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Role of electrostatics on membrane binding, aggregation and destabilization induced by NAD(P)H dehydrogenases. Implication in membrane fusion

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ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is considered a classical glycolytic protein that can promote the fusion of phospholipid vesicles and can also play a vital role on *in vivo* fusogenic events. However, it is not clear how this redox enzyme, which lack conserved structural or sequence motifs related to membrane fusion, catalyze this process. In order to detect if this ability is present in other NAD(P)H dehydrogenases with available structure, spectroscopic studies were performed to evaluate the capability of alcohol dehydrogenase (ADH), glutamic dehydrogenase (GDH) and sorbitol dehydrogenase (SDH) to bind, aggregate, destabilize and fuse vesicles. Based on finite difference Poisson–Boltzmann calculations (FDPB) the protein–membrane interactions were analyzed. A model for the protein–membrane complex in its minimum free energy of interaction was obtained for each protein and the amino acids involved in the binding processes were suggested. A previously undescribed relationship between membrane destabilization and crevices with high electropositive potential on the protein surface was proposed. The putative implication of the non-specific electrostatics on NAD(P)H dehydrogenases induced membrane fusion is discussed.

1. Introduction

Phospholipid bilayers form the barriers that define and partition all living cells and thus, membrane remodeling through fusion and fission is crucial in numerous intra and intercellular events such as: membrane trafficking, myotube formation, secretory exocytosis, fertilization and virus infection. Phospholipid membranes do not fuse spontaneously since the process is energetically unfavorable because biological membranes are subjected to strong repulsive hydration electrostatic and steric barriers [1]. These barriers can be overcome mainly by the presence of different agents such as divalent cations and/or proteins.

Protein-mediated membrane fusion was extensively studied and the best characterized systems are those involved in the enveloped virus infection [2] and membrane trafficking [3]. The biophysics of membrane fusion is dominated by the stalk hypothesis. According to this view, fusion of pure lipid membranes requires at least five distinct steps: approach to small distances; local perturbation of the lipid structure and merger of proximal monolayers; stalk formation; stalk expansion, which in some variants of the stalk model is associated with a hemi fusion diaphragm; and, finally, pore formation [4].

Structural and functional analyses have revealed similarities between viral and intracellular fusion where a four helical bundle folding leads to the apposition of two membranes and provides the energy for the fusion reaction [4].

During viral infection, the protein interaction with the target membrane involves an hydrophobic stretch of about 15 residues called "the fusion peptide" [5]. This segment was initially identified either at the N-terminus, as in most orthomyxoviruses, paramyxoviruses and several retroviruses [5,6]; or in the interior of the fusion proteins, as in *Rous sarcoma* virus [7], Vesicular Stomatitis virus [8], or Ebola virus [9]. It was generally accepted that each viral fusion protein contains a single fusion peptide and this segment was the sole responsible for the destabilization of the target cell membrane. However, new evidences indicate that in addition to classical fusion peptides, other regions from viral fusion

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glutamic dehydrogenase; SDH, sorbitol dehydrogenase; ADH, alcohol dehydrogenase; DOPC, dioleyl phosphatidylcholine; DOPS, dioleyl phosphatidylserine; SUV, small unilamellar vesicles; DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; ANTS, 8-aminonaphthalene-l,3,6-trisulfonic acid; DPX, *p*-xylene-bispyridinium bromide; HMM, hidden Markov Model; PDB, protein data bank; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; Rh-PE, N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine.

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proteins interact with membranes, contributing to their merging. Nevertheless, the precise function for each region is still unclear [10].

In addition to the viral and SNARE proteins, GAPDH is a cytoplasmic redox protein that can also promote the fusion of phospholipid vesicles [11,12]. Moreover, several independent studies demonstrated that GAPDH also plays a vital role on *in vivo* fusogenic events [13].

In the present work, the study is extended over some other NAD(P)H dehydrogenases with structural data available. In this way, spectroscopic studies were performed to evaluate the capability of ADH, GAPDH, GDH and SDH to bind, aggregate, destabilize and fuse vesicles. Based on computational tools, along with the availability of structural data, a model that could explain the molecular basis of these phenomena was constructed. Using FDPB calculations, the most probable orientation of each protein in relation to the membrane was obtained and the putative amino acids involved in the binding process were proposed. The two-dimensional representation of the binding free energy as a function of the protein rotation angles provides information about its vesicle aggregation capability. The protein–membrane complex obtained also suggested that these enzymes expose to the membrane a concave surface with high electropositive potential which could be related to the bilayer destabilization.

2. Materials and methods

2.1. Chemicals

Bovine liver glutamic dehydrogenase (EC 1.4.1.3), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), baker's yeast alcohol dehydrogenase (EC 1.1.1.1), sheep liver sorbitol dehydrogenase (EC 1.1.1.14) from Sigma Chem. Co, were dissolved in 20 mM Tris–HCl buffer pH 7.4 and used immediately. DOPC and DOPS were obtained from Avanti Polar lipids. NBD-PE and Rh-PE were purchased from Molecular Probes Inc.

