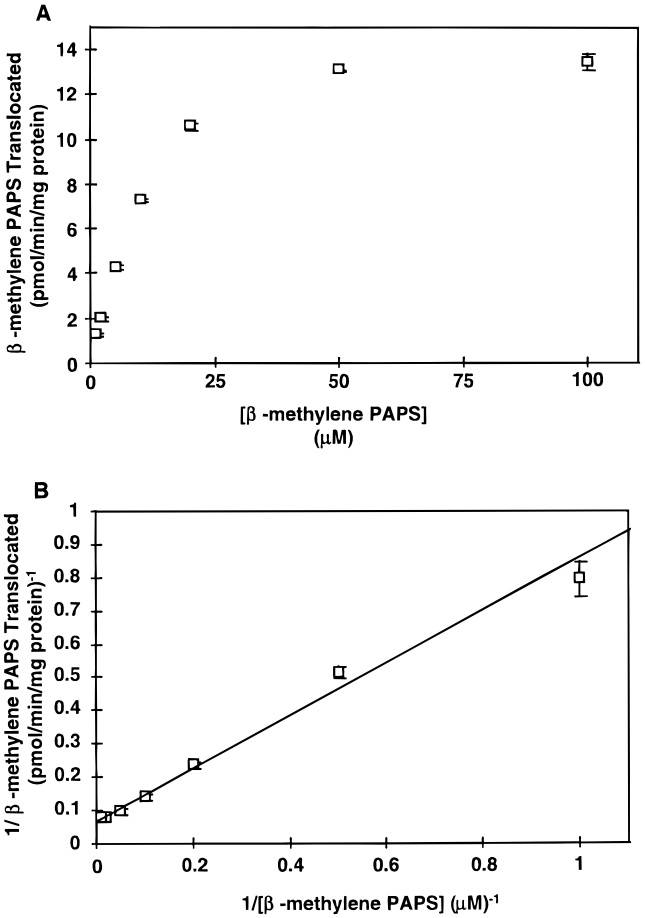

FIGURE 5: PAPS translocase uptake of β-methylene PAPS. (A) Hyperbolic plot of initial velocities of PAPS translocase over a range of β-methylene PAPS concentrations from 1.0 to 100 μM. (B) Initial velocity pattern in the form of a Lineweaver−Burke double-reciprocal plot of the same data as in A. The line shown is modeled using the kinetic constants provided by nonlinear fitting of the entire range of data using the MacEnzkin program.

than a simple pattern describing inhibition of transport (as seen in *zero-trans* studies) or even stimulation (as seen with PAPS in equilibrium), a mixed pattern is observed. This pattern is characteristic of transporters (Devés & Krupka, 1987) interacting with competitive inhibitors and provides additional evidence that the activity studied is clearly that of transport.

DISCUSSION

Transporters of cellular (or subcellular) metabolites are often considered to be a specialized class of membrane receptors where ligand, substrate, and effector are one (Racker, 1985). A transport protein had previously been proposed to exist for PAPS, as well as for several of the nucleotide sugars, based upon the effect on transport of the nucleotide sugars by preincubation of Golgi vesicles with other nucleotide derivatives (Capasso & Hirschberg, 1984). Although no direct evidence had been presented for the particular case of PAPS transport (Capasso & Hirschberg, 1984), it was also hypothesized on the basis of some double-label substrate studies that these transporters in general behave as antiports utilizing the corresponding nucleoside monophosphate. Because the activity of a transport system is dependent upon the return of the reciprocating transporter and on the conditions within each of the compartments with which it interacts, analyses of transport must consider the effect of *trans* concentrations of substrates, inhibitors and other effectors. In fact, studies of solublilized membrane proteins and reconstituted receptors and transporters may yield similar results when investigated using only *zero-trans* conditions. This is because, under these conditions, one is only looking at the effects within a single compartment.

