Rapid Plasmenylethanolamine-Selective Fusion of Membrane Bilayers Catalyzed by an Isoform of Glyceraldehyde-3-Phosphate Dehydrogenase: Discrimination between Glycolytic and Fusogenic Roles of Individual Isoforms†

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ABSTRACT: Recently we demonstrated that the unique stereoelectronic relationships inherent in the structure of plasmenylethanolamine facilitate membrane fusion, and we postulated the existence of a membrane fusion protein which could exploit the propensity of plasmenylethanolamine molecular species to adapt an inverted hexagonal phase [Glaser & Gross (1994) *Biochemistry 33*, 5805−5812]. We now report a cryptic membrane fusion activity in rabbit brain cytosol, which requires separation from an endogenous inhibitor to express its activity, and demonstrate that vesicle fusion catalyzed by this protein is highly selective for membrane vesicles containing plasmenylethanolamine. The cytosolic protein catalyzing membrane fusion activity was purified to apparent homogeneity by sequential column chromatographies, revealing a single 38-kDa protein band after sodium dodecyl sulfate−polyacrylamide gel electrophoresis and silver staining. Automated Edman degradation demonstrated that the purified protein is an isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was confirmed by Western blot analysis utilizing polyclonal antibodies and by solution-state inactivation of membrane fusion activity by a monoclonal antibody directed against GAPDH. Both GTP-affinity and Mono Q chromatographies resolved GAPDH isoforms that catalyzed dehydrogenase activity from the GAPDH isoform that catalyzed membrane fusion activity. The purified fusion protein was calcium-independent, resistant to treatment with *N*-ethylmaleimide, and possessed an obligatory requirement for plasmenylethanolamine and cholesterol. High-resolution stopped-flow kinetic analysis of plasmenylethanolamine-facilitated membrane fusion demonstrated that one tetramer of the GAPDH isoform catalyzed one fusion event between two vesicles containing plasmenylethanolamine every millisecond (on average). Collectively, these results constitute the first description of a protein which can catalyze the fusion of vesicles at a rate which satisfies the mathematical constraints imposed by the observed rates of fusion of synaptic vesicles with the presynaptic membrane *in vivo*.

The fusion of membrane bilayers represents a critical event in cellular growth (Wilson et al., 1989; Diaz et al., 1989; Stamnes & Rothman, 1993), regulation (von Wedel et al., 1981; Almers & Tse, 1990; Sollner et al., 1993), and intercellular communication (Poste & Allison, 1973; Pollard et al., 1992). Through the translocation and delivery of both intravesicular and membrane-associated constituents to their specifically targeted intracellular destinations, membrane fusion facilitates the appropriate response of each cell to alterations in its chemical (Wilson et al., 1991; Turk et al., 1993), electrical (Rutecki, 1992; Hessler et al., 1993), or mechanical environment (Lang et al., 1985; Oliet & Bourque, 1993). Thus, the temporally and spatially orchestrated execution of these events is necessary for cellular adaptation and represents a *sine qua non* for survival of the organism as a whole.

In some cases, fusion between membrane bilayers is so rapid (e.g., fusion of neurotransmitter vesicles with the presynaptic membrane is complete within 1 ms after depolarization) (Kelly 1993) and the uncatalyzed energetic barrier for membrane fusion is so high that it has been difficult to envisage how this process is mediated *in vivo*. Thus, the satisfactory resolution in reconstituted systems of the kinetic constraints mandated by the observed rates of membrane fusion *in vivo* has been a long-revered goal in membrane chemistry (Duzgunes, 1985; Papahadjopoulos et al., 1990; Vogel et al., 1993). Although substantial progress has been made in identifying cellular constituents which facilitate the appropriate docking of predestined fusion partners (Sollner et al., 1993; Stamnes & Rothman, 1993), identification of the critical chemical constituents which catalyze the actual fusion of previously apposed membrane bilayers has remained elusive (Schekman, 1992). One potential reason underlying the prior inability to identify proteins which facilitate rapid bilayer fusion has been the utilization of model systems which do not incorporate critical chemical information central to the membrane fusion process *in vivo*. Recently, we demonstrated that plasmenylethanolamine, a major chemical constituent of synaptic vesicle membranes and plasma membranes, facilitates non-enzyme-catalyzed membrane fusion by virtue of its propensity to adopt an inverted hexagonal phase (Lohner et al., 1984; Han & Gross, 1992), thereby decreasing the activation energy of the rate-determining step in the fusion process (Glaser & Gross, 1992). Accordingly, we proposed the utility of a protein(s) which could catalyze the fusion process by

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exploiting the unique stereoelectronic relationships inherent in the structure of plasmenylethanolamine. One corollary of this hypothesis was that the identity of such a protein(s) could be ascertained only through utilization of fusion systems which contain chemical characteristics essential to the fusion of phospholipid bilayers *in vivo* (e.g., putatively, plasmenylethanolamine molecular species). To this end, we utilized physiologically modeled vesicles composed of mixtures of phospholipids and cholesterol present in the salient membrane compartments to systematically search for the protein(s) that could exploit the unique chemical characteristics inherent in plasmenylethanolamine molecular species. Although initial efforts were confounded by the failure of crude brain cytosol to facilitate plasmenylethanolamine-assisted membrane fusion, we hypothesized that fusion activity in the cytosolic compartment was latent until resolved from an endogenous inhibitor. We now report the identification of an endogenous protein present in rabbit brain cytosol which specifically catalyzes the rapid fusion [>1 vesicle fusion ms^{-1} (molecule of fusion protein)$^{-1}$] of vesicles containing plasmenylethanolamine (but not phosphatidylethanolamine) after its separation from an endogenous inhibitor and demonstrate that this protein is an isoform of GAPDH by employing chemical, immunologic, and chromatographic criteria. Collectively, these results constitute the first description of a protein which can catalyze the fusion of vesicles composed of physiologic mole fractions of lipids with kinetic parameters which satisfy the mathematical constraints imposed by the exocytotic fusion process.

MATERIALS AND METHODS

Bovine brain ethanolamine glycerophospholipids, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol was obtained from Nu Chek Prep (Elysian, MN). Octadecylrhodamine (R_{18}),[1] $TbCl_3$, and dipicolinic acid (DPA) were purchased from Molecular Probes (Eugene, OR). DE-52 anion-exchange resin was purchased from Whatman (Maidstone, England). DTT was purchased from Calbiochem (San Diego, CA), while GTP and GMP were purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Multiphor 2D electrophoresis supplies including Immobilines II were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). HPLC-grade solvents were purchased from Baxter Scientific (McGaw Park, IL). Most other chemicals were obtained from Sigma (St. Louis, MO).

Purification of Phospholipids Utilized in Membrane Fusion Assays. Phospholipids were purified to remove trace contaminants on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) utilizing a mobile phase composed of hexane/2-propanol with a 1−7% H_2O gradient (Geurts van Kessel et al., 1977). Plasmenylethanolamines (16:0−18:1 plasmenylethanolamine and 18:0−20:4 plasmenylethanolamine) were synthesized as described previously (Glaser & Gross, 1994; Han et al., 1992). Octadecylrhodamine was purified on an Altex Ultrasphere-CN column (4.6 mm × 25 cm) utilizing a mobile phase composed of acetonitrile and a gradient of triethanolamine (20−50 mM, pH 7.0 with acetic acid) over 40 mL at a flow rate of 2.0 mL/min. Purified R_{18} fractions were extracted using a modified Bligh and Dyer technique, and the organic layer was collected. All lipids were stored under nitrogen at −20 °C in chloroform.

Stopped-Flow Kinetic Analysis of Protein-Catalyzed Fusion of Vesicles Composed of Physiologic Mixtures of Lipids. Samples were screened for membrane fusion activity employing a modified version of the R_{18} fusion assay previously described by Hoekstra et al. (1984). Phospholipids, cholesterol, and lipid fluorescent probes were first codissolved from individual chloroform stock solutions, evaporated under nitrogen, and finally evacuated at 100 mTorr for 1 h. Vesicles were composed of phosphatidylcholine (POPC) (27%), ethanolamine glycerophospholipid (27%), phosphatidylserine (POPS) (6%), and cholesterol (40%) and were utilized at a final lipid concentration of 200 μM. Separate populations of vesicles containing, in addition, 4% R_{18} were also prepared. Vesicle components were resuspended in assay buffer (typically 5 mL) composed of 100 mM sodium chloride, 5 mM sodium MES, and 0.1 mM EGTA, pH 6.0 (some studies employed pH 7.4 liposome fusion buffer as indicated). After vigorous vortexing to form multilamellar vesicles, small unilamellar vesicles (SUVs) were formed by sonication for 4 min at 37 °C utilizing a 40% duty cycle at a power level of 1.5 on a Vibra Cell sonicator equipped with a medium tip. For the preparation of large unilamellar vesicles (LUVs), lipid components were resuspended in the above buffer, vortexed rigorously, and subjected to 5 cycles of freeze−thaw followed by 5 cycles of extrusion through a 0.1-μm polycarbonate filter in a French press as described previously (Chen & Gross, 1994). Liposomes were maintained under a nitrogen atmosphere and were used immediately after preparation.

