Physicochemical Characterization of Progressive Changes in the *Xenopus laevis* Egg Envelope following Oviductal Transport and Fertilization†

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ABSTRACT: Previous studies have shown that the *Xenopus laevis* egg envelope exists in three forms with differing ultrastructural, macromolecular, and sperm penetrability properties. The coelomic envelope (CE) is derived from eggs released from the ovary into the body cavity of the female, the vitelline envelope (VE) from eggs which have passed through the oviduct, and the fertilization envelope (FE) from fertilized eggs. In the present study, the physicochemical characteristics of these three envelope types were differentiated. Investigation of envelope solubility, deformability, sulfhydryl reactivity, and hydrophobic dye and ferritin binding capacity demonstrated that profound physicochemical changes occur in envelope conversions CE → VE → FE. The physical strength of the envelopes, as evidenced by deformability studies, ranked FE > CE > VE. These differences were not accountable by differences in the number of disulfide bonds, although the CE sulfhydryl groups were significantly less accessible than those in the VE or FE. All three envelope forms were hydrophilic in nature, exhibiting little ability to bind 1-anilino-8-naphthalenesulfonic acid. The CE bound greater amounts of ferritin in comparison to the VE and FE, indicating the presence of a basic domain, presumably in the 43-kDa glycoprotein, which is lost upon proteolysis to 41 kDa during the CE → VE conversion. The envelope integrity of all three forms was maintained by both noncovalent and covalent (disulfide) bonds. Measurements of the effect of pH on envelope solubilization indicated the involvement of an ionizable group with pK_a of 8.0 in maintaining envelope structure.

The *Xenopus laevis* egg is surrounded by a glycoprotein envelope which exists in one of three forms according to the biological state of the egg. Coelomic envelopes are derived from body cavity eggs, vitelline envelopes from oviposited eggs, and fertilization envelopes from fertilized eggs. These forms have distinct ultrastructures (Grey et al., 1974, 1977; Yoshizaki & Katagiri, 1984; Larabell & Chandler, 1988, 1989), sperm penetrability properties (Grey et al., 1976, 1977), and macromolecular compositions (Gerton & Hedrick, 1986a,b).

Morphologically, the coelomic envelope (CE)[1] consists of a network of bundled fibers interconnected by smaller fibrils and filaments (Grey et al., 1977; Larabell & Chandler, 1989). As the coelomic egg passes through the oviduct, the bundled fibers of the CE are splayed apart into individual strands (Grey et al., 1977; Larabell & Chandler, 1988). In addition to the rearrangement of fibers, the prefertilization layer, an amorphous electron-dense layer, is deposited on the external face of the envelope (Yoshizaki & Katagiri, 1984). This restructuring of envelope morphology to form the VE is essential to fertilization as sperm are only capable of penetrating the VE (Grey et al., 1977).

At fertilization, the amphibian envelope undergoes yet another morphological transformation. Fertilization or parthenogenetic activation of the *Xenopus laevis* egg induces an exocytotic event referred to as the cortical reaction (Grey et al., 1974; Steinhardt et al., 1978; Elinson, 1980). As a consequence of this reaction, the envelope elevates away from the plasma membrane of the egg, and the fibers comprising the envelope segregate into multiple concentric layers (Larabell & Chandler, 1988). The entire envelope becomes encased by an electron-dense shell termed the fertilization layer (Grey et al., 1974; Larabell & Chandler, 1988). The FE thus formed is impenetrable to sperm and therefore serves as a block to polyspermy (Grey et al., 1976; Elinson, 1980). The FE is often referred to as a "hardened" envelope as it is relatively resistant to chemical and enzymatic degradation (Wolf et al., 1976; Urch & Hedrick, 1981). The developing embryo is thus suitably separated and protected from its external environment by this envelope barrier.

Differences in the macromolecular compositions of the CE, VE, and FE have been defined by Gerton and Hedrick (1986a,b). The *X. laevis* egg envelope is composed of six major glycoproteins ranging in size from 120 to 37 kDa. In the CE to VE conversion, a 57-kDa protein is added to the envelope, and a 43-kDa glycoprotein undergoes limited proteolytic processing to 41 kDa. In the VE to FE conversion, additional limited proteolytic events take place as a 69-kDa component is converted to a 66-kDa component and a 64-kDa component is converted to a 61-kDa component. A cortical granule lectin (539 kDa) and prefertilization layer components (>200 kDa) also become associated with the FE which results in the formation of the fertilization layer (Nishihara et al., 1986).

