# The amphiphilic character of glycogenin

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Received 18 October 2001; revised 7 November 2001; accepted 7 November 2001

First published online 22 November 2001

Edited by Thomas L. James

**Abstract This study describes for the first time the amphiphilicity of the protein moiety of proteoglycogen. Glycogenin but not proteoglycogen associates to phospholipid vesicles and forms by itself stable Gibbs and Langmuir monolayers at the air–buffer interface. The adsorption free energy (−6.7 kcal/mol) and the glycogenin collapse pressure (47 mN/m) are indicative of its high surface activity which can thermodynamically drive and retain the protein at the membrane interface to a maximum equilibrium adsorption surface pressure of 21 mN/m. The marked surface activity of glycogenin is further enhanced by its thermodynamically favorable penetration into zwitterionic and anionic phospholipids with a high cut-off surface pressure point above 30 mN/m. The strong association to phospholipid vesicles and the marked surface activity of glycogenin correspond to a high amphiphilic character which supports its spontaneous association to membrane interfaces, in which the de novo biosynthesis of glycogen was proposed to initiate. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Glycogenin; Proteoglycogen; Glycogen; Amphiphilicity; Surface activity; Surface pressure

## 1. Introduction

Glycogenin, the autoglucosyltransferase that initiates the polymerization of glucose to prime the de novo biosynthesis of glycogen [1–4] is found at the end of the biosynthetic pathway as a constituent of the newly synthesized proteoglycogen molecule. The first evidence for protein covalently bound to glycogen was described in the trichloroacetic acid-soluble proteoglycogen fraction of bovine retina [5]. A trichloroacetic acid-insoluble proteoglycogen fraction was also characterized [6], which behaved as a biosynthetic precursor of glycogen [7]. This acid-insoluble polysaccharide fraction was found firmly associated with retina membrane vesicles, leading us to postulate that its protein moiety is bound to membranes during the initial polymerization of glucose. The membrane bound proteoglycogen would dissociate from membranes after further glucosylation and growth of its polysaccharide moiety [7]. These results were consistent with the association of newly formed glycogen particles to elements of the smooth endoplasmic reticulum, that had been revealed in rat liver by electron microscopic radioautography using a labeled glycogen precursor [8].

In the present work we show the amphiphilic character of glycogenin, which associates with lipid vesicles and monolayers, thus providing strong support to the proposal of a membrane-associated initiation of the de novo glycogen biosynthesis [7].

## 2. Materials and methods

### 2.1. Materials

Vector pTYB1 and chitin columns were from New England Biolabs, Beverly, MA, USA. Phospholipids were from Avanti Polar-Lipids Inc., Albaster, AL, USA. Na [$^{125}$I]I (carrier free; 17.4 Ci/mg) was from Comisión Nacional de Energía Atómica, Buenos Aires, Argentina. Isopropylthiogalactopyranoside (IPTG), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), N$\alpha$-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), $\alpha$-amylase, glucoamylase, leupeptin, pepstatin and Sephacryl S-500 were from Sigma-Aldrich, St. Louis, MO, USA.

### 2.2. Isolation of retina proteoglycogen and preparation of recombinant rabbit muscle glycogenin

The acid-soluble proteoglycogen fraction was isolated from bovine retina as described before [6]. In order to obtain recombinant glycogenin, primers (forward primer, 5′-GGTGGTCATATGACAGATCAGGCCTTT-3′; reverse primer, 5′-GGTGGTTGCTCTTCCGCACTGGAGGT-AAGTGTCAAG-3′) containing an *Nde*I site (upstream) and a *Sap*I site (downstream) were used to polymerase chain reaction amplify rabbit muscle glycogenin cDNA for in frame insertion upstream of the intein/chitin binding domain encoding sequence in the vector pTYB1. The resultant plasmid, which was free of mutations in the glycogenin coding region based on DNA sequencing, was then transformed into *Escherichia coli* strain ER2566. Typically, 1 l of culture was grown at 37°C to optical density values of 0.6–0.8, then induced with 0.3 mM IPTG for 3 h at 30°C. The cells were collected by centrifugation, resuspended in 20 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl, 0.01% Triton X-100 and 20 μM PMSF and sonicated. The clarified lysate was passed through a chitin column (20 ml) at a flow rate of 0.5 ml/min. The column was then washed with 250 ml of 20 mM Tris–HCl buffer/0.5 M NaCl (column buffer), pH 8.0, and treated with 70 ml of column buffer containing 50 mM DTT at 2 ml/min. After 24–48 h standing at room temperature, glycogenin was eluted with the column buffer, and dialyzed against 20 mM Tris–HCl buffer, pH 7.5, at 4°C to remove the excessive DTT. The recovered glycogenin was active for auto- and transglucosylation and showed only a 38 kDa Coomassie-blue stainable band when analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; results not shown).