Equimolar amounts of labeled and unlabeled vesicles were loaded into one chamber of an SLM stopped-flow apparatus equipped for use with an SLM Aminco 4800 spectrofluorometer (Model FP-052). The other chamber was loaded with either sample dilution buffer alone (containing 100 mM sodium chloride, 50 mM sodium MES, and 0.1 mM EGTA, pH 6.0) or with protein-containing fractions diluted with buffer in the indicated ratios. The contents of the chambers were rapidly mixed (dead time = 7 ms) in a 1:1 (v/v) ratio. Fusion was monitored by the temporal dependence of R_{18} dequenching observed at 590 nm utilizing an excitation wavelength of 560 nm. The 0% fusion level was assessed by monitoring fluorescence when the vesicles were mixed with sample dilution buffer only. The 100% fusion level was assessed by preparing liposomes composed of the mixture which would result if all vesicles fused (e.g., 27% PC, 27% PE, 6% PS, 40% cholesterol, and 2% R_{18}) by quantifying the resultant R_{18} fluorescence. Fluorescence tracings were normalized utilizing the 0% and 100% fusion levels, and initial rates were calculated and expressed as a

[1] Abbreviations: DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); HEPES, 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; LPE, lysoplasmenylethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; POPC, 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine; POPE, 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoserine; R_{18}, octadecylrhodamine; SUV, small unilamellar vesicle; TLC, thin-layer chromatography; 16:0−18:1 (plasmal), 1-*O*-[(*Z*)-hexadec-1′-enyl]-2-octadec-9′-enoyl-*sn*-; 16:0−18:1 (diacyl), 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-; 18:0−20:4 (plasmal), 1-*O*-[(*Z*)-octadec-1′-enyl]-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-; 18:0−20:4 (diacyl), 1-octadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-.

percentage of maximum fusion per second ($F_{max\%}$, s^{-1}).

Contents mixing was assessed using the Tb/DPA method as described previously by Wilschut et al. (1980). Large unilamellar vesicles were prepared utilizing 5 cycles of freeze−thaw and 5 cycles of extrusion through a 0.1-μm polycarbonate filter [as described in Chen and Gross (1994)] in either 20 mM sodium chloride, 50 mM DPA, and 5 mM sodium HEPES (pH 7.0) or 2.5 mM terbium chloride, 50 mM sodium citrate, and 5 mM sodium HEPES (pH 7.0) at a concentration of 2.0 mM lipid. LUVs (0.8 mL) containing entrapped terbium chloride or DPA were separated from unencapsulated probe utilizing Sephadex G-50 2-mL spin columns eluted with 0.1 mL of buffer (100 mM sodium chloride, 5 mM sodium HEPES, and 1.0 mM EDTA, pH 7.0). At this juncture, aliquots of the vesicle-containing fraction were subjected to Bligh and Dyer extraction, acid methanolysis, and capillary gas chromatography to quantify the liposomal lipid concentration for use in subsequent fluorescence assays. LUVs containing either Tb or DPA in equimolar concentrations were mixed in the stopped-flow apparatus, and protein-mediated fusion was monitored by the formation of the fluorescence Tb/DPA complex as ascertained by the increase in fluorescence intensity at wavelengths >470 nm after excitation at 276 nm. Final total lipid concentrations in the assay were approximately 1.0 mM. In these studies, 1 mM EDTA was always present to chelate any Tb^{3+} that leaked from the vesicles. The 100% fusion level was assessed by measuring the fluorescence of vesicles prepared in a buffer containing 10 mM NaCl, 25 mM sodium citrate, 1.25 mM Tb, 25 mM DPA, and 5 mM Na HEPES (pH 7.0) and processed as described above. All fusion assays were performed at 37 °C.

To verify that differences in observed fusion rates were not due to selective breakdown of one subclass of ethanolamine glycerophospholipid, aliquots of liposomes employed in the fusion studies were extracted by the method of Bligh and Dyer (1959) and analyzed by TLC and straight-phase HPLC. No lysophospholipids or other contaminants were detected within the time frame of the assay. For higher sensitivity detection of potential breakdown products, 16:0−18:1 plasmenylethanolamine was synthesized with a tritium-labeled *sn*-2 oleoyl group (Glaser & Gross, 1994). Radiolabeled plasmenylethanolamine was added to a liposome preparation containing unlabeled PC and PE. No significant (<2%) generation of radiolabeled fatty acid or lysophospholipid was observed during the procedures employed.

Measurement of Glyceraldehyde-3-Phosphate Dehydrogenase Activity. The activity of GAPDH was measured spectrophotometrically utilizing modifications of the methods of Cori et al. (1948) and Steck et al. (1973). Briefly, sample was added to a 1-cm path-length semimicrocuvette containing 50 mM triethanolamine (pH 7.6), sodium arsenate (50 mM) (pH 8.8), 2.4 mM glutathione (reduced), 0.5 mM NAD, and water in a final volume of 990 μL. After a baseline was established (1 min), the reaction was initiated with 10 μL of 10 mM D-glyceraldehyde 3-phosphate (prepared according to Sigma product bulletin G-8007) and absorbance at 340 nm was measured for 5 min. Units of GAPDH activity represent the mass (micromoles) of D-glyceraldehyde-3-phosphate converted per minute. The mass of NADH generated was calculated utilizing $\epsilon = 0.622$.

Purification of the Membrane Fusion Protein from Rabbit Brain Cytosol. New Zealand White rabbits (typically, 10/preparation) were sacrificed by cervical dislocation, and brains were harvested and placed in ice-cold homogenization buffer (30% w/v) consisting of 250 mM sucrose, 30 mM Tris·HCl, 10 mM EGTA, 2 mM EDTA, and 1 mM DTT (pH 7.4, measured at 25 °C). Brains were homogenized using three 10-s pulses from a Brinkman PT 10/35 Polytron apparatus at incremental output settings of 4.5, 5, and 5.5. The homogenate was initially centrifuged at 10000*g* for 20 min, and the resultant supernatant was centrifuged at 100000*g* for 60 min. The crude cytosol was twice dialyzed for 6 h against 500 volumes of buffer A (50 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, pH 7.0 at 4 °C). The dialyzed cytosol was loaded onto a DE-52 column (2.6 cm × 20 cm) previously equilibrated with buffer A at a flow rate of 2 mL/min. Fractions from the DE-52 column were assayed for their ability to catalyze membrane fusion. Activity was quantified in units per milliliter, where 1 unit represents 1 nmol of lipid fused/s in an assay containing 200 μM lipid in a final volume of 100 μL. Fractions from the void volume containing fusion-catalyzing activity were pooled, filtered utilizing a Millipore GS 0.22-μm filter, and loaded onto a HiLoad SP Sepharose column (1.6 cm × 10 cm, Pharmacia) previously equilibrated with 20 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT (pH 7.0 at 4 °C) (buffer B) at 3.0 mL/min. After the column was washed with 3 column volumes of buffer B, membrane fusion activity was eluted utilizing a nonlinear sodium chloride gradient from 0 to 0.5 M NaCl in buffer B. Active fractions from the HiLoad SP Sepharose column were pooled, diluted 3-fold with buffer B, and loaded onto a GTP−agarose column (Sigma, G-9768, lot 70H9545, 1 cm × 5 cm) at a flow rate of 0.3 mL/min. After the affinity matrix was washed with 5 column volumes of buffer B, bound proteins were sequentially eluted with 5 mM GMP in buffer B, 3 mM GTP in buffer B, and finally, an additional 5 column volumes of buffer B alone. Membrane fusion activity was eluted with 20 mM tetrasodium tripolyphosphate and 10 mM NAD^+ in buffer B. Column eluents were assayed for GAPDH activity and membrane fusion activity.