The purpose of this investigation was to further establish physicochemical differences between the CE, VE, and FE in order to better understand the mechanisms of egg envelope

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[1] Abbreviations: CE, coelomic envelope; VE, vitelline envelope; FE, fertilization envelope; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; ANS, 1-anilino-8-naphthalenesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFMS, trifluoromethanesulfonic acid.

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conversion. Physicochemical analyses included (1) solubilization behavior, (2) deformation in response to pressure, (3) reactivity of cysteinyl residues and their oxidation states, (4) hydrophobic dye binding, (5) anionic protein binding, and (6) chemical characterization of the CE to VE conversion process as it relates to the 43-kDa glycoprotein and its hydrolysis to 41 kDa.

Materials and Methods

Eggs. *Xenopus laevis* were purchased from South African Snake Farm (Fish Hoek, South Africa). Coelomic eggs were collected from females whose oviducts were ligated prior to the inducement of ovulation (Bakos, 1987). Oviposited and fertilized eggs were obtained as described by Gerton and Hedrick (1986b).

Envelopes. CE, VE, and Fe were obtained from coelomic, oviposited, and fertilized eggs according to methods described by Gerton and Hedrick (1986a,b). FE were also collected from oviposited eggs which had been parthenogenetically activated with 5 μM calcium ionophore A23187 (Gerton & Hedrick, 1986b).

Solubilization Behavior. CE, VE, and FE were suspended at a concentration of 0.5 mg/mL envelope protein in 20 mM sodium borate, pH 9.3 (unless otherwise indicated), and incubated for a period of 5 min at a series of temperatures ranging from 35 to 65 °C. A previous study (Bakos, 1987) determined that the solubilization end point is reached following 5-min incubation at a given temperature. After centrifugation (1500*g*) at 4 °C to pellet undissolved material, the intrinsic fluorescence of solubilized envelope protein was measured at excitation and emission wavelengths of 290 and 340 nm, respectively. All fluorescence values were recorded as a percentage of the maximum obtainable for each experiment. This normalization of fluorescence units allowed direct comparison of solubilization curves. The temperature at the midpoint of the transition curve was designated as the solubilization temperature (T_m). In order to determine the absolute amount of envelope protein solubilized at the end of each experiment, the supernatant solution (solubilized envelope protein) was separated from the pellet (undissolved envelope protein), and both fractions were evaporated to dryness. Each of the dried fractions was taken up in 70 μL of 0.1% SDS, heated to 100 °C for 10 min, and assayed for protein according to the fluorescamine method of Undenfriend (1962) using BSA as a standard.

Deformability Studies. The deformabilities of the CE, VE, and FE were measured in situ with a cell elastimeter according to the method of Drobnis et al. (1988). Briefly, eggs were placed in a Tris-buffered DeBoer's solution (10 mM Tris, 110 mM NaCl, 1.3 mM KCl, and 1.3 mM $CaCl_2$, pH 7.8) filled chamber created by a glass slide and a silicone grease supported coverslip. A glass micropipet (84.6-μm internal diameter) also filled with Tris-HCl-buffered DeBoer's solution, pH 7.8, was inserted at one end of the chamber. The pipet was connected via tygon tubing to a movable reservoir of additional buffer. With the use of a microscope and a micromanipulator, the pipet was brought into contact with an egg and gentle suction applied such that the egg was drawn onto the end of the pipet, with the envelope of the egg bulging slightly up into the pipet. Gradual increments of negative hydrostatic pressure (suction) were applied to the egg, and the extent to which the envelope was drawn into the pipet was recorded by videomicroscopy and measured from the televised image. The microscopic image of a stage micrometer was also video-recorded in order to convert centimeter distances measured from the televised image to micrometers. A manometer column was used to measure the negative pressure applied to the egg. The slope of a plot of envelope deformation vs increasing negative hydrostatic pressure [μm $(10^3$ dyn$)^{-1}$ cm^{-2}] was used as a measure of envelope deformability.

Sulfhydryl Titration. Envelopes were suspended in 20 mM sodium borate buffer, pH 8.2, at a protein concentration of 0.4 mg/mL as determined by the method of Lowry et al. (1951). The protein content of envelopes determined by the Lowry method using BSA as a standard was identical with that obtained by amino acid analysis (Wolf et al., 1976). Quantitation of total envelope sulfhydryl was carried out by adding SDS and DTT to final concentrations of 0.1% and 3.0 mM, respectively. The envelopes were heated at 100 °C for 2 min and allowed to cool at room temperature for 2 h. [^{14}C]Iodoacetic acid (23 mCi/mmol) previously diluted 25-fold with unlabeled iodoacetic acid was then added to a final concentration of 15 mM and the reaction allowed to proceed in the dark at 25 °C for 20 min. The reaction mixture was dialyzed extensively against borate buffer prior to determination of envelope protein specific activity.

