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CHARACTERIZATION OF THE FUSOGENIC PROPERTIES OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE: FUSION OF PHOSPHOLIPID VESICLES[1]

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A fluorescence assay based on resonance energy transfer has been used to characterize the fusogenic properties of glyceraldehyde-3-phosphate dehydrogenase. The extent of phospholipid vesicles fusion induced by the protein increased with decreasing pH, being maximum at pH 4.5-5.0. Fusion reaction was temperature dependent with an activation energy of 10 Kcal/mol, and virtually completed within 1 min. at pH 5.0. Fusion is most efficient with vesicles bearing negative charge, however uncharged and even positively charged vesicles were fused. The negatively charged and uncharged vesicles showed the same pH dependence. These observations suggest the importance of hydrophobic interaction in the process of fusion, which was supported by a correlation between extent of fusion and exposure of hydrophobic region of the protein.

Membrane fusion is a key event in many biological processes,still not well understood in spite of intensive research. Due to the complexity of biological membranes, the phenomenon was studied in model membrane systems such as liposomes. The most extensively studied fusion agent in this system was the divalent cation Ca2+. In this respect, investigations of the interaction of calcium with acidic phospholipids in well-defined model systems are particularly useful to understand the mechanism of fusion (1).This mechanism was extended to biological membranes (2,3), since cytoplasmic calcium concentration rise precedes many events that involve membrane fusion (4). Nevertheless, more recently, it was show that exocytosis in plateles (5) and in neutrofils (6), can be triggered without an increase of the intracellular Ca2+ concentration, indicating that Ca2+ may not be required for the fusion

[1]Dedicated to Dr. Luis F. Leloir on the occasion of his eightieth birthday.

Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; N-NBD-PE, N-7-nitro-2 1.3 benzoxadiazol 4-yl phosphatidylethanolamine; CA9C, cholesterol antracene 9 carboxylate; ANS, 1-anilino-8-naphtalene sulfonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SA stearylamine.

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reaction itself. In addition, the Ca2+ concentration necessary to induce fusion of liposomes is very high compared with the intracellular Ca2+ concentration.

The potencial role of proteins in membrane fusion has been repeatedly stressed. Recently, soluble proteins and peptides have been shown to induce fusion of liposome systems (7-11). In a previous paper we described the capacity of some proteins to induce fusion,of phospholipid vesicles one of them, glyceraldehyde-3- phosphate dehydrogenase, a well characterized soluble enzyme,is of special interest since it was able to induce fusion at very low concentration (11).

The objective of the present study was to characterize the fusogenic activity of glyceraldehyde-3-phosphate dehydrogenase. The results revealed that fusion induced by glyceraldehyde-3-phosphate dehydrogenase was temperature and pH dependent being particularly active at physiological pH. Fusogenic activity was observed with uncharged as well as negatively and positively charged vesicles. The importance of hydrophobic and electrostatic interactions on fusion process are briefly discussed.

MATERIALS AND METHODS

Rabbit muscle GAPDH (from Sigma Chem.Co) was desalted by filtration through a Sephadex G-25 column (1 x 10 cm). Eluted with 20 mM Tris-malate buffer, pH 7.4,the enzyme was maintained at 4°C and used within a day. Protein concentration was determined spectrophotometrically employing A280= 1.0 cm for the holoenzyme (12).

Phosphatidylcholine was purified from egg yolk (13). Phosphatidic acid was obtained by the action of phospholipase D on purified PC (14) and isolated by a silicic acid column chromatography. N-NBD-PE was prepared as described by Monti et al. (15) but the reaction was carried out at 60° C to maintain dipalmitoil phosphatidylethanolamine in solution. CA9C was purchased from Molecular Probes Inc. The purity of the lipids was checked by thin layer chromatography on silica gel H and stored in chloroform-methanol 9:1 at -20°C. Phospholipid concentrations were estimated by the Ames phosphate assay (16).