GTP−agarose affinity eluents were further purified either by reverse-phase HPLC (in preparation for protein sequencing) or by Mono Q chromatography. Anion-exchange chromatography was accomplished utilizing a Mono Q column equipped for a Smart System FPLC (Pharmacia). Active fractions were pooled, diluted 10-fold into buffer C (20 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, pH 8.5 at 4 °C) and loaded onto a Mono Q PC 1.6/5 column previously equilibrated with buffer C at a flow rate of 200 μL/min. Fusion-catalyzing activity was eluted using a continuous gradient from 0 to 500 mM NaCl over 6 mL. Aliquots of column eluents were assayed for membrane fusion activity or subjected to ^{125}I-Bolton−Hunter labeling as previously described (Hazen et al., 1990).

The GTP affinity column active fraction (0.1 mL) utilized for sequencing was diluted 1:1 with buffer containing 14% acetonitrile and 0.2% trifluoroacetic acid and loaded onto a C18 HPLC column (Vydac, 300-Å pore size, 4.6 mm × 25 cm) preequilibrated with 10% mobile phase B at 500 μL/min (mobile phase A, water containing 0.1% TFA; mobile phase B, 70% acetonitrile and 0.12% TFA). Proteins were eluted with a gradient of 10−70% mobile phase B over 50

min at a flow rate of 500 μL/min. Homogeneity of the 38-kDa band eluting as the major UV-absorbing peak from the RP-HPLC was verified by SDS−PAGE prior to submission for protein sequencing.

Preparation and Purification of Monoclonal Antibodies. Four mice were initially injected with purified membrane fusion protein from the GTP−agarose column, and subsequent booster injections were made with rabbit muscle GAPDH. Hybridomas were formed from mouse spleen as described previously (Harlow & Lane, 1988) and supernatants were screened for anti-GAPDH antibodies by automated particle-concentration fluorescence immunoassays. Samples possessing activity after the initial screen were verified by Western blot analysis of rabbit brain cytosol. Hybridomas were used to generate ascites fluid by traditional methods (Harlow & Lane, 1988) and the resultant IgG was purified from ascites fluid utilizing protein A−agarose affinity chromatography.

Two-Dimensional Electrophoresis. Two-dimensional electrophoresis was performed utilizing a Multiphor system (Pharmacia) with the first-dimension isoelectric phast-gels cast utilizing Immobilines II in a 4% polyacrylamide matrix containing a pH gradient from pH 7.0 to 10.0 over 10 cm. Following polymerization, washing, and drying of the gel, 3-mm strips were cut and reswelled in buffer containing 8 M urea, 1% NP-40 (Pierce SurfactAmps), 1 mM DTT, and 0.25% Pharmalyte 7−9 for 24 h. Protein samples were diluted into buffer containing 10 M urea, 1% NP-40, 0.1% β-mercaptoethanol, and 0.25% Pharmalyte 7−9 and were concentrated utilizing Microcon-10 ultrafiltration units. First-dimension gels were electrophoresed for 26 h using a discontinuous voltage gradient (5 h at 500 V, 18 h at 2500 V, and 2.5 h at 3500 V). After equilibration of 1D gels with SDS−PAGE buffer, samples were electrophoresed on either ExcelGel SDS 8−18% or ExcelGel SDS homogeneous 12.5% gels (Pharmacia Biotech Inc.). Gels were subsequently either silver-stained (Bio-Rad Silver Stain Plus system) or transferred to PVDF paper for subsequent Western blotting.

Miscellaneous Procedures. Protein content was determined utilizing the Bio-Rad or Quanti-Gold protein assay with bovine serum albumin as standard. Polyacrylamide gel electrophoresis, silver staining, and Western blotting were performed as previously described (Hazen et al., 1990). Polyclonal antibodies against GAPDH were raised in chickens using standard procedures (Jensenius et al., 1981) and purified on an affinity column containing covalently linked SDS−PAGE-purified and electroeluted rabbit skeletal muscle GAPDH. Protein sequencing was performed by the Protein Chemistry Center of Washington University using N-terminal Edman degradation.

RESULTS

Identification of a Latent Membrane Fusion Activity in the Cytosolic Fraction of Rabbit Brain Homogenates. To determine whether a protein present in rabbit brain homogenates catalyzes the fusion of membrane bilayers by exploiting the unique stereoelectronic characteristics of plasmenylethanolamine, an assay system utilizing vesicles comprised of physiologic mole fractions of phospholipids [i.e., phosphatidylcholine (27%), plasmenylethanolamine (27%), phosphatidylserine (6%), and cholesterol (40%)] was employed. Neither crude rabbit brain cytosolic nor microsomal proteins facilitated the fusion of membrane vesicles containing selected mole fractions of plasmenylethanolamine. Remarkably, void volume eluents from anion-exchange chromatography of rabbit brain cytosol catalyzed the rapid fusion of vesicles containing plasmenylethanolamine but did not facilitate fusion utilizing vesicles which contained phosphatidylethanolamine (Figure 1, panel A).

Characterization of the membrane fusion-catalyzing activity in the void volume demonstrated that it was trypsin-sensitive, calcium-independent (full activity at 5 mM EGTA), neutral-active (maximal activity was present between pH 6 and 7), heat-labile (activity was ablated by heating for 3 min at 90 °C), and inactivated by DTNB (1 mM for 30 min at 37 °C) but was not inhibited by *N*-ethylmaleimide (1 mM for 60 min at 37 °C) (data not shown). Membrane fusion activity catalyzed by void volume eluents was not due to the fusion of labeled R_{18} vesicles with endogenous lipid carried through in the void volume since dequenching of R_{18} fluorescence did not occur in the absence of added acceptor vesicles. Furthermore, the observed dequenching of R_{18} fluorescence catalyzed by the protein in the void volume represented bona fide membrane fusion and did not result from interventricular lipid exchange (*vide infra*). Examination of the tissue specificity of the fusion activity revealed appreciable levels only in brain and muscle (rabbit hind leg muscle contained approximately 25% of the specific activity at the DE void stage) with only diminutive amounts of activity present in liver and kidney (100-fold and 50-fold less than that manifest in brain, respectively). Finally, a crude surgical separation of rabbit brain white matter from gray matter demonstrated that fusion activity in void volume eluents derived from gray matter was 3-fold higher than those derived from white matter (data not shown). Thus, the void volume from anion-exchange chromatography of rabbit brain cytosol contains a tissue-specific protein that catalyzes the calcium-independent fusion of membrane bilayers containing plasmenylethanolamine but not phosphatidylethanolamine.

Purification of the Protein Constituent Catalyzing Plasmenylethanolamine-Facilitated Membrane Fusion Activity in Rabbit Brain Cytosol. To elucidate the molecular identity of the protein constituent catalyzing membrane fusion activity in the void volume, the fusion protein was purified by sequential column chromatographies. The void volume from DE-52 anion-exchange chromatography was filtered and loaded onto a cation-exchange column (HiLoad SP Sepharose), and bound proteins were eluted by application of a nonlinear salt gradient as described in Materials and Methods. The major peak of fusion activity eluted at ≈100 mM NaCl (Figure 1, panel B). Since G-proteins play a prominent role in modulating membrane fusion in several systems, affinity chromatography employing a GTP−agarose affinity matrix was attempted. The active fractions from HiLoad SP Sepharose chromatography were diluted with equilibration buffer and directly loaded onto a 1- × 5-cm GTP−agarose affinity column. The protein catalyzing membrane fusion activity (or other regulatory proteins associated with it) bound to the affinity matrix. Accordingly, the column was first washed with buffer containing 5 mM GMP and subsequently with buffer containing 3 mM GTP. The majority of adsorbed proteins eluted during these washes while membrane fusion activity remained bound to the matrix (Figure 1, panel C). The majority of membrane fusion activity was subsequently desorbed by elution with 20 mM P_{i_3} and 10 mM NAD^+, demonstrating a predominant 38-kDa protein constituent after