To fully characterize the PAPS translocase activity of intact Golgi membranes and reconstituted liposomes, initial velocities of *zero-trans* translocation were determined as well as initial velocities for equilibrium exchange and *zero-trans* in the presence of equilibrium concentrations of a competitive inhibitor of transport. Inhibition of PAPS uptake by intact vesicles and reconstituted liposomes was also analyzed to identify possible structural characteristics recognized by the PAPS binding site(s). Varying concentrations of β-methylene PAPS, PAP, AMP, ADP, and ATP versus a range of PAPS concentrations generated initial rate values and double-reciprocal patterns as shown in Figures 2−4. Slope and intercept analysis as a function of inhibitor concentration allowed the determination of inhibition type and apparent K_I for each inhibitor (Table 3). An additional series of experiments utilizing a double-labeled system of [^{35}S]PAPS and 3′-[^{32}P]-β-methylene PAPS preloaded into reconstituted vesicles is under development in order to provide direct evidence for the stoichiometry of PAPS transport.

West (1983) defines three essential functions for a mobile carrier. First, a carrier must contain a substrate-specific binding site. Second, this binding site must alternate between two states generally assumed to be accessible to the external compartment and alternately to the internal compartment. Third, the transition between these two states is a discrete kinetic event which can be assigned a rate constant. The strong competitive inhibition seen in *zero-trans* uptake experiments versus β-methylene PAPS and PAP (K_I = 7.61 and 5.38 μM, respectively) and the much weaker inhibition seen versus ATP, ADP and AMP (K_I's are ∼2.5−6.6-fold greater), assign a significant degree of specificity to the transporter and help define the 3′-phosphate as the major structural component recognized in substrates, fulfilling the first of West's criteria.

The demonstration of "*trans* acceleration" [as defined by Lieb (1982)] by PAPS translocase, with the observation of a 5-fold stimulation of activity under exchange conditions, supports the identification of the PAPS translocase entity as a carrier with distinct binding sites accessible from the opposite faces of the membrane, thus fulfilling the second of West's criteria. The third criterion for the definition of a mobile carrier is addressed by considering PAPS translocase as a simple mobile carrier. In this case, a four-state system exists with two states of the free carrier which can interact with substrate at either of the two membrane faces (Figure 13). The maximum rate of transport under *zero-trans* conditions depends on the rate constant for inward translocation of the carrier−substrate complex (f_2) *and* the rate constant for the outward movement of the empty carrier returning it to the *cis* face (f_{-1}). Under exchange modes of analysis, however, the maximum rate of transport depends exclusively on the rates of translocation of the carrier−substrate complexes (f_2 and f_{-2}). The definitions of the maximal rates of transport (V_{max}) and half-saturation constants (K_m) for this simple model are presented in Table 4. Based on the analysis of the available data, and allowing for the