The total number of accessible sulfhydryl groups (cysteinyl and cystinyl residues) in native envelopes was determined by incubating the initial suspension of envelopes in 3.0 mM DTT at room temperature for 2 h. [^{14}C]Iodoacetic acid was added as before, and the reaction mixture was dialyzed and then heated at 100 °C in the presence of 0.1% SDS for 2 min.

The total number of accessible free sulfhydryl groups in native envelopes was determined by reaction with [^{14}C]iodoacetic acid at 25 °C for 20 min. The reaction mixture was subsequently dialyzed against borate buffer and denatured with SDS, and the specific activity of envelope protein was determined.

Hydrophobic Dye Binding. Envelopes were solubilized in 20 mM sodium phosphate, pH 9.0, by heating at 60 °C for 10 min. The pH of the solution was adjusted to 7.0 and the protein concentration to 2.5×10^{-6} M (utilizing an average component molecular weight of 48K). Aliquots of ANS dissolved in sodium phosphate buffer, pH 7.0, were added, and the fluorescence of bound ANS was measured at excitation and emission wavelengths of 370 and 470 nm, respectively. Concentrations of ANS stock solutions were determined spectrophotometrically using a molar absorptivity of 4.95×10^3 M^{-1} cm^{-1} at 350 nm (Weber & Young, 1964). As it was not possible to saturate the envelopes with ANS under practical experimental conditions, the fluorescence yield of protein-bound ANS was determined by titrating a solution of BSA to the point of ANS saturation. The maximum fluorescence value so obtained was divided by the known concentration of ANS binding sites on BSA (Weber & Young, 1964). This factor was then used to determine the relative amount of ANS that bound to the envelopes.

Anionic Protein (Ferritin) Binding. Approximately 250 envelopes were placed in 550 μL of 0.25 M Tris-HCl, pH 6.8. A 50-μL aliquots of the suspension was withdrawn for protein quantitation. Horse spleen ferritin was added to the remaining envelope suspension to a final concentration of 10 mg/mL. The mixture was incubated for 1 h at room temperature. Unbound ferritin was removed from the envelope suspension by six cycles of centrifugation (1500*g*) and washing with Tris buffer. The rinsed envelope pellet was taken up in 50 μL of water, and the amount of ferritin that associated with the envelopes was determined by using the ferrozine colorimetric assay for iron (Tietz, 1976). Varying amounts of a commercially prepared iron standard solution (Sigma Chemical Co., St. Louis, MO) were analyzed in order to determine the

range of linearity of the assay. A standard curve relating nanomoles of Fe to micrograms of ferritin was also prepared.

Purification and S-Carboxamidomethylation of the 43- and 41-kDa Glycoproteins. The glycoprotein components of the CE and VE were separated by SDS–PAGE according to the method of Laemmli (1970) in 6 cm × 8 cm × 0.075 cm slab gels for analytical purposes or in 10 cm × 12.5 cm × 0.3 cm slab gels for preparative purposes. Electrophoresis was performed in separating gels of 8% acrylamide using a constant current of 12 mA per analytical gel or 40 mA per preparative gel. The envelope components were visualized in the analytical gels with Coomassie R-250 staining (Fairbanks et al., 1971). The location of the separated components in the preparative gels was determined by UV visualization of dansylated components, prepared according to the protocol of Seiler (1970) and run in marker lanes on both sides of the gel. The 43- and 41-kDa glycoproteins were electroeluted from preparative gels of the CE and VE, respectively, as described by Gerton et al. (1982). In order to rid the electroeluted components of contaminating SDS, Extracti-gel D (Pierce Chemical Co., Rockford, IL) was added to the glycoprotein-containing eluant (400 μL of Extracti-gel D suspension/mL of eluant). The mixture was gently shaken for 10 min and centrifuged to separate the Extracti-gel from the eluant. Tris and glycine were subsequently removed from the eluted glycoproteins by gel filtration using a Bio-Gel P-10 column equilibrated with 0.1% v/v triethylamine adjusted to pH 8.0 with formic acid.