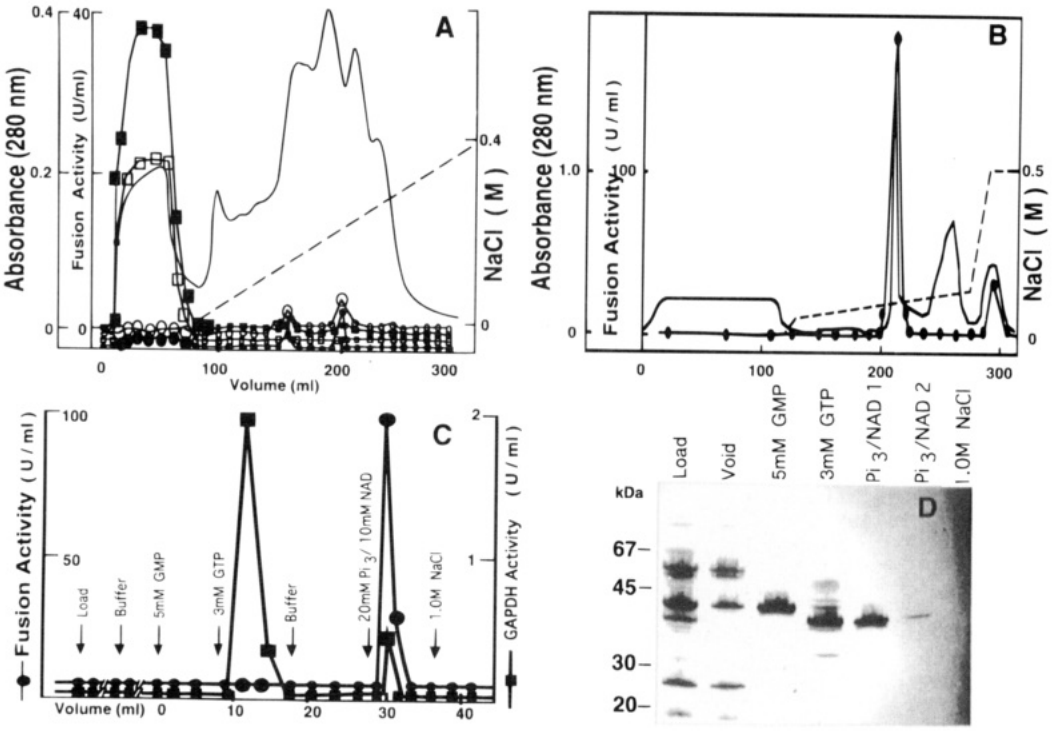

FIGURE 1: Purification of a protein catalyzing membrane fusion through sequential chromatography steps. Panel A: Anion-exchange chromatography of dialyzed rabbit brain cytosol and characterization of the substrate selectivity of the protein catalyzing membrane fusion. Rabbit brain cytosol was prepared and loaded onto a previously equilibrated DE-52 column as described in Materials and Methods. The column was developed with a linear NaCl gradient (0−400 mM NaCl), and column eluents were assayed for their ability to catalyze membrane fusion utilizing physiologically modeled small unilamellar vesicles (SUVs) as described in Materials and Methods. Vesicles were comprised of 16:0−18:1 phosphatidylcholine (27%), 16:0−18:1 phosphatidylserine (6%), cholesterol (40%), and 27% each of the following ethanolamine glycerophospholipids: 18:0−20:4 plasmenylethanolamine (■), 16:0−18:1 plasmenylethanolamine (□), 18:0−20:4 phosphatidylethanolamine (●), or 16:0−18:1 phosphatidylethanolamine (○). (—) UV absorbance at 280 nm; (− − −) NaCl gradient. Panel B: Cation-exchange chromatography of the protein catalyzing membrane fusion. Active fractions from DE-52 chromatography were pooled, filtered, and loaded onto a previously equilibrated HiLoad SP Sepharose column as described in Materials and Methods. The column was developed with a nonlinear sodium chloride gradient as indicated. Aliquots of column eluents were assessed for their ability to catalyze membrane fusion (●) utilizing physiologically modeled vesicles as described in Materials and Methods. (—) UV absorbance at 280 nm; (− − −) NaCl gradient. Panel C: Active fractions from HiLoad SP Sepharose chromatography were pooled, diluted with equilibration buffer, and loaded onto a GTP−agarose column (1 cm × 5 cm) at a flow rate of 0.3 mL/min as described in Materials and Methods. After washing, bound proteins were eluted with equilibration buffer containing, in succession, 5 mM GMP, then 3 mM GTP, and finally 20 mM tetrasodium tripolyphosphate and 10 mM NAD^+. Fractions were assayed for glyceraldehyde-3-phosphate dehydrogenase activity (■) and membrane fusion-catalyzing activity (●) as described in Materials and Methods. Panel D: Proteins in GTP−agarose column eluents were visualized after SDS−PAGE and silver staining.

Table 1: Purification of GAPDH and the Membrane Fusion Protein[a]

	protein (mg)	GAPDH activity (units)	fusion activity (units)	specific activity (units/mg)		purification (x-fold)		yield (%)	
				GAPDH	fusion	GAPDH	fusion	GAPDH	fusion
dialyzed cytosol	675	21.9	nd	0.032	nd	1	nd	100	nd
DE void	84	10.7	2160	0.127	25.7	4.0	1	48.9	100
HiLoad SP Sepharose	20.6	6.15	1500	0.299	72.8	9.3	2.8	28.1	69
GTP-Ag, GTP eluent	3.5	7.38	0	2.11	0	65.9	0	33.7	0
GTP-Ag, P/N eluent	0.072	0.03	420	0.42	5830	0	227	0	19
Mono-Q	0.014	0	284	0	20300	0	790	0	13

[a] Membrane fusion activity and glyceraldehyde 3-phosphate dehydrogenase activities were quantified during the sequential chromatographic purification described in Materials and Methods. nd, not detected.

SDS−PAGE and silver staining (Figure 1, panel D). To elucidate the molecular identity of the 38-kDa protein constituent, it was concentrated and further purified by reverse-phase HPLC prior to sequencing by automated Edman degradation. The N-terminal sequence of 23 amino acids was identical to the N-terminal amino acid sequence of glyceraldehyde-3-phosphate dehydrogenase (Table 2). No protein in the gene bank other than GAPDH contained either this N-terminal sequence or other highly homologous sequences.

After the major protein constituent was identified as glyceraldehyde-3-phosphate dehydrogenase, the glyceraldehyde-3-phosphate dehydrogenase mass and activity eluting from the GTP−agarose affinity column was assessed. The majority of the glyceraldehyde-3-phosphate dehydrogenase activity (assessed spectrophotometrically) and mass (assessed by SDS−PAGE and subsequent silver staining) eluted in the GTP eluent from the GTP−agarose affinity column (Figure 1, panel C). However, the GTP eluent did not possess demonstrable membrane fusion activity. In contrast, the 38-

Table 2: Sequence of the Membrane Fusion Protein and Homology with GAPDH[a]

VKVGVNGFGRIGRLVTRAAFNSG	Memb.Fusion Protein
VKVGVNGFGRIGRLVTRAAFNSG	Human GAPDH (muscle)
VKVGVNGFGRIGRLVTRAAFNSG	Human GAPDH (liver)
VKVGVNGFGRIGRLVTRAAFNSG	Bovine GAPDH
1 5 10 15 20 aa	

[a] The membrane fusion protein was purified as described in Materials and Methods and applied to a Vydac reverse-phase HPLC column. A single homogeneous 38-kDa band was obtained and submitted for N-terminal Edman degradation sequencing. Twenty-three amino acids of N-terminal sequence were obtained and found to be homologous to GAPDH.

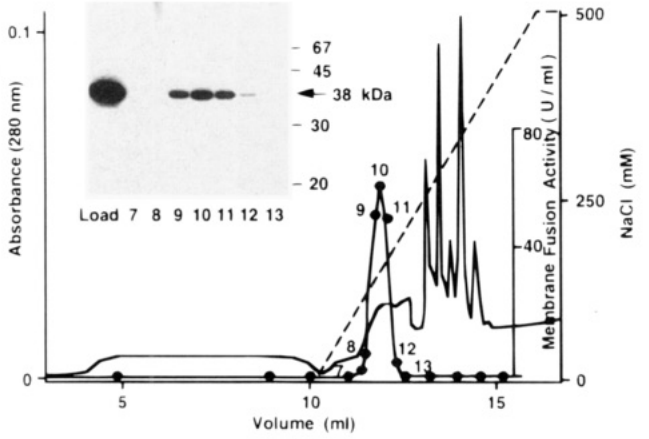

FIGURE 2: Mono Q chromatography of eluents from GTP−agarose affinity chromatography containing membrane fusion-catalyzing activity. Active fractions from the GTP−agarose column were pooled, diluted 10-fold with equilibration buffer, and loaded onto a Mono Q column as described in Materials and Methods. The column was developed with a sodium chloride gradient (− − −), and aliquots of column eluents were assayed for fusion-catalyzing activity utilizing physiologically modeled vesicles as described in Materials and Methods. The UV-absorbing peaks eluting between 13 and 15 mL result from the elution of metabolites, NADH, and other nonprotein moieties. (—) UV absorbance at 280 nm. Inset: Autoradiograph of ^{125}I-Bolton−Hunter-labeled and SDS−PAGE-separated proteins from both the Mono Q column load and column eluents containing membrane fusion-catalyzing activity.