### 2.3. Preparation of $^{125}$I-labeled retina proteoglycogen and isolation of $^{125}$I-labeled glycogenin

The retina proteoglycogen fraction was iodinated with [$^{125}$I]iodide as described before [6]. The radioiodinated proteoglycogen (270 μg in glycogen) was subjected to amylolysis for 48 h in a system containing

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*Abbreviations:* IPTG, isopropylthiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TLCK, N$\alpha$-*p*-tosyl-L-lysine chloromethyl ketone; PC, phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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PII: S0014-5793(01)03192-1

0.86 μg of α-amylase, 0.65 μg of glucoamylase, 1 mM $CaCl_2$, 100 mM sodium acetate buffer, pH 5.0, 1.0 mM TLCK, 60 μM PMSF, 0.05% sodium azide, and 20 μg/ml each of leupeptin and pepstatin, in a total volume of 125 μl. Under these conditions the released glycogenin was insoluble, thus allowing its separation from the amylolytic enzymes by centrifugation for 60 min at 100 000 × *g*. The precipitated protein was solubilized in 50 mM ammonium bicarbonate and showed a single radioactive band corresponding to $^{125}$I-labeled glycogenin by SDS–PAGE.

### 2.4. Preparation of vesicles of phosphatidylcholine in the presence of labeled glycogenin or proteoglycogen

The preparation of lipid vesicles was carried out essentially as described elsewhere [9]. Briefly, a solution of egg phosphatidylcholine (PC) in chloroform (16.5 mg in 165 μl) was dried as a thin film in a conical tube by rotatory evaporation under water aspirator vacuum and submitted to high vacuum for 3 h. The dried lipid was hydrated in 0.6 ml of 0.1 M sodium phosphate buffer pH 7.5 containing 0.02% sodium azide, at 30°C for 3 h. The lipid vesicles were obtained by sonication for 10 min (40 W output), kept overnight at 4°C and the suspension centrifuged at 1000 × *g* for 3 min prior to gel filtration (see below). Where indicated, labeled glycogenin (0.14 μg) or proteoglycogen (0.17 μg in protein; 11 μg in glycogen) was included in the buffer–azide solution for hydration.

### 2.5. Gel filtration

The association of labeled glycogenin with lipid membranes was ascertained by passage of the vesicles suspension through a column of Sephacryl S-500 (10 ml), equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.5, containing 0.02% sodium azide, at a rate of 3 ml/h. Fractions (0.5 ml) were collected and monitored for lipid vesicles (absorbance at 450 nm) and radioactivity.

### 2.6. Monomolecular films

Spread phospholipid monolayers and protein films formed by adsorption or spreading were prepared in a specially designed apparatus as previously described [10]. Surface pressure- and surface potential-molecular area compression isotherms were performed in one compartment (76 ml of subphase, 90 $cm^2$ of surface area) of the Teflon through filling with 20 mM Tris–HCl buffer, pH 7.5, and 150 mM NaCl. Measurements were automatically recorded as described elsewhere [11]. Surface potential was measured with a high impedance millivoltmeter through a surface ionizing electrode plate ($^{241}$Am) and a calomel reference in the subphase [10]. In the lipid monolayer penetration experiments the amount of protein injected in the subphase buffer (17 ml, 18 $cm^2$ of surface area) was 13.7 μg (in 50 μl) in all adsorption experiments. The molecular weight taken for glycogenin was 38 kDa.

## 3. Results and discussion

### 3.1. Association of glycogenin to bilayer phospholipid vesicles

From previous results in bovine retina it was proposed that the biogenesis of glycogen starts on membrane-associated glycogenin to form a trichloroacetic acid-insoluble proteoglycogen precursor. Growing of the polysaccharide moiety of the precursor would result in the dissociation from membrane and formation of the mature proteoglycogen form [7]. This suggestion implies that the glycogenin constituent of the mature