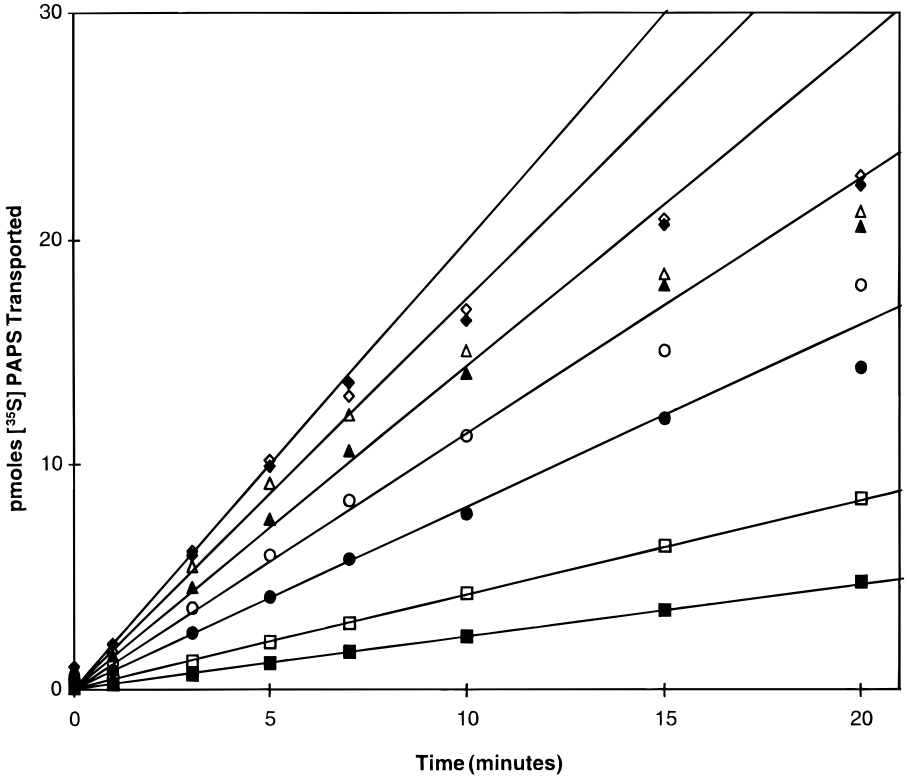

FIGURE 6: Dependence of reconstituted PAPS translocase activity on time. Solubilized, detergent-depleted Golgi membrane proteins (~1 mg) were reconstituted into egg yolk phosphatidylcholine liposomes as described in Materials and Methods. Samples of reconstituted vesicles containing 1.0 mg of protein were assayed as described, with [^{35}S]PAPS concentrations of 0.1 (■), 0.2 (□), 0.5 (●), 1.0 (○), 2.0 (▲), 5.0 (△), 10 (◆), and 20 μM (◇). Following 0, 1, 3, 5, 7, 10, 15, and 20 min of incubation at 37 °C, samples were loaded onto Bio-Spin 30 columns and spun in an IEC clinical benchtop centrifuge at top speed (~1300 rpm) for 4 min. [^{35}S]PAPS which had been taken up by liposomes eluted in the void volume.

Table 4: Kinetics of Membrane Transport

experiment type	V_{max}[a]	K_m[a]
	Expression	
zero-trans entry	$\frac{f_2 f_{-1} 4C_t}{f_2 + f_{-1}}$	$\frac{K_{So}(f_1 + f_{-1})}{(f_{-1} + f_2)}$
equilibrium exchange[b]	$\frac{f_2 f_{-2} C_t}{f_2 + f_{-2}}$	$\frac{K_{So}\left(1 + \frac{f_1}{f_{-1}}\right)}{\left(1 + \frac{f_2}{f_{-2}}\right)}$
	Transport Kinetic Constants of PAPS Translocase	
zero-trans	21.59 ± 1.52	0.86 ± 0.05
equilibrium exchange	87.81 ± 7.89	7.89 ± 0.32

[a] For simplicity, it is assumed that $f_1 = f_{-1}$ and $f_2 = f_{-2}$ (Devés, 1991). C_t represents the concentration of the carrier. [b] Labeled substrate is in the external compartment.

assumption that under the conditions employed in activity measurements $f_1 = f_{-1}$ and $f_2 = f_{-2}$ (Devés, 1991), the corresponding constants for PAPS translocase are also given in Table 4.

The striking effect on the results of experiments utilizing vesicles which were preloaded with unlabeled PAP and assayed for the uptake of [^{35}S]PAPS from the *cis* compartment (Figure 12) is explained as follows. A competing substrate will always behave as an inhibitor at the *cis* face of the membrane but can act as an activator from the *trans* side by speeding the return of the binding site to the *cis* face of the membrane (Devés & Krupka, 1987) and by causing an isotope dilution effect on the transported substrate. This