2.2. Vesicle preparation

DOPC with appropriate amount of DOPS was dissolved in chloroform:methanol (2:1, v/v) and dried under nitrogen onto the wall of a Corex glass tube and then placed in a vacuum oven to completely remove any remaining solvent. The lipid was then rehydrated in 20 mM Tris–HCl buffer pH 7.4, and the large multilamellar vesicles formed were sonicated on ice under nitrogen with probe-type sonifier. Cycles of sonication (1-min pulse) and cooling (1 min) were repeated up to 20 times until the initially cloudy lipid dispersion became clear. In order to obtain small unilamellar vesicle (SUV) suspension free of titanium particles, the suspension was centrifuged for 15 min at 1100 ×g [14].

2.3. Protein–membrane binding assays

The accessibility of the protein Trp to aqueous quenchers was studied by monitoring the changes in the Trp fluorescence emission spectra upon addition of SUV [15]. Corrected spectra were obtained by subtracting the light scattering of SUV alone. The quenching experiments were carried out by the addition of KI on a 0.1 μM protein solution in the absence or presence of 50 μM SUVs. The lipid–protein mixtures (molar ratio of 500:1) were incubated for 1 h at room temperature prior to the measurements. The quenching constants were obtained from the slope of the Stern–Volmer plots of F_0/F vs. [KI], where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively [16]. The slope of the Stern–Volmer plot, obtained from the quenching experiments, is the Stern–Volmer constant (K_{SV}). This parameter (K_{SV}) is related to the exposure degree (accessibility) of Trp residues to the water soluble quencher. In general, when the slope increases a high degree of exposure is implied, assuming that there is not a large difference in fluorescence lifetime.

2.4. Aggregation measurements

Liposome aggregation was estimated as an increase in light scattering after protein addition, measured in an ISS spectrofluorometer with both monochromators set at 450 nm. The protein:lipid molar ratio used was the same as for the protein–membrane binding assays.

2.5. Leakage measurements

The release of the liposomal content was measured by following the fluorescence quenching of pre-encapsulated Tb/DPA complex upon release into the external medium containing 0.1 mM EDTA [17]. To prepare vesicles containing Tb–DPA complex trapped within, the dried lipid film was resuspended in the appropriated buffer (20 mM Tris–HCl pH 7.40) containing a 1:1 mixture of 15 mM $TbCl_3$ and 150 mM DPA (sodium salt). After sonication, non-encapsulated material was removed by molecular sieve chromatography on Sephadex G-75. Excitation and emission wavelengths were 278 and 545 nm respectively, while a high-pass cutoff filter (>490 nm, a 3–71 filter, Corning, Corning, NY) was placed before the emission monochromator to minimize contributions from light scattering. The fluorescence scale was calibrated so that 0% and 100% leakage corresponded to the Tb/DPA fluorescence intensities in the original vesicles and in the presence of 0.2 % Triton X_{100}, respectively. The measurements were done in an ISS spectrofluorometer.

2.6. Fusion measurements

Lipid mixing assays were performed following a modification of the method of Struck et al. [18]. Two liposomes population were prepared with either 1.0 mol% of NBD-PE (donor) or 2.0 mol% of Rh-PE (acceptor). Lipid mixing between both populations resulted in a decrease of the relative N-NBD-PE fluorescence due to resonance energy transfer between the two probes. The fluorescence of a third liposome population containing 0.5 and 1 mol% of NBD-PE and Rh-PE respectively was taken as a control for 100% of fusion. The fluorescence emission of N-NBD-PE was monitored at 530 nm with an excitation wavelength of 460 nm. The percent of fusion was calculated according to the following equation:

$$\%\text{Fusion} = [(F_t - F_0)/(F_\infty - F_0)] \times 100 \quad (1)$$

where F_t is the fluorescence intensity at time t, F_0 is the fluorescence at time 0 and F_∞ is the final fluorescence determined with the "mock fused" vesicles.

2.7. Sequence and structure analysis

In order to detect the presence of any putative conserved sequence motif related to fusion among GAPDH, GDH, SDH and ADH the following procedure was applied. First, homologue sequences for each protein were retrieved from UniprotKB sequence database using PSI-BLAST [19]. Multiple sequence alignments were built using CLUSTALX [20], and Hidden–Markov profiles [21] were extracted using the HMMER package and compared to each other with LogoMat-P [22]. Alternatively, conserved motifs among each group were extracted using MEME System [23] and compared using LAMA software [24]. With the aim of searching any common structural motif, protein structure comparison was performed using OPAAS web server [25].

2.8. Electrostatic potentials and free energies calculations

Both, electrostatic potential and free energies were obtained from a modified version of the DelPhi program [26] that solves the nonlinear Poisson–Boltzmann equation for protein/membrane systems [27]. In the calculations described in this work, protein molecule was represented in atomic detail, mixed phosphatidylcholine:phosphatidylserine bilayers