kDa band present in the P_{i_3} eluate possessed only diminutive amounts of GAPDH catalytic activity (the specific activity was reproducibly <15% of GAPDH activity in the GTP eluent) yet catalyzed membrane fusion activity. These results suggested that separate and distinct isoforms of GAPDH were responsible for GAPDH activity and membrane fusion activity. Accordingly, the fractions catalyzing membrane fusion activity after GTP affinity chromatography (i.e., the 20 mM P_{i_3}/10 mM NAD^+ eluent) were further purified utilizing Mono Q anion-exchange chromatography. After loading, the Mono Q column was eluted with a linear salt gradient (0−500 mM NaCl) as described in Materials and Methods (Figure 2). A symmetrical peak of membrane fusion activity eluted that precisely cochromatographed with a 38-kDa protein band as assessed by SDS−PAGE after iodination with ^{125}I-Bolton−Hunter reagent and subsequent autoradiography (Figure 2) or after silver staining (Figure 3, left panel). Western blotting demonstrated that the 38-kDa band from Mono Q chromatography was recognized by polyclonal immunopurified antibodies directed against GAPDH (Figure 3, right panel). However, although protein

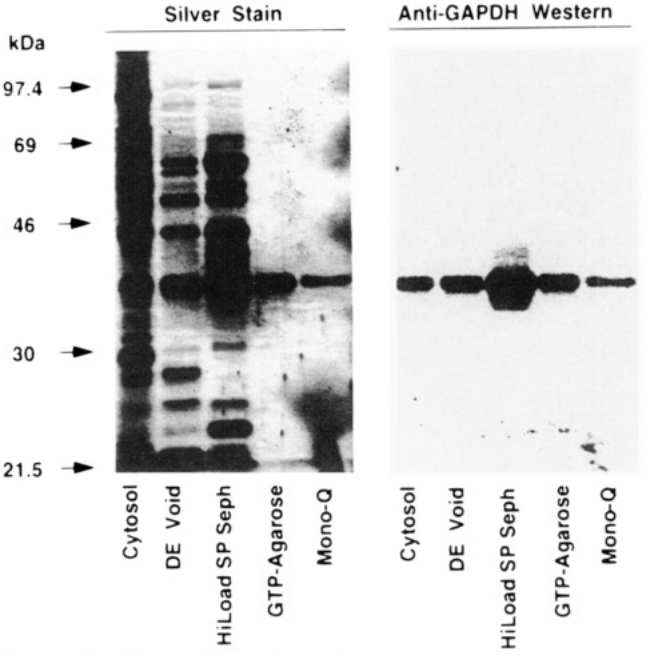

FIGURE 3: Comparison of protein composition and GAPDH immunoreactive proteins during the purification of the GAPDH isoform catalyzing membrane fusion. Protein constituents in column eluents at each stage of the purification procedure were separated by SDS−PAGE (11% acrylamide) and visualized by silver staining. Proteins electrophoresed in an identical gel were subjected to Western blot analysis utilizing a chicken anti-rabbit GAPDH immunoaffinity-purified monospecific antibody as described in Materials and Methods.

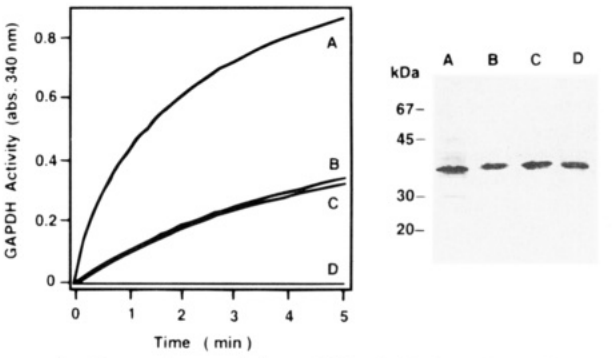

FIGURE 4: Comparisons of glyceraldehyde-3-phosphate dehydrogenase activity and membrane fusion activity in fractions from GTP−agarose and Mono Q chromatographies. Left panel: GAPDH activity was assessed spectrophotometrically by production of NADH as described in Materials and Methods. Enzyme aliquots were obtained from either the GTP−agarose affinity column [3 mM GTP eluent (A) or the 20 mM tripolyphosphate/10 mM NAD^+ eluent (B)] or from the concentrated void volume of the Mono Q column (C) (concentrated using an Amicon Microcon-10) or from the Mono Q eluents catalyzing membrane fusion activity (D) (also concentrated utilizing an Amicon Microcon-10). Right panel: SDS−PAGE (10−15% acrylamide PhastGel) analysis of aliquots of enzyme fractions utilized in samples A−D, visualized by silver staining.

from the Mono Q column eluents catalyzed membrane fusion, they did not contain demonstrable dehydrogenase activity (Figure 4). This purification scheme resulted in a 790-fold purification of membrane fusion activity in 13% overall yield (Table 1) and was remarkable for the isolation of a protein which catalyzed membrane fusion and was immunologically related to GAPDH but did not possess demonstrable GAPDH catalytic activity. To further distinguish between immunoreactive species possessing and those

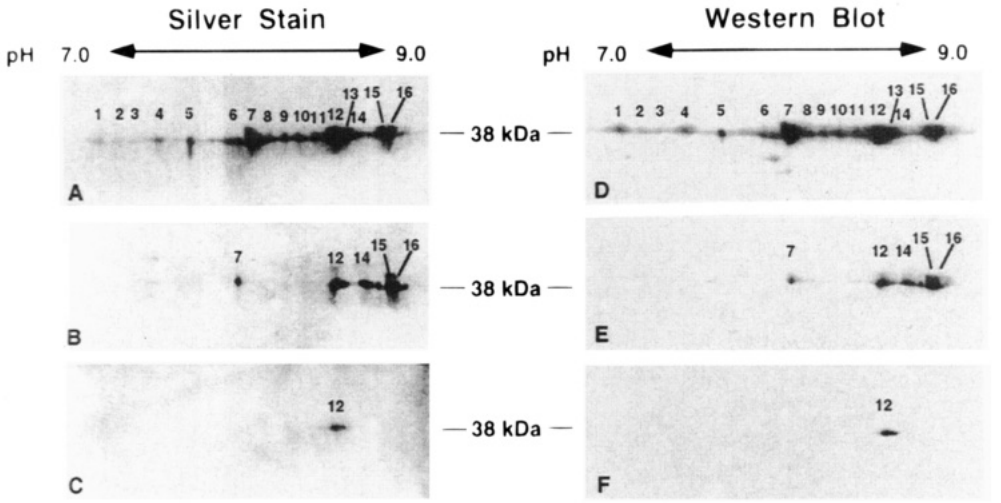

FIGURE 5: Two-dimensional electrophoresis of glyceraldehyde-3-phosphate dehydrogenase isoforms in column eluents from various steps in the purification of the polypeptide catalyzing membrane fusion activity. Left panel: Immobiline strips containing a pH gradient from pH 7.0 to 10.0 were utilized for isoelectric focusing in the first dimension, and SDS−12.5% PAGE (Excel Gel) was utilized for the second dimension as described in Materials and Methods. Aliquots of fractions from GTP−agarose chromatography [3 mM GTP eluent (lacking membrane fusion activity)] (sample A) and the 20 mM tripolyphosphate/10 mM NAD^+ eluent (corresponding to the fraction containing maximal membrane fusion activity) (sample B) as well as the Mono Q fraction containing maximal membrane fusion activity (sample C) were analyzed to determine the distribution of GAPDH isoforms present. Right panel: Western blots were performed on 2D gels run in parallel and proteins were transferred to PVDF paper and subsequently visualized utilizing mouse monoclonal antibody 155.D2.2 (directed against rabbit skeletal muscle GAPDH) as primary antibody and ^{125}I-radiolabeled rabbit anti-mouse IgG as secondary antibody. Western blot panels correspond to the 3 mM GTP eluent from the GTP−agarose column (D), the 20 mM tripolyphosphate/10 mM NAD^+ eluent from the GTP−agarose column (E), and fraction 10 from the Mono Q column (F).

lacking GAPDH catalytic activity, two-dimensional electrophoresis was performed.