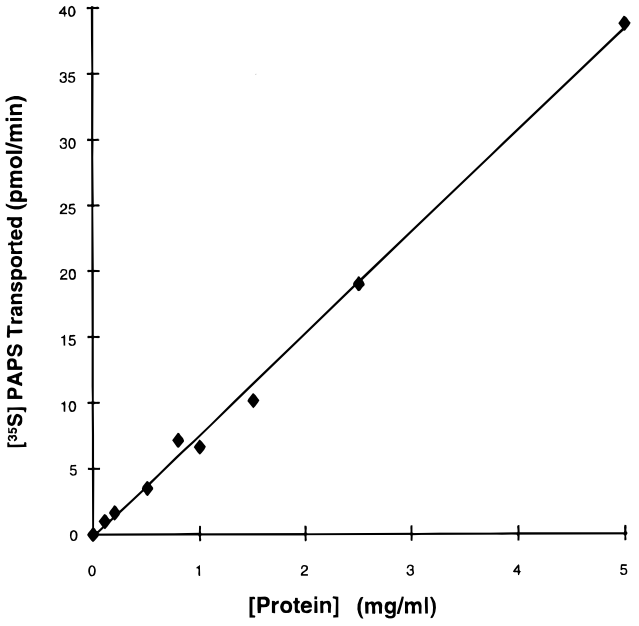

FIGURE 7: Dependence of reconstituted PAPS translocase activity on protein concentration. Samples of reconstituted vesicles containing 0.1−5.0 mg of protein were assayed, and PAPS transport was determined as described (Figure 6).

behavior suggests that the PAPS translocase is not merely a mobile carrier but one which is operating as an antiport, vectorially transferring two substrates in opposite directions between two compartments (Stein, 1986; Devés, 1991), which may or may not be tightly coupled. Due to the vectorial nature of transport, kinetic analyses must take into account particular relationships such as these which simply

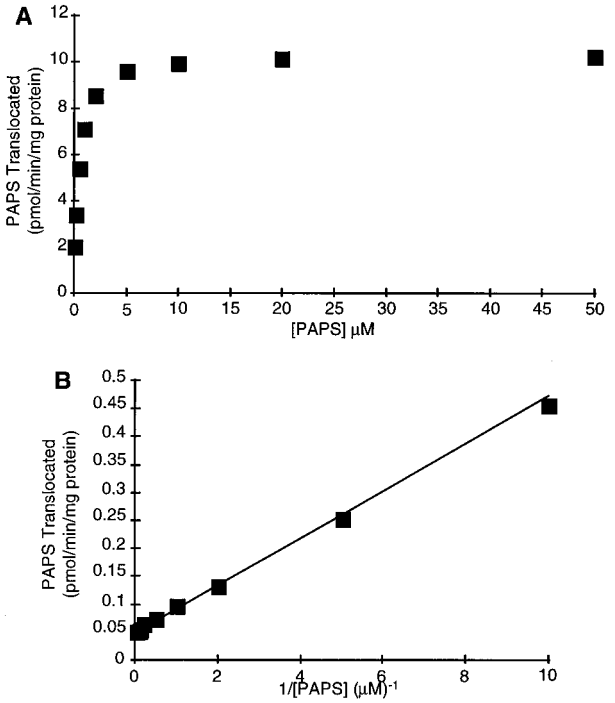

FIGURE 8: Dependence of reconstituted PAPS translocase activity on PAPS concentration. (A) Hyperbolic plot of initial velocities of PAPS translocase over the range of PAPS concentrations from 0.1 to 50.0 μM. (B) Initial velocity pattern in the form of a Lineweaver−Burke double-reciprocal plot of the same data as in A. The line shown is modeled using the kinetic constants provided by nonlinear fitting of the entire range of data.