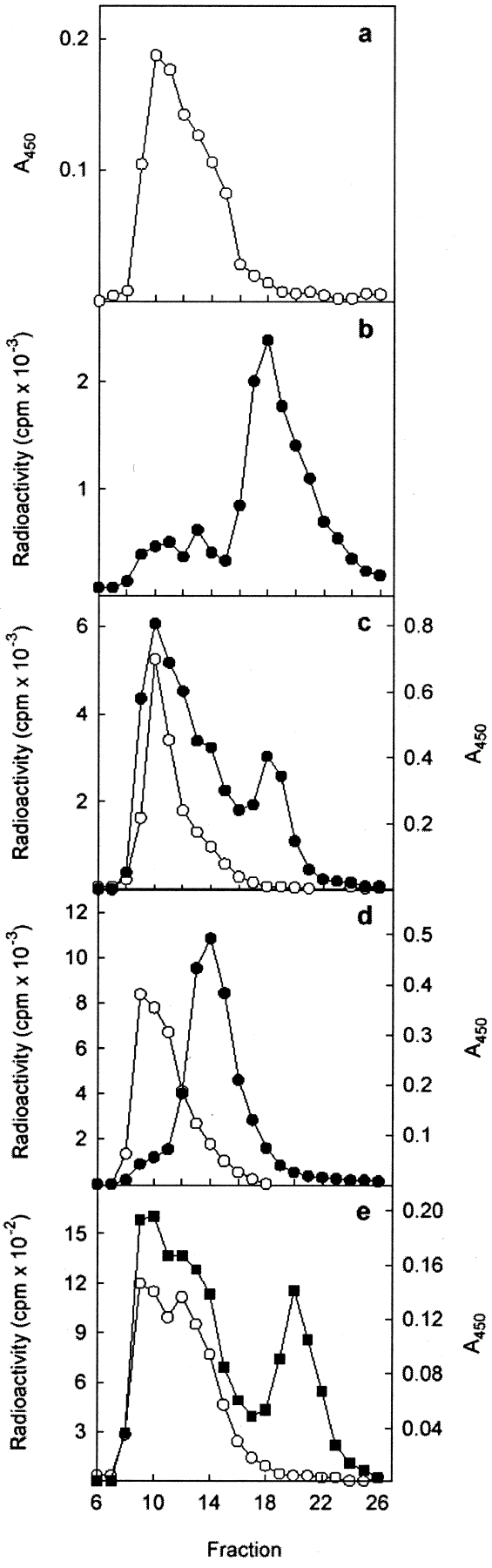


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Fig. 1. Gel filtration in Sephacryl S-500 of $^{125}$I-labeled glycogenin associated with phospholipid vesicles. The lipid vesicles were prepared as indicated in Section 2, in the absence (a), or in the presence of (c) $^{125}$I-labeled glycogenin (64 000 cpm), or (d) $^{125}$I-labeled proteoglycogen (65 000 cpm); (b) labeled glycogenin (75 000 cpm) in the absence of vesicles; (e) the same as in (c) with the following exceptions: half the amount of labeled glycogenin was present during vesicles formation and before the passage through Sephacryl the vesicles suspension was made 1 M NaCl and the column was equilibrated and eluted with the buffer–azide solution containing 1 M NaCl.

acid-soluble proteoglycogen should have, under the non-glucosylated state, the amphiphilic properties required for its association with membranes.

The amphiphilicity of retina glycogenin was examined by assessing its elution profile on Sephacryl S-500 after attempting to associate the labeled retina protein with phospholipid vesicles. In the absence of protein, the bulk of the unilamellar lipid vesicles prepared to test for association was excluded from the column (Fig. 1a). Glycogenin, in the absence of lipid vesicles, was included in the column and eluted well separated from the vesicles (Fig. 1b). The recovery of labeled glycogenin from the column was poor (26%) contrasting with a better recovery (76%) when associated with the lipid vesicles (see below), suggesting that it sticks to the column matrix or aggregates on the top of the column. When glycogenin was included in the hydration step during the formation of the vesicles, the labeled protein predominantly coeluted (80% of recovered radioactivity) with the vesicles (Fig. 1c). The greater recovery (76%) and the pattern of elution of glycogenin under these conditions clearly indicated its association to the vesicles. No association with phospholipid was observed when the glycogen-free glycogenin was replaced by proteoglycogen glycogenin during vesicles formation (Fig. 1d). The stability of the glycogenin-phospholipid association to high ionic strength (Fig. 1e) was consistent with hydrophobic effects being fundamental in the lipid–protein interaction.