Analysis of GAPDH Isoforms Catalyzing Fusion Activity by Two-Dimensional Electrophoresis. Fractions derived from salient steps in the membrane fusion protein purification scheme were further analyzed by two-dimensional electrophoresis utilizing a first-dimensional pH gradient from pH 7−10 (initial runs of pH 3−10 demonstrated no acidic proteins after the DE-52 chromatography purification step) and a second dimension consisting of a homogeneous SDS−12.5% polyacrylamide gel. Silver staining of two-dimensional gels of fractions derived from GTP−agarose chromatography demonstrated enrichment of the 38-kDa molecular species possessing a more basic p*I* in the P_{i_3}/NAD eluent (p*I* = 8−9) while the load (data not shown) and GTP eluents contained additional protein constituents with more acidic p*I*'s (p*I* = 7−8) (Figure 5). After Mono Q chromatography, the active fraction (which possessed fusion activity but no dehydrogenase activity) demonstrated a spot with a p*I* = 8.5 (labeled spot 12 in Figure 5). Western blots probed with a monoclonal antibody to GAPDH (Figure 5, right panel) as well as those probed with monospecific polyclonal antibodies directed against GAPDH (data not shown) demonstrated that the protein catalyzing fusion activity (spot 12 in Figure 5) is immunologically indistinguishable from the other GAPDH isoforms present (compare left- and right-hand panels in Figure 5).

Independent Substantiation of the Identity of Membrane Fusion Activity as a GAPDH Isoform through Inhibition of Fusion Activity by Monoclonal Antibodies Directed against GAPDH and by Glyceraldehyde 3-Phosphate. Due to the surprising nature of these results, additional independent criteria were employed to substantiate the identity of the protein catalyzing membrane fusion as an isoform of GAPDH and to clarify the existence of separate and distinct GAPDH isoforms which catalyze fusion activity and dehydrogenase activity. First, membrane fusion activity was inhibited by D-glyceraldehyde 3-phosphate with a K_i (≈100 μM) similar to the association constant of glyceraldehyde 3-phosphate with GAPDH (data not shown). Second, a monoclonal antibody directed against GAPDH (i.e., antibody 155.D2.2) specifically inhibited membrane fusion in a dose-

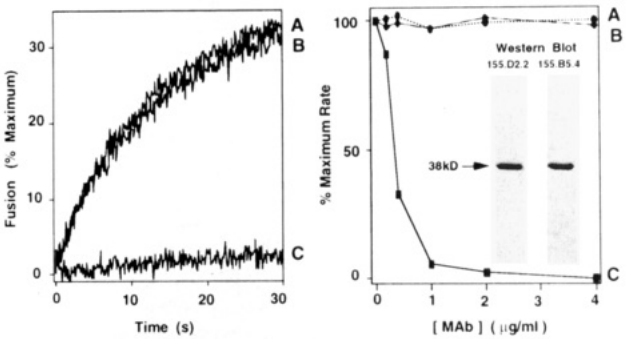

FIGURE 6: Solution-state inhibition of GAPDH isoform-catalyzed membrane fusion by the monoclonal antibody 155.D2.2. Left panel: Physiologically modeled small unilamellar vesicles were prepared (200 μM) and placed in one chamber of a stopped-flow apparatus as described in Materials and Methods. The other chamber contained the P_{i_3}/NAD^+ eluate from GTP−agarose chromatography (0.004 mg/mL) previously incubated at 37 °C for 1 min with either nonrelevant antibody (anti-actin, 100 μg/mL) (A), monoclonal 155.B5.4 (100 μg/mL) (B), or monoclonal 155.D2.2 (1 μg/mL) (C). Samples were rapidly mixed in a stopped-flow apparatus, and membrane fusion was quantified as described in Materials and Methods. Right panel: Concentration dependence of the inhibition of membrane fusion activity by the 155.D2.2 monoclonal antibody, and concentration independence of the inhibition of membrane fusion by anti-actin (A) and anti-GAPDH 155.B5.4 (B) monoclonal antibodies. Inset: Demonstration of the specificity of both 155.D2.2 and 155.B5.4 for GAPDH when used to probe a Western blot of rabbit brain cytosol separated on SDS−11% PAGE. The specificity of monoclonal antibodies was determined by visualizing the distribution of bound primary monoclonal antibody after treatment with ^{125}I-labeled rabbit anti-mouse IgG (secondary antibody) and subsequent autoradiography.

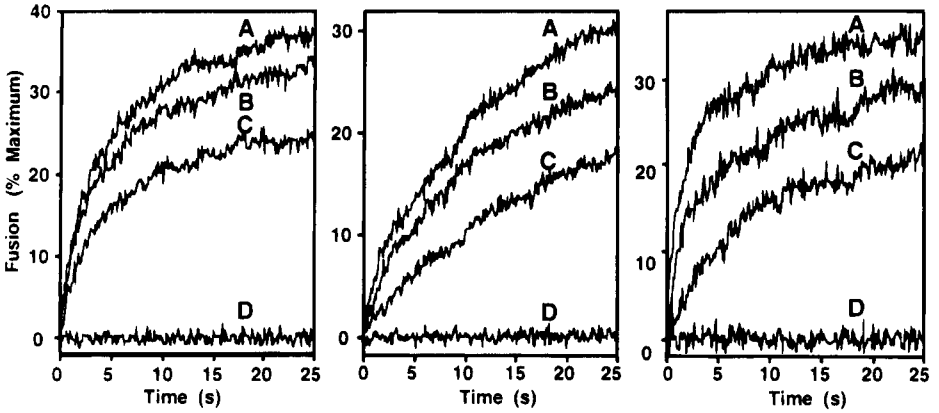

FIGURE 7: Characterization of GAPDH-catalyzed membrane fusion. Left panel: Membrane fusion was quantified in sequential column chromatographic fractions utilizing SUVs (400 μM total lipid) as described in Materials and Methods. Vesicles from one chamber of a stopped-flow apparatus were mixed with the contents of the second chamber, which contained either the DE-52 void volume (0.03 mg/mL), (curve A), the GTP−agarose active fraction (0.002 mg/mL) (curve B), the Mono-Q active fraction (0.1 μg/mL) (curve C), or no added protein (curve D). Middle panel: Fusion of large unilamellar vesicles (400 μM total lipid) was measured utilizing the R_{18} fusion assay as described in Materials and Methods. The protein fractions corresponding to curves A−D are described above. Right panel: Large unilamellar vesicles (LUVs) comprised of 16:0−18:1 phosphatidylcholine (27%), 18:0−20:4 plasmenylethanolamine (27%), 16:0−18:1 phosphatidylserine (6%), and cholesterol (40%) containing either entrapped terbium chloride or dipicolinic acid (DPA) were prepared by extrusion through polycarbonate membranes as described in Materials and Methods. After separation of liposome-entrapped ions from nonencapsulated probe by gel-filtration chromatography, liposomes (at 1.0 mM total lipid) were mixed in equimolar ratios with protein fractions in curves A−D as described above. Results represent the mean of 8 recordings (left and middle panels) and 10 recordings (right panel) obtained from two independent preparations.

dependent manner, while other monoclonal antibodies [e.g., either other antibodies directed against GAPDH (e.g., 155.B5.4) or those directed against other nonrelevant proteins (e.g., actin)] did not inhibit membrane fusion activity even at concentrations of monoclonal antibody that were 100-fold higher than those which inhibited fusion activity (Figure 6). Finally, koningic acid, a known inhibitor of GAPDH enzyme activity (Sakai et al., 1991) that covalently binds to the active-site cysteine (cysteine 149), failed to inhibit membrane fusion activity at concentrations (up to 200 μM) which maximally inhibited GAPDH enzyme activity (data not shown). Collectively, these results unambiguously demonstrate that membrane fusion activity is catalyzed by a GAPDH isoform (modulated by glyceraldehyde 3-phosphate and blocked by a monoclonal antibody directed against GAPDH) and that the isoforms which mediate dehydrogenase activity and membrane fusion activity represent separate and distinct GAPDH isoforms (e.g., koningic acid blocks dehydrogenase but not fusion activity).

Characterization of Glyceraldehyde-3-Phosphate Dehydrogenase Isoform-Catalyzed Fusion of Large Unilamellar Vesicles. To characterize the dependence of GAPDH isoform-catalyzed membrane fusion on vesicle size and to unambiguously identify the observed dequenching of R_{18} fluorescence as *bona fide* membrane fusion, additional experiments were performed. First, two populations of large unilamellar vesicles (LUVs) were individually prepared which contained either 0 or 4 mol % R_{18} as described in Materials and Methods. Coincubation of fluorophore-labeled LUVs with unlabeled LUVs with active fractions from chromatography (including the GAPDH isoform purified to homogeneity by Mono Q chromatography) resulted in the rapid dequenching of R_{18} fluorescence demonstrating the ability of the enzyme to catalyze the fusion of LUVs as well as SUVs (Figure 7, left and middle panels). In the absence of enzyme, both SUVs and LUVs were stable for >60 min under the conditions employed (Figure 7). To confirm that the observed dequenching of R_{18} fluorescence reflected *bona fide* membrane fusion and did not result from the transfer of R_{18} probe between vesicles after membrane apposition, the rate of enzyme-catalyzed internal contents mixing of vesicles was quantified utilizing a Tb-DPA assay. Incubation of either the void volume from anion-exchange chromatography, the GTP−agarose active fraction, or the Mono Q active fraction with vesicles containing individual constituents of the charge transfer complex resulted in a rapid increase in fluorescence emission, demonstrating internal contents mixing from vesicle fusion (Figure 7, right panel).