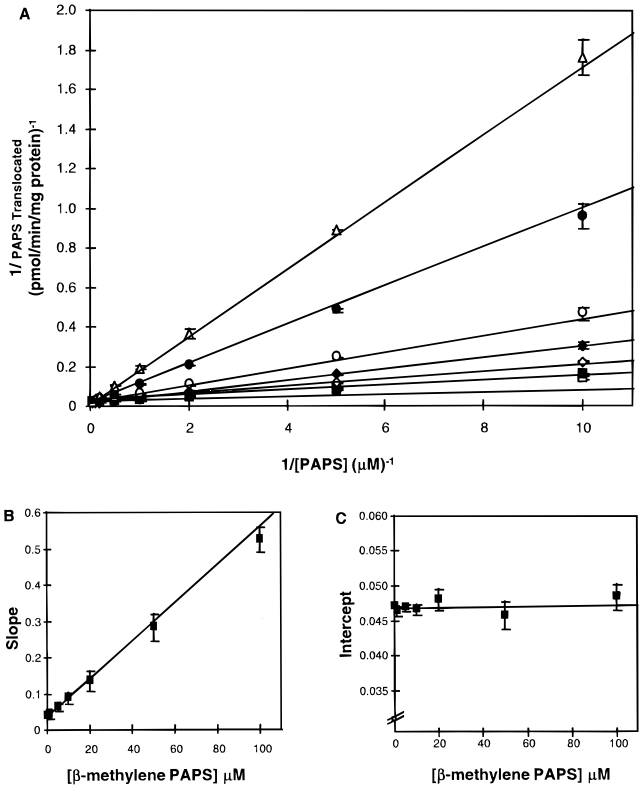

FIGURE 9: Inhibition of reconstituted PAPS translocase activity by β-methylene PAPS. (A) Lineweaver−Burke double-reciprocal plot of the inhibition pattern for reconstituted PAPS translocase in the presence of the following concentrations of β-methylene PAPS: (□), uninhibited; (■), 1 μM; (◊), 5 μM; (♦), 10 μM; (○), 20 μM; (●), 50 μM; (△), 100 μM. The secondary plot of primary slopes versus β-methylene PAPS concentration. (C) The secondary plot of primary intercepts versus β-methylene PAPS concentration.

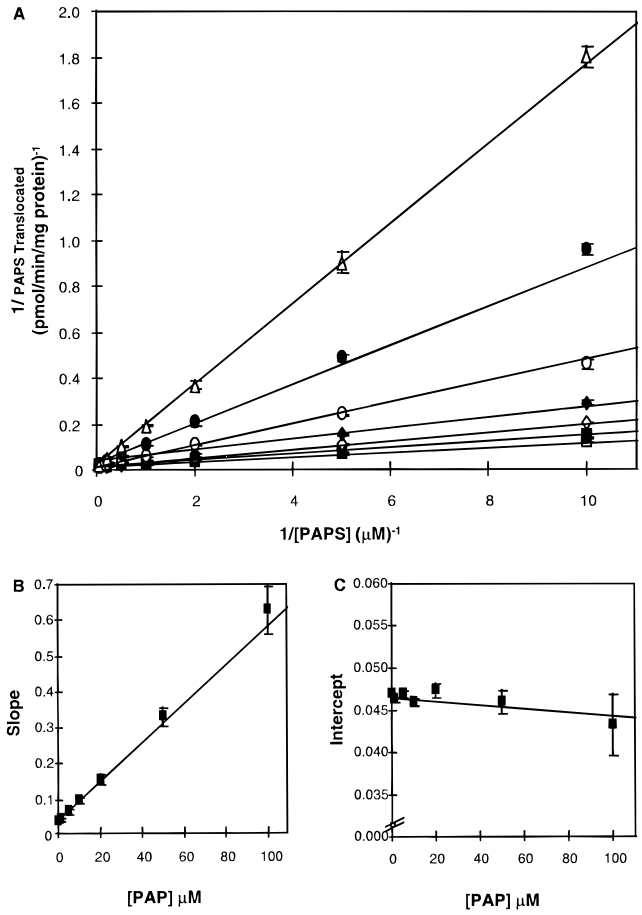

FIGURE 10: Inhibition of reconstituted PAPS translocase activity by adenosine 3′,5′-bisphosphate. (A) Lineweaver−Burke double-reciprocal plot of the inhibition pattern for reconstituted PAPS translocase in the presence of the following concentrations of PAP: (□), uninhibited; (■), 1 μM; (◊), 5 μM; (♦), 10 μM; (○), 20 μM; (●), 50 μM; (△), 100 μM. (B) The secondary plot of primary slopes versus β-methylene PAPS concentration. (C) The secondary plot of primary intercepts versus PAP concentration.