The 43- and 41-kDa glycoproteins were reduced with dithiothreitol and S-carboxamidomethylated with iodoacetamide in order to promote unfolding of the polypeptide backbone and facilitate subsequent digestions by carboxypeptidase P and endoglycosidase F. The lyophilized components (1.5 mg) were denatured for 1 h at 50 °C in 500 μL of 0.5 M Tris-HCl, pH 8.2, containing 6 M guanidine hydrochloride and 2 mM EDTA. N_2 gas was bubbled through the solutions for 20 min, and all subsequent reactions were carried out under nitrogen. DTT (380 μg) was added, and the samples were stirred for 60 min. Iodoacetamide (1.26 mg) was then added and the mixture reacted for 20 min in the dark. The alkylation was quenched by the addition of 1.5 μL of 2-mercaptoethanol. The glycoproteins were separated from excess reagents by chromatography through a Bio-Gel P-10 column equilibrated with 0.1% v/v triethylamine adjusted to pH 8.0 with formic acid.

Amino Acid Analysis of Envelope Glycoproteins. For amino acid analysis, 10-μg aliquots of S-carboxamidomethylated 43- and 41-kDa glycoproteins were lyophilized and hydrolyzed in vacuo in 500 μL of 5.7 N HCl for 24, 48, or 72 h at 107 °C. The amino acids in the hydrolysate were quantitated with a Beckman 6300 automated amino acid analyzer. Values for serine and threonine were obtained by extrapolating the time-course hydrolyses to zero time, while the values for isoleucine and valine were obtained from their 72-h values. Cysteine was determined as (carboxymethyl)cysteine.

Hexosamine Analysis of Envelope Glycoproteins. S-Carboxamidomethylated 43- and 41-kDa glycoproteins (30 μg) were hydrolyzed in vacuo in 500 μL of 4 N HCl at 100 °C for 12 h prior to analysis using a Beckman 121M amino acid analyzer (Johnston et al., 1989).

Chemical Deglycosylation of Envelope Glycoproteins. Glycosidic moieties present on the S-carboxyamidomethylated 43- and 41-kDa glycoproteins were cleaved with trifluoromethanesulfonic acid according to the protocol of Karp et al. (1982).

Enzymatic Deglycosylation of Envelope Glycoproteins. Endo-β-*N*-acetylglucosaminidase F (endoglycosidase F, New England Nuclear, Boston, MA) was used to remove N-linked oligosaccharides from S-carboxamidomethylated 43- and 41-kDa glycoproteins. The lyophilized glycoproteins (30 μg) were solubilized in 30 μL of 100 mM sodium phosphate buffer, pH 6.1, containing 50 mM EDTA, 1.0% NP-40, and 0.3% SDS, by incubating at 25 °C for 1 h. One unit of endoglycosidase F was added and the mixture incubated at room temperature for 12 h. Another unit of enzyme was added and the digestion continued for an additional 12 h. The reaction mixture was frozen until analysis by SDS–PAGE.

Carboxypeptidase Digestion of Envelope Glycoproteins. Reduced and S-carboxamidomethylated 43- and 41-kDa components (200 μL) were digested with 0.2 μg of carboxypeptidase P (Boehringer-Mannheim, Indianapolis, IN) and carboxypeptidase Y (Pierce Chemical Co., Rockford, IL) in 250 μL of 0.03 M acetic acid adjusted to pH 4.2 with pyridine. At 0, 2, 5, 10, and 15 min, 50-μL aliquots of the digests were withdrawn and placed in a boiling water bath for 5 min. The samples were centrifuged under vacuum to dryness and taken up in 600 μL of 0.3 N lithium citrate buffer, pH 2.2, containing 3.75% sulfosalicylic acid and 40 μM *S*-(4-pyridylethyl)cysteine. The precipitated protein was removed by centrifugation, and the supernatant solution containing released C-terminal amino acids was applied to a Beckman 6300 amino acid analyzer.

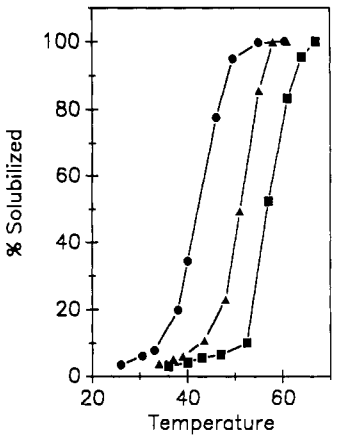

FIGURE 1: CE, VE, and FE solubilization curves. CE (▲), VE (●), and FE (■) were incubated in 20 mM sodium borate buffer, pH 9.3, at a concentration of 0.5 mg/mL envelope protein for 5 min at the indicated temperatures. The extent of envelope solubilization was determined by measuring the intrinsic fluorescence of the solubilized envelope protein.