Stopped-Flow Kinetics of Vesicle Fusion Catalyzed by the GAPDH Fusion Protein. To quantify the kinetics of the initial rate of GAPDH isoform-catalyzed membrane fusion, the initial rate of R_{18} fluorescence dequenching was examined utilizing a stopped-flow apparatus. The initial rate of membrane fusion catalyzed by the Mono Q active fraction (initial concentration = 2 μg/mL, which was diluted 40-fold to a final concentration = 0.05 μg/mL for assay) was quantified utilizing a vesicle concentration of 400 μM. On the basis of the assumptions that (1) $\approx 2 \times 10^3$ molecules of lipid are present in each vesicle (for ~200-Å SUVs), (2) the active form of GAPDH is a tetramer (similar to the active form of the dehydrogenase; Harris & Waters, 1976), and (3) $\approx$1% of vesicle fusion was manifest within 5 ms after mixing, the purified GAPDH isoform catalyzed the fusion of one pair of vesicles in $\approx$1 ms (on average). Since many rounds of vesicle fusion are catalyzed during each assay, it is evident that the fusion protein represents a true catalyst of vesicle fusion and does not mediate the fusion of vesicles in a stoichiometric fashion (e.g., >100 pairs of vesicles undergo fusion mediated by one GAPDH tetramer in seconds).

Substrate Specificity of the Membrane Fusion Protein. To identify critical structure−activity relationships which facilitate GAPDH isoform-catalyzed membrane fusion, additional experiments were performed. Vesicles composed of different phospholipid classes and subclasses, as well as those containing differing mole fractions of cholesterol, were

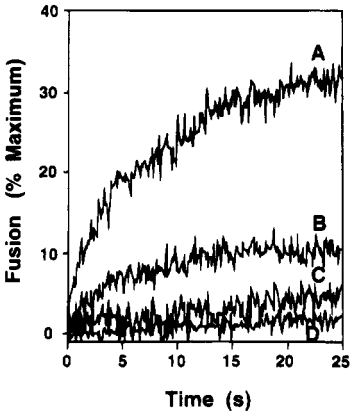

FIGURE 8: Ethanolamine glycerophospholipid selectivity of the GAPDH isoform mediating membrane fusion. Physiologically modeled vesicles comprised of phosphatidylcholine (27%), ethanolamine glycerophospholipids (27%), phosphatidylserine (6%), and cholesterol (40%) (200 μM total lipid) were prepared and placed in one chamber of a stopped-flow apparatus as described in Materials and Methods. The other chamber contained aliquots of protein samples from the active fraction from GTP–agarose chromatography (20 mM tripolyphosphate/10 mM NAD^+ eluent, 0.001 mg/mL). Ethanolamine glycerophospholipid selectivity was assessed by incorporating four different molecular species of ethanolamine glycerophospholipid into vesicles: 18:0–20:4 plasmenylethanolamine (A), 16:0–18:1 plasmenylethanolamine (B), 18:0–20:4 phosphatidylethanolamine (C), or 16:0–18:1 phosphatidylethanolamine (D). Results represent the mean of 6 recordings from two separate preparations expressed as a percentage of maximal fusion.

prepared, and the effects of altering the vesicular membrane lipid composition on the kinetics of membrane fusion were explored. First, the ethanolamine glycerophospholipid subclass requirements of the fusion protein at each stage of the purification procedure were examined. Membrane fusion activity was highly selective for vesicles containing plasmenylethanolamine (in comparison to phosphatidylethanolamine) at each stage of the purification procedure (Figures 1 and 8). Furthermore, a 2–4-fold selectivity for plasmalogens containing arachidonic acid in comparison to oleic acid at the *sn*-2 position (synaptic membranes are substantially enriched in arachidonic acid-containing molecular species; Glaser and Gross, unpublished observations) was manifest (Figure 8). To quantify the dependence of membrane fusion activity on the fractional percent of plasmenylethanolamine present in the vesicles undergoing membrane fusion, the mole fraction of ethanolamine glycerophospholipids represented by plasmenylethanolamine was varied from 0–100%. Membrane fusion activity increased as the mole fraction of plasmenylethanolamine in the vesicles was increased up to 75% plasmenylethanolamine with no additional increment at higher plasmenylethanolamine mole fractions (Figure 9, left panel).

To determine the importance of serine glycerophospholipids and cholesterol in facilitating fusion mediated by the purified protein, fusion rates were quantified in vesicles containing selected concentrations of phosphatidylserine (0–12 mol %) and cholesterol (0–40 mol %). In vesicles in which the serine glycerophospholipid content was varied

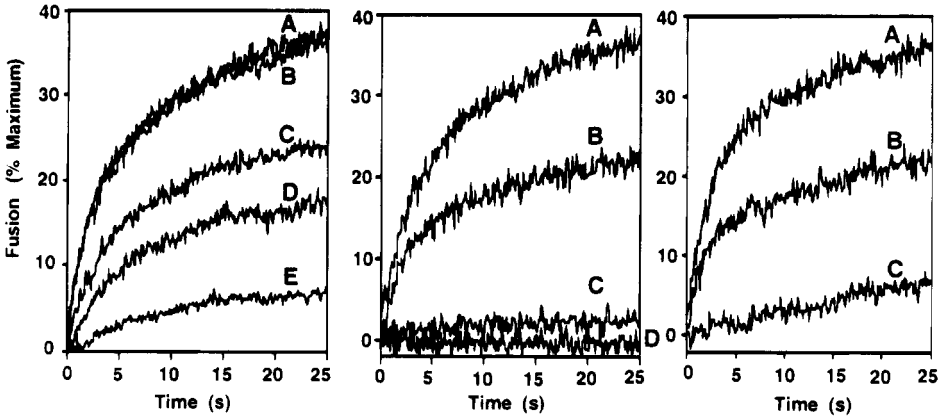

FIGURE 9: Selectivity of the GAPDH isoform catalyzing membrane fusion for plasmenylethanolamine, cholesterol, and phosphatidylserine. Left panel: Selectivity of the GAPDH isoform catalyzing membrane fusion for physiologically modeled vesicles containing distinct mole fractions of plasmenylethanolamine and phosphatidylethanolamine. Physiologically modeled small unilamellar vesicles consisting of 16:0–18:1 phosphatidylcholine (27%), ethanolamine glycerophospholipid (27%), 16:0–18:1 phosphatidylserine (6%), and cholesterol (40%) were prepared (final lipid concentration of 200 μM) and placed in one chamber of a stopped-flow apparatus as described in Materials and Methods. Aliquots of the HiLoad SP Sepharose column eluent (protein concentration = 0.2 mg/mL) were placed in the other chamber, samples were rapidly mixed in a stopped-flow apparatus, and membrane fusion was quantified as described in Materials and Methods. Ethanolamine glycerophospholipids were comprised of either 18:0–20:4 plasmenylethanolamine (A), 18:0–20:4 plasmenylethanolamine (75%) and 18:0–20:4 phosphatidylethanolamine (25%) (B), 18:0–20:4 plasmenylethanolamine (50%) and 18:0–20:4 phosphatidylethanolamine (50%) (C), 18:0–20:4 plasmenylethanolamine (25%) and 18:0–20:4 phosphatidylethanolamine (75%) (D), or 18:0–20:4 phosphatidylethanolamine (E). Middle panel: Dependence of GAPDH isoform-catalyzed membrane fusion on the phosphatidylserine content of vesicles. Physiologically modeled SUVs were prepared (final lipid concentration = 200 μM) and placed in one chamber of a stopped-flow apparatus. The other chamber contained aliquots of the P_{i_3}/NAD^+ eluent from the GTP–agarose column (0.004 mg/mL). Curves represent the fusion of small unilamellar vesicles consisting of 16:0–18:1 phosphatidylcholine/18:0–20:4 plasmenylethanolamine/cholesterol (27/27/40 molar ratio) and varying molar percentages of phosphatidylserine [6% (A), 12% (B), 3% (C), or 0% (D)]. Right panel: Dependence of GAPDH isoform-catalyzed membrane fusion on the cholesterol content of vesicles. Physiologically modeled SUVs containing phosphatidylcholine/18:0–20:4 plasmenylethanolamine/phosphatidylserine (45/45/10 molar ratio) and selected molar percentages of cholesterol [40% (A), 20% (B), or 0% (C)] were prepared (final lipid concentration of 200 μM) and placed in one chamber of a stopped-flow apparatus. The other chamber contained aliquots of the active fraction from GTP–agarose chromatography [i.e., 20 mM tripolyphosphate/10 mM NAD^+ (P_{i_3}/NAD^+)], at a protein concentration of 0.004 mg/mL. Samples were rapidly mixed and membrane fusion was quantified as described in Materials and Methods. Results represent the average of 8 (left panel) or 6 (middle and right panels) recordings from two independent preparations expressed as a percentage of maximal fusion.