### 3.2. Surface activity of glycogenin

The adsorption of rabbit muscle glycogenin from the subphase as a Gibbs monolayer was monitored by the increase of surface pressure as a function of time (Fig. 2). When injected into the subphase under continuous stirring, at a concentration of 200 nM, the protein induced a rapid increase of the surface pressure, reaching 14 mN/m in 1.2 min and a concomitant variation of 280 mV in the surface potential; the extrapolated value of surface pressure at infinite time is 21 mN/m (the changes were the same at 300 nM; at 100 nM the final increase in surface pressure was within 5 mN/m of the value shown in Fig. 2 but the adsorption kinetics was slower). The maximum surface pressure remained constant, indicating that a stable monolayer was formed. The adsorbed film could be compressed and decompressed reversibly between 1 mN/m and 47 mN/m by reduction and expansion

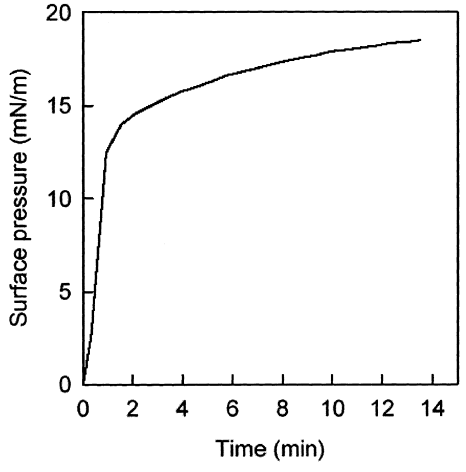


Fig. 2. Adsorption of glycogenin as a Gibbs monolayer. The protein concentration in the subphase was 200 nM.

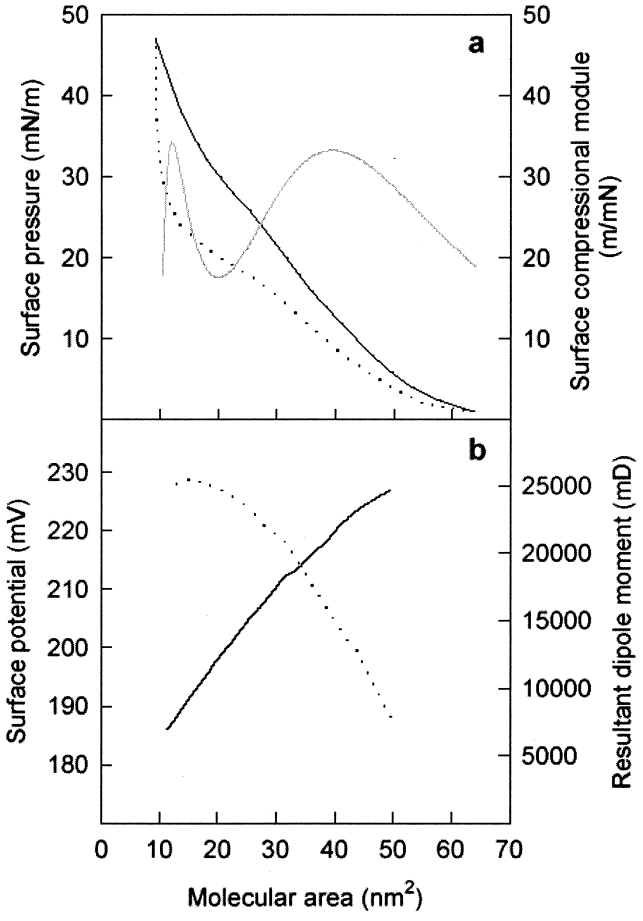


Fig. 3. Compression and decompression isotherms, surface potential and dipole moment of the glycogenin monolayer. a: Compression isotherm (black line) with the corresponding surface compressional modulus (gray line), and decompression isotherm (dotted line) of a Langmuir monolayer of glycogenin. b: Surface potential (dotted line) and the resultant dipole moment perpendicular to the interface (black line) of the compression isotherm shown in (a).

of the available area to the initial value, further indicating the high film stability. This behavior is comparable to that shown for the interfacial adsorption of membrane-active proteins and highly amphipathic peptides [12,13].