Table I: Summary of *Xenopus laevis* Egg Envelope Properties

property	envelope stage CE	VE	FE
T_m (°C)[a]	51	42	56
deformability [μm (10^3 dyn)$^{-1}$ cm^{-2}]	1.35	2.51	0.15
accessible sulfhydryl groups (nmol/mg of protein)	92	140	130
ANS affinity (K_d × 10^{-7} M)	7.3	47	74
ANS binding sites (mol/mol of protein)	0.43	0.51	0.65
ferritin binding (μg/mg of protein)	275	59	41

[a] In 20 nM sodium borate, pH 9.3.

RESULTS

Solubilization Behavior. Figure 1 illustrates the solubilization curves obtained for the CE, VE, and FE in 20 mM sodium borate, pH 9.3. The curves are steeply sigmoidal, indicating that the transition of the envelopes from an insoluble to soluble state is a highly cooperative process. The curves are also symmetrical, possessing only one inflection point,

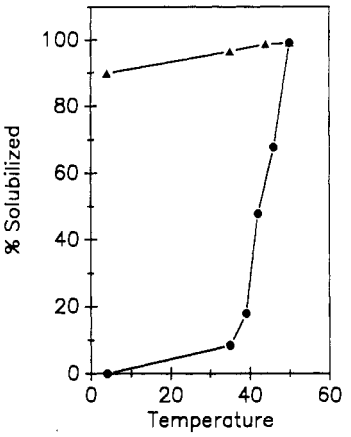

FIGURE 2: Reversibility of VE solubilization. (●) VE solubilization curve. VE were incubated in 20 mM sodium borate, pH 9.3, at a concentration of 0.5 mg/mL envelope protein for 5 min at each of the indicated temperatures. The extent of envelope solubilization was determined by measuring the intrinsic fluorescence of the solubilized envelope protein. (▲) VE renaturation curve. Heat-solubilized VE were incubated for 5 min at a series of decreasing temperatures. The amount of envelope protein remaining in solution was determined fluorometrically.

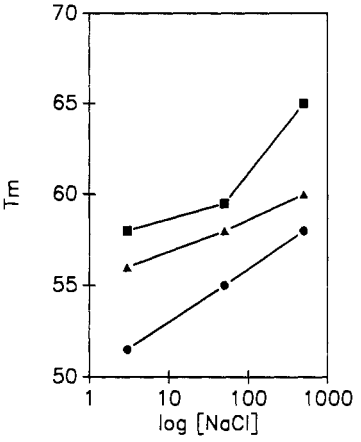

FIGURE 3: Effect of NaCl concentration on the T_m of egg envelopes. Suspensions of CE (▲), VE (●), and FE (■) were incubated in 20 mM sodium borate buffer, pH 9.3, containing 3, 50, or 500 mM NaCl for 5 min at a series of increasing temperatures until envelope dissolution was complete. The temperature at which 50% dissolution had occurred (T_m) was recorded and plotted as a function of the log of the concentration of NaCl.

defined as the T_m, the temperature at which the transition is 50% complete (Table I). The shape of the solubilization curves reflected a simple two-state transition, A → B, in which no conformational intermediates between the initial insoluble and final soluble states were significantly populated. Gerton and Hedrick (unpublished data) have shown that the solubilized envelope material is a high molecular weight complex, >200K, which retains all of the envelope components. The envelopes therefore represented a unified polycomponent system in which the states of all the elements were mutually connected.

The results of an experiment to determine the extent to which envelope solubilization was reversible are shown in Figure 2. It was apparent that the dissolution process was irreversible as the dissolved envelopes remained soluble upon cooling.