from 0 to 12%, the most rapid rates of membrane fusion were present in vesicles containing 6 mol % PS (Figure 9, middle panel) [note that this approximates the percentage of PS found in synaptosomal membranes (Westhead, 1987; Cullis & Hope, 1991)]. Importantly, vesicles containing only 3 mol % PS or less could not be induced to fuse by the membrane fusion protein under the conditions employed. Vesicles containing 12 mol % PS fused at rates that were considerably less than those manifest at 6 mol % PS, demonstrating that neither bulk alterations in membrane surface charge nor alterations in physical properties of the vesicle is the sole mechanism through which changes in PS content facilitate protein-mediated membrane fusion (in contrast to non-protein-mediated Ca^{2+}-dependent vesicle fusion). Next, the concentration of cholesterol in the vesicles was varied from 0 to 40 mol %. The membrane fusion protein possessed an obligatory requirement for cholesterol, with little or no fusion manifest in vesicles lacking cholesterol (Figure 9, right panel). Protein-facilitated membrane fusion activity induced by cholesterol increased in a dose-dependent fashion, with the most rapid rates of membrane fusion manifest in vesicles containing a physiologic complement of cholesterol (i.e., 40 mol %). Collectively, these results demonstrate that the membrane fusion activity has an obligatory requirement for physiologic complements of plasmenylethanolamine, phosphatidylserine, and cholesterol and will not rapidly catalyze membrane fusion in the absence of any one of these constituent lipids of synaptic vesicles and the plasma membrane.

DISCUSSION

This study reports the purification of a calcium-independent fusion protein which possesses an obligatory requirement for vesicles comprised of physiologic mole fractions of plasmenylethanolamine, cholesterol, and phosphatidylserine. Through sequential column chromatographic purification, a final preparation was obtained which was 790-fold purified and catalyzed the fusion of two vesicles every millisecond (on average). The identity of the fusion protein as an isoform of GAPDH was substantiated through chemical, immunologic, chromatographic, and pharmacologic criteria. The column chromatographic resolution of discrete GAPDH isoforms catalyzing independent functional events (i.e., dehydrogenase and membrane fusion activities) identifies the likely chemical and evolutionary rationale underlying the plethora of previously demonstrated GAPDH isoforms in mammalian cells.

The obligatory dependence of the fusion protein on vesicles containing plasmenylethanolamine is unprecedented. The importance of plasmenylethanolamine in facilitating membrane fusion was previously suggested from experiments examining the spontaneous (i.e., non-enzyme-catalyzed) fusion of vesicles in which accelerated rates of spontaneous membrane fusion were manifest in vesicles containing plasmenylethanolamine in comparison to phosphatidylethanolamine (Glaser & Gross, 1994). These subclass-specific differences in membrane fusion were attributed to the fact that plasmenylethanolamines exhibit a marked propensity for adopting the hexagonal phase (their aliphatic volume vastly exceeds the volume occupied by their polar head group), thereby lowering the activation necessary for formation of the critical H_{II}-like intermediate (Han & Gross, 1990). On the basis of the high content of plasmenylethanolamine in synaptic vesicles and plasma membranes, we hypothesized that evolutionary selection exploited the unique stereoelectronic properties of plasmenylethanolamine molecular species to facilitate enzyme-catalyzed membrane fusion.

The demonstration that membrane fusion catalyzed by this GAPDH-isoform possesses an absolute requirement for cholesterol is intriguing from several perspectives. First, although cholesterol does not have profound effects on the rate of spontaneous vesicle−vesicle fusion (i.e., non-protein-catalyzed fusion) in several model systems (Bental et al., 1987; Stamatatos & Silvius, 1987), physiologic amounts of cholesterol dramatically accelerate membrane fusion catalyzed by this enzyme. Accordingly, there are unique aspects of the enzyme-catalyzed fusion which do not appear to be mechanistic features of traditionally employed vesicle−vesicle fusion systems. Furthermore, since cholesterol is only present in substantial quantities in plasma membranes or in membrane fractions derived from the plasma membrane (e.g., recycled synaptic vesicles), these results suggest the importance of this fusion protein in cellular exocytosis.

Prior demonstration that synaptic vesicle fusion with plasma membranes occurs within 1 ms after presynaptic depolarization has placed strict kinetic constraints on the properties of the proteins, lipids, and regulatory factors participating in this process. It is now well recognized that a highly specific docking system present in the presynaptic membrane facilitates the appropriate synaptosomal plasma membrane apposition of membrane fusion partners (Stamnes & Rothman, 1993). However, vesicle docking is not the rate-determining step in membrane fusion, since electron micrographs demonstrate many synaptic vesicles poised ready for fusion at the plasma membrane interface in resting cells. Thus, the rate-determining step of the fusion process appears to reflect the intrinsic rate of membrane fusion of preassembled synaptic vesicle−plasma membrane fusion partners. The demonstration that the GAPDH isoform purified herein catalyzes one fusion event every 1 ms (on average) satisfies the mathematical constraints poised by the exocytotic (i.e., synaptic vesicle) fusion problem. The identification of this highly active fusion protein changes the thrust of the problem from how vesicle fusion can occur so rapidly to rather what tonically inhibits synaptic membrane fusion in the basal (i.e., resting) state.

Although the mathematical constraints of membrane fusion are satisfied by the kinetic characteristics of this GAPDH isoform, they do not, by themselves, demonstrate that this polypeptide mediates fusion of synaptic vesicles with the presynaptic membrane *in vivo*. The polypeptide purified in this report possesses multiple kinetic characteristics which make it an attractive candidate as the enzymic mediator of synaptic vesicle fusion *in vivo*, including (1) its ability to catalyze many rounds of vesicular fusion per molecule of protein, (2) a turnover rate on the millisecond time scale, (3) a mechanism for regulation (i.e., deinhibition) that can facilitate the extraordinarily rapid initiation of membrane fusion, (4) an obligatory requirement for cholesterol and plasmenylethanolamine, two prominent constituents of the synaptic vesicle and plasma membrane compartments (i.e., suggesting that it functions to mediate membrane fusion events between these compartments *in vivo*), and (5) optimal function at physiologic pH and calcium ion concentration. We point out that although the fusion protein does not possess an obligatory requirement for calcium ion in the

catalysis of membrane fusion, calcium ion may nonetheless serve an important role in the modulation of the activity of this enzyme.

It is well-known that a wide variety of genes (over 200 in some species) contain sequences which can encode proteins with strong similarity to GAPDH (Piechaczyk et al., 1984; Arcari et al., 1989). The demonstration that distinct isoforms of GAPDH exist in mammalian cells which possess widely disparate catalytic properties suggests that one potential explanation underlying the transcription of nominally "non-functional" GAPDH messages is that the polypeptides encoded by those messages are utilized for a separate and distinct catalytic or regulatory function. Since the oligomeric state of GAPDH is of known importance in regulating GAPDH dehydrogenase activity, the possibility exists that non-full-length transcripts of GAPDH isoforms may participate in the regulation of membrane fusion and membrane trafficking in intact cells.

Collectively, the results of the present study identify a GAPDH isoform as a potent and specific enzymic mediator of membrane fusion *in vitro*. The identification of the remarkable substrate selectivity, catalytic efficiency, and regulatory motif (i.e., relief of inhibition) employed by this polypeptide render it an attractive candidate as the enzymic mediator of exocytotic fusion events. Proof of the principles suggested by these experiments awaits identification of the *in vivo* roles and functional significance of the protein−lipid and lipid−lipid interactions elaborated herein.

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