Phospholipid vesicles were prepared by the ethanol-microinjection method of Batzri and Korn (17). Phospholipids were dissolved in ethanol, and aliquots of 10 µl were injected into a stirred 10 ml solution of a desired buffer. The lipid concentration was 50 µM, and the ethanol concentration did not exceed 2 %. The fluorescent probe CA9C (donor) and N-NBD-PE(acceptor) were incorporated into different vesicle populations.The probes accounted for 1 mole % or 3 mole % of total lipid respectively. "smock fused" vesicles (18) were prepared injecting lipids and fluorescent probes together into the buffer in order to obtain both probes in the same vesicle population.

The assay of membrane intermixing was based on the resonance energy transfer described in detail by Hoekstra (19). Equal volumes(1.0 ml)of CA9C-labeled vesicles and N-NBD-PE vesicles were mixed and fusion was initiated by addition of 5-40 µl aliquots of enzyme solution. The results expressed as percent of fusion were calculated according to the following equation.

$$\% \text{ Fusion} = \frac{(1 - F/Fo)}{(1 - F/FoN)} \times 100$$

Where Fo is the CA9C fluorescence (Ex. wavelength: 380 nm; Em.wavelength 460 nm) of the donor in presence of the acceptor in a different vesicle population; F is the CA9C fluorescence after fusion; and FoN is the CA9C fluorescence of "smock fused" vesicles.

1 - Anilino-8-naphtalene sulfonate was purchased from Sigma Chem. Co. and recrystallized twice in water before used. ANS fluorescence was determined by exciting at 360 nm and measuring emission at 490 nm. All the fluorescence

measurements were made in an Aminco Bowman spectrofluorometer equipped with a thermostated cuvette holder.

The isoelectric point of GAPDH was estimated by a method based upon the pH-dependent binding affinity of amphoteric molecules developed by Yang and Langer (20).

RESULTS

pH Dependence of vesicle fusion

Previous studies from our laboratory showed that fusion of phospholipid vesicles by GAPDH occurs at neutral pH, and in the absence of calcium (11). In this work we show that the process is critically dependent upon the pH. Fig. 1-A shows the extend of fusion of (PC/PA, 9:1) vesicles incubated at various pH in the presence of GAPDH. There was a good level of fusogenic activity at neutral pH as described previously. With decreasing pH the extend of fusion increased , reaching a maximum at pH 4.5-5.0. Upon a further lowering of the pH, a relative decrease of the activity was observed, being almost null at pH 3.5, probably due to denaturation of the protein. A similar pH profile was noted for the fusion of pure PC vesicles by GAPDH. The arrow indicate the isoelectric point of the protein (6.2).

Kinetic analysis showed that increasing fusion occurred during the first 5 min of incubation at 37°C and pH 7.4 (Fig. 1-B). However, when vesicles were incubated at pH 5.0 , a higher extend of fusion (80%) was obtained, in less than 1 min.

The rate of fusion between phospholipid vesicles was dependent on the concentration of GAPDH. Fig. 1-C shows a comparison of the dose-response curve