do not have analogies in the field of enzyme kinetics. Thus, the behavior of the PAPS translocase in the presence of equilibrium concentrations of PAP strongly supports the presence of a loosely coupled antiport mechanism for PAPS translocase, as was originally proposed for the transport of all of the nucleotide sugars and PAPS (Capasso & Hirschberg, 1984). However, data from inhibition studies, as summarized in Table 3, suggest that PAP, rather than AMP [as previously proposed by Capasso and Hirschberg (1984)], is the likely antiport ligand. This may also have certain implications for the overall behavior of the sulfate activation pathway, such that synthesis of PAPS by the ATP sulfurylase/APS kinase system may, in fact, be regulated through a product inhibition feedback mechanism controlled ultimately by the availability of acceptor molecules for the PAPS-dependent sulfotransferases. Lack of sulfate acceptors could result in an accumulation of free PAPS in the Golgi lumen which would cause futile cycling of PAPS translocase activity. The decrease in removal of PAPS from the cytoplasm then could lead to direct product inhibition of the APS kinase activity by the accumulated PAPS (Lyle *et al.*, 1994a).

Additionally, the ability to reconstitute PAPS translocase activity was instrumental in the identification of the PAPS translocase protein (Ozeran *et al.*, 1996) and allows consid-

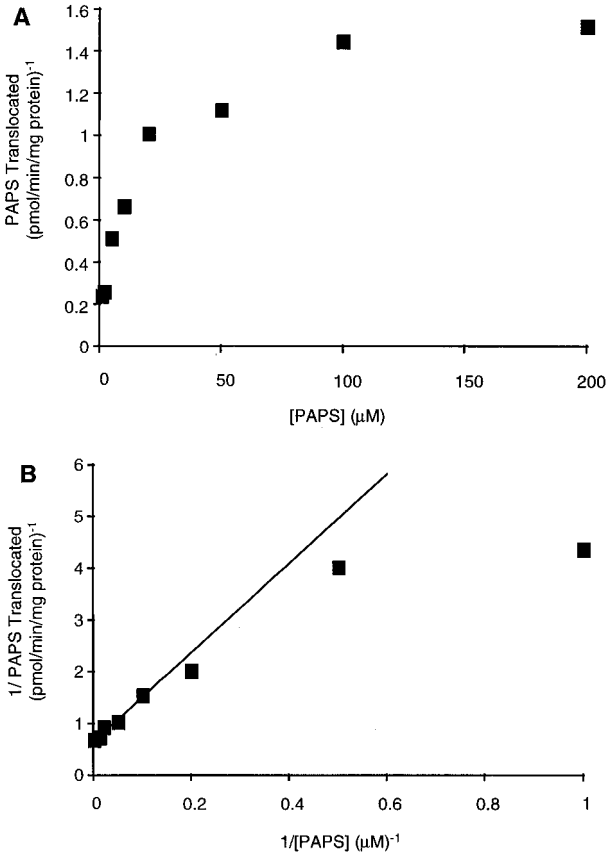

FIGURE 11: Equilibrium exchange measurements of PAPS translocase activity. Soluble Golgi proteins (~1 mg) were reconstituted with phosphatidylcholine liposomes (10 mg of lipid) in a final volume of 1 mL containing unlabeled PAPS at concentrations ranging from 1 to 200 μM as described. Assays for PAPS translocase activity were initiated by the addition of 1.0 μL containing ~250 000 cpm [^{35}S]PAPS (>400 Ci/mmol). Assays were terminated, and translocated [^{35}S]PAPS was determined as described. (A) Hyperbolic plot of initial velocities of reconstituted PAPS translocase in the presence of trans-membrane equilibrium concentrations of PAPS over the range 1−200 μM. (B) Initial velocity pattern in the form of a Lineweaver−Burke double-reciprocal plot. The line shown is modeled using the kinetic constants provided by nonlinear fitting of the entire data using the MacEnzkin program.