Glycogenin can also spread as a stable Langmuir monolayer directly from aqueous solution. The high surface stability of the protein film is reflected in the fact that surface pressure- and surface potential-molecular area isotherms are fully reproducible after expansion and recompression cycles (Fig. 3a). The hysteresis shown in the isotherms is common for membrane lipids and surface-active proteins [11,14,15]; in the case of glycogenin it amounts to a rather high compression free energy gap of 27.3 kcal/mol. This indicates that the protein intermolecular organization at the surface is capable, over the long range, of conserving information of the molecular state acquired initially under spreading, and after it has reached the compressed packing state. The monolayer collapse pressure is also very high, again revealing the remarkable surface activity of glycogenin, and occurs at about 45 mN/m with a cross-sectional molecular area of 10 nm$^2$ and a surface potential of about 230 mV. On the basis of the average diameter of a protein α-helix (1.3–1.5 nm, depending on amino acid side chains [16]), the closest packing area of

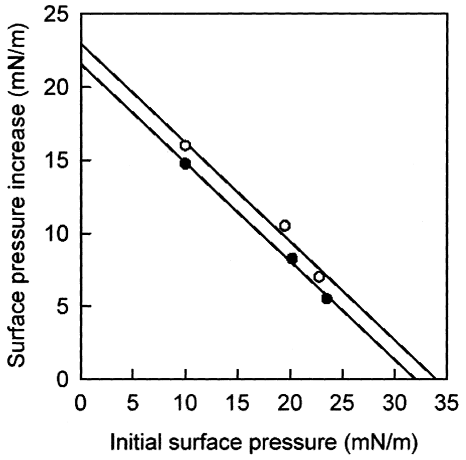


Fig. 4. Penetration of glycogenin in phospholipid films. Dependence of the surface pressure increase with the initial surface pressure (cut-off plot) induced by injection of 200 nM glycogenin beneath phospholipids monolayers, PC (●) and dilauroylphosphatidic acid (○).

glycogenin would correspond to a bundle of about six α-helical structures perpendicular to the interface. On the basis of the surface molecular density at 21 mN/m, the overall free energy of adsorption is −6.7 kcal/mol, a value comparable to that of highly membrane-active proteins [12,13,17]. A marked reversible rearrangement of the intermolecular organization of glycogenin occurs under compression between about 15 and 30 mN/m and molecular packing areas of 37 and 20 $nm^2$ (under expansion the reorganization is shifted to the left by about 8 $nm^2$ along the molecular area axis). The protein rearrangement is clearly marked by the variation of the surface compressibility modulus (Fig. 3a) whose maximum and minimum indicate the beginning and ending of the surface phase change, all within values corresponding to liquid-expanded states [18]. The change of surface organization is also evidenced, at approximately the same packing areas at which is found the inflexion point in the surface compressional modulus, by the variations of the tendency of the slope of the resultant dipole moment as a function of the molecular area (Fig. 3b). This is due to defined reorientation of the protein resultant dipole moment perpendicular to the interface, bearing a positive end pointing away from the hydrophilic (aqueous) subphase and into the hydrophobic (air) phase. Qualitatively, this is also similar to the intermolecular reorganization of membrane proteins in simple or complex monolayer interfaces [19].

### 3.3. Association of glycogenin to phospholipid monolayers

According to the Gibbs adsorption equation, increases of surface pressure indicate a stabilization of the surface (in proportion to the decrease in surface free energy, measured by the surface pressure increase) and are correlated to increases in the interfacial concentration of molecules that are incorporated in the interface [20]. Apart from showing self-surface activity, glycogenin is also capable of readily penetrating monolayers of liquid-expanded zwitterionic (egg PC) or anionic phospholipids (dilauroylphosphatidic acid), at initial surface pressures that are above the maximum equilibrium surface pressure attained by the protein adsorption to lipid-free interfaces (Fig. 4). Similar to the behavior of other membrane and soluble proteins able to penetrate lipid interfaces [11,12,17], for a given amount of glycogenin injected into the subphase the protein penetration is inversely dependent on the initial surface pressure of the lipid film. Again in accordance with its surface activity, the high value of surface pressure cut-off for glycogenin penetration in the phospholipid interface is in the entour of 30 mN/m, an average value likely to occur in biomembranes [21] with fluctuations that can be within ±15 mN/m depending on temperature and film compressibility [22].

The two independent approaches used in our work concur in demonstrating directly the amphiphilic nature and marked surface active properties of glycogenin, and provide further support of our proposal that glycogen biogenesis begins on membrane-associated glycogenin.

*Acknowledgements:* We thank Dr. P. Roach (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine) for the gift of the glycogenin clone. This work was supported by grants from CONICET, FONCyT, CONICOR and SECyT-UNC. M.E.C. was Fellow of SECyT-UNC and M.C.M., J.A.C. and B.M. are Career Investigators of CONICET.

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