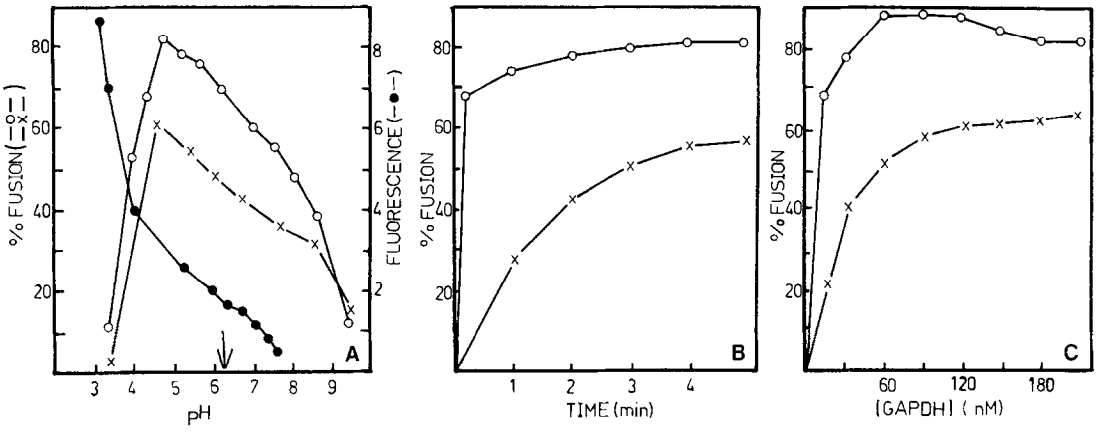

Figure 1. A) Effect of medium pH on vesicle fusion and on ANS fluorescence. Fusion of PC (-x-), and (PC/PA , 9:1) vesicles (-○-) was measured by the resonance energy transfer method described in Materials and Methods. Fusion was induced by 0.1 uM glyceraldehyde-3-phosphate dehydrogenase.
Relative fluorescence of 10 µM ANS (-●-) in the presence of 0.15 mg GAPDH in 20 mM Tris-HCl buffer which was brought with HCl to a desired pH. Excitation wavelength: 360 nm. Emission wavelength: 490 nm.

B) The time dependence of vesicle fusion as induced by GAPDH. The percent of fusion of (PC/PA, 9:1) vesicles (50 uM lipid) was measured by resonance energy transfer assay as a function of time. Fusion was induced by 0.1 mM GAPDH at pH 5.0 (-○-) and pH 7.4 (-x-).

C) Effect of GAPDH concentration on vesicle fusion. Fusion of (PC/PA, 9:1) vesicles was measured by the resonance energy transfer assay described under Materials and Methods at pH: 5.0 (-○-), and 7.4 (-x-).

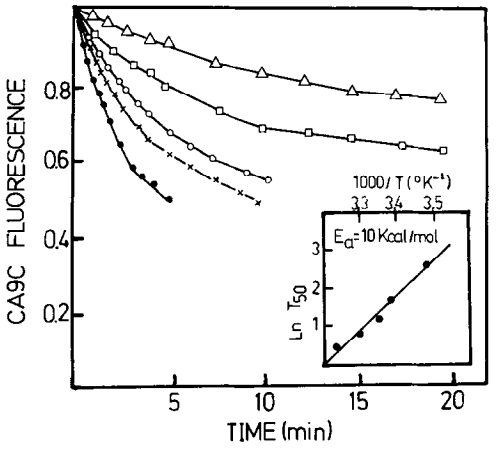

Figure 2. Temperature dependence of vesicles fusion induced by GAPDH. Vesicles were prepared as described in Materials and Methods. The fusion mixture (2.0 ml final volume, 20 mM Tris-HCl, pH 7.4) contained n nmol of phospholipids (PC/PA, 9:1) nmol CA9C, and 3 nmol N-NBD-PE. Fusion was initiated by the addition of 0.1 mg GAPDH and carried out at 37°C (-●-), 30°C (-x-), 26°C (-○-) 22°C (-□-) and 16°C (-△-). Fluorescence of CA9C (ex.wavelength: 370; em. wavelength: 450 nm) was recorded as a function of time. Inset: the rate of fusion was estimated at the indicated temperatures and the results were presented as an Arrhenius plot.

at pH 7.4 and pH 5.0; the enzyme concentration needed to induce half of the maximal fusion is lower at pH 5.0 (7.5 nM) than at pH 7.4 (23 nM).

Temperature dependence

The fusion of (PC/PA ,9:1) vesicles by GAPDH was also studied at various temperature (Fig. 2). The initial rate of fusion was very low at temperature below 15° C, and as expected, increased with increasing temperature. the Arrhenius Plot of fusion rate vs. temperature(Inset Fig.2) shows a single linear slope between 10°and 37°C. This indicates that there was no change in membrane structure in the temperature range that affects fusion. The calculated energy of activation was 10 KCal/mol.

Vesicle charge dependence

The relation between fusion induced by GAPDH and vesicles net charge was studied at pH 7.4 (Table I). A comparative experiment was performed with calcium. Phospholipid vesicles bearing different negative charge density were obtained by increasing the PA/PC ratio. Positively charged vesicles were made replacing PA by SA. As can be seen, GAPDH was capable of inducing fusion of negatively, neutral and even positively charged vesicles. However a maximum was obtained with vesicles containing 10 % of phosphatidic acid. On the other hand, calcium was unable to induce fusion of neutral and positively charged vesicles but with negatively charged vesicles its ability to induce fusion increased proportionally to the negative density of the vesicles.