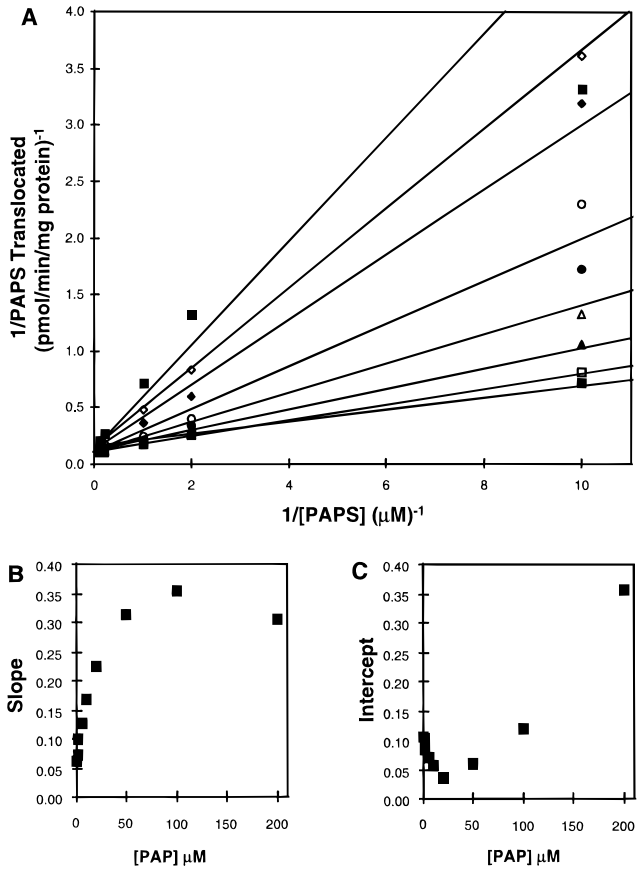

FIGURE 12: PAPS translocase activity in the presence of equilibrium concentrations of adenosine 3′,5′-bisphosphate. The inhibition pattern is displayed for reconstituted PAPS translocase in the presence of the following concentrations of PAP in equilibrium across the membrane: (■), uninhibited; (□), 1 μM; (▲), 2 μM; (△), 5 μM; (●), 10 μM; (○), 20 μM; (◆), 50 μM; (◇), 100 μM; (■), 200 μM. (B) The secondary plot of primary slopes versus adenosine 3′,5′-bisphosphate concentration. (C) The secondary plot of primary intercepts versus PAP concentration.

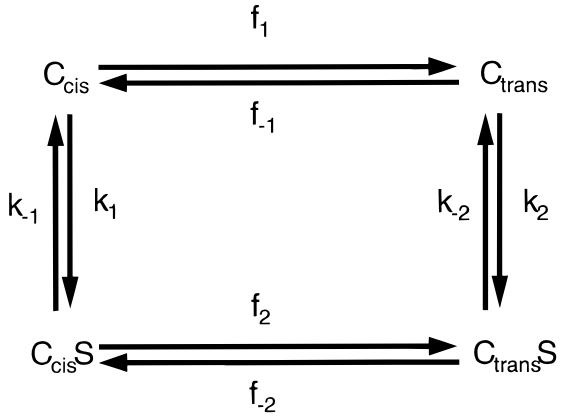

FIGURE 13: Simple model for carrier behavior.

eration of the entire sulfate activation and utilization pathway as a single system. For example, ATP sulfurylase/APS kinase from the rat chondrosarcoma has been purified and characterized as a single bifunctional enzyme (Lyle *et al.*, 1994c) which exhibits intermediate channeling (Lyle *et al.*, 1994b). In the brachymorphic mouse, the sulfate-activating enzymes exhibit a defect in this channeling mechanism (Lyle *et al.*, 1995). Geller (1987) and Ozeran (1995) have reported an apparent association between the sulfurylase/kinase and the PAPS translocase in the form of Golgi-associated ATP sulfurylase/APS kinase activities. The phenomenon may be exploited by combining liposomes containing reconstituted PAPS translocase with a normal or mutant sulfurylase/kinase or with specific sulfotransferases in the presence or absence of sulfurylase/kinase activities. This will provide a model system in which to study the entire sulfate activation and utilization pathway and to assess the effects of various inhibitors, drugs, and other compounds in order to enhance our understanding of this critical pathway of chemical modification.

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