Effect of pH on the exposure of protein hydrophobic regions.

Evaluation of the effect of pH upon GAPDH hydrophobicity is important for the understanding of the fusion process. ANS becomes highly fluorescent upon

TABLE 1. Dependence of the extent of fusion induced by GAPDH on the vesicle charge

Vesicle Composition %			Relative	Fusion %	
PC	PA	SA	Vesicle Charge	GAPDH(0.1 μM)	Ca2+(5mM)
70	30	0	- - -	42	58
80	20	0	- -	48	40
90	10	0	-	61	30
100	0	0	uncharged	35	0
90	0	10	+	27	0
80	0	20	+ +	20	0
70	0	30	+ + +	8	0

Vesicle bearing negative, positive and no charge and containing CA9C or N-NBD-PE were prepared as described in Materials and Methods. Fusion mixture (2.0 ml final volume, 20 mM Tris-HCl, pH 7.4) containing 50 μM phospholipids was incubated 5 min. at 37°C, in the presence of 0.1 μM GAPDH or 5 mM calcium.

binding to the hydrophobic sites of proteins (21). Since negatively charged and uncharged vesicles showed the same pH dependence on fusion (see Fig 1-A), we reasoned that the increased rate of fusion found at low pH might be explained by the appearance of hydrophobic regions hidden in the native structure of GAPDH at neutral pH.The ability of GAPDH to bind ANS was determined at various pHs . Although a weak fluorescence of ANS was detected in the presence of enzyme at neutral pH, acidification of the solution enhanced the ANS fluorescence almost 20-fold. Fig. 1-A shows a smooth increment of the fluorescence occurring between pH 7.5 to 4.0, and a sharp increment at pH lower than 4.0. These changes parallels quite well the increment of the fusogenic activity and the abrupt inactivation of GAPDH respectively.

DISCUSSION

In this paper we have utilized a fluorescence assay based on resonance energy transfer to characterize the fusion of phospholipid vesicles by GAPDH, an amphipotential protein (22) which can exist in a water-soluble or membrane associate state (23-24).

GADPH fused either uncharged (PC) and negatively (PC:PA) or positively (PC/SA) charged phospholipid vesicles, which suggests that the charge of the polar head group is not critical for this process. Modification of the ionic strength of the medium did not change the fusogenic activity of the protein (not shown). The established pH dependence of fusion activity showed a maximum at pH: 4.5-5.0, just below the isoelectric point of the protein (PI:6.2). However, at pHs above the isoelectric point GAPDH was particularly active.

Moreover the pattern of fusion rate induced by GADPH as a function of pH was almost identical when (PC)- vesicles or (PC/PA)- vesicles were used.These results argues against the role of electrostatic interactions between the protein and the polar head groups of vesicles. On the contrary, it seems more likely that some hydrophobic region of the protein which intereact with the hydrocarbon region of the lipid bilayer acquire some relevance. This hydrophobic region of GAPDH became exposed at low pHs (4.5 - 5.0), which is supported by a good correlation between the increased fluorescence of ANS, and the rate of fusion induced by GAPDH (Fig. 1-A).

It is well known that the presence of charged residues on the polar head group of lipid bilayer modifies its interfacial conformation, electrical potential, and molecular packing (25). Our results do not discard the importance that these factors might play in the fusogenic process induced by GAPDH.

The entry of several viruses like the influenza (26), Semliki Forest (27) and vesicular stomatitis (28), into the cells requires virus and cell membrane fusion induced by viral proteins. This fusion was showed to be clearly dependent on pH, being maximum at pH 4.5 - 5.0. Several mechanism can explain the pH-dependent activation fusion, one is a pH dependent conformational change in the protein leading to the exposure of hydrophobic peptide (29).

The results showed in the present study suggest that GAPDH is a protein with fusogenic characteristic similar to the viral proteins, where exposure of hydrophobic regions of protein could be an important feature of the process. Therefore, this system may serve as a model to study the mechanism of fusion of viral envelopes with target cell membranes.

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