By measuring T_m as a function of NaCl concentration, the effect of a neutral salt on the stability of native envelope structure was determined. Figure 3 illustrates the fact that increasing the ionic strength of the solubilization buffer increased the T_m of envelope melting. $CaCl_2$ was more effective

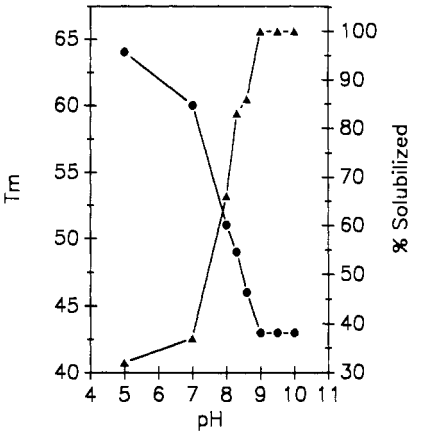

FIGURE 4: Effect of pH on VE solubilization. VE (0.5 mg/mL envelope protein) were incubated in 20 mM sodium acetate, pH 5.0, 20 mM sodium phosphate buffer, pH 7.0, or 20 mM sodium borate buffer, pH 8–10, for 5 min at a series of increasing temperatures. The T_m (●) and the percent soluble envelope protein (▲) were determined as described under Materials and Methods.

at increasing the T_m than NaCl at comparable ionic strengths. For example, the T_m's for the CE, VE, and FE were 56, 52, and 58 °C, respectively, in borate buffer containing 3 mM NaCl (μ = 0.013), in comparison to 58, 51, and 61 °C in borate buffer containing 1 mM $CaCl_2$ (μ = 0.013).

The influence of pH on VE solubilization was also investigated. Figure 4 indicates that changes in T_m mirrored changes in envelope solubility as a function of pH. The curves profiling changes in T_m and solubility as a function of pH both had inflection points at pH 8.0, indicating that a prototropic group with pK_a of about 8.0 was involved in envelope dissolution.

Envelope Deformation Studies. The deformability values of the CE, VE, and FE are recorded in Table I. The FE had the lowest deformability value; i.e., it exhibited the greatest resistance to the negative pressure within the pipet. The VE, in contrast, was drawn further into the pipet per given pressure than the FE; hence, it was considered to be the least stiff, or most deformable. The CE was intermediate between the FE and VE in terms of stiffness.

Sulfhydryl Titration Studies. The CE, VE, and FE denatured in SDS did not exhibit significant differences in total sulfhydryl content (cysteine and cystine). Values of 252 ± 5 (SD), 291 ± 7, and 271 ± 5 nmol of SH/mg of protein were obtained for the three classes of envelopes, respectively. These values were in excellent agreement with values of 295 nmol of SH/mg for the VE and 296 nmol of SH/mg for the FE as determined by amino acid analysis following performic acid oxidation (Wolf et al., 1976).

The total number of reactive sulfhydryl groups (cysteine and cystine) in the native envelope in the absence of SDS was determined to be 92 ± 3, 140 ± 4, and 130 ± 3 nmol of SH/mg of protein the for the CE, VE, and FE, respectively. The total number of accessible sulfhydryl groups in the CE was significantly lower ($p \leq 0.01$) from the total number of accessible sulfhydryl groups in either the VE or the FE. The amount of free sulfhydryl groups in native CE, VE, and FE was also determined. Values of 3.6 ± 0.1, 3.0 ± 0.1, and 5.4 ± 0.1 nmol of free SH/mg of protein were obtained for the three classes of envelopes, respectively, indicating that the majority of envelope sulfhydryl groups were in disulfide linkages.

ANS Binding Studies. The relative affinities of the CE, VE, and FE for ANS were determined in order to compare their hydrophobic properties. Figure 5 illustrates a typical

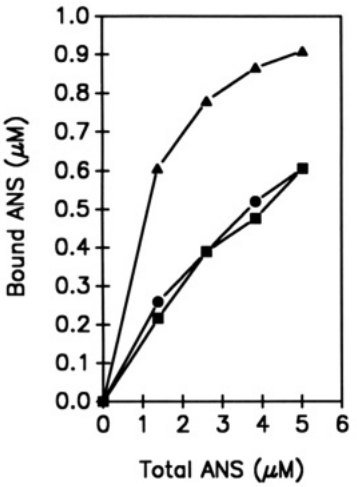

FIGURE 5: Binding of ANS to envelopes. Increasing amounts of ANS were added to a 2.5 μM solution of heat-solubilized CE (▲), VE (●), or FE (■), and the amount of envelope-bound ANS was determined by fluorometric analysis.

Table II: Amino Acid Composition of the 43- and 41-kDa Glycoproteins

amino acid	43 kDa (mol/mol of protein)[a]	41 kDa (mol/mol of protein)[b]
Asp	36	36
Thr	23	22
Ser	32	27
Glu	28	28
Pro	53	52
Gly	23	22
Ala	27	25
Val	35	32
Met	7	6
Ile	13	11
Leu	20	16
Tyr	9	8
Phe	13	13
Trp	ND[c]	ND
Lys	15	12
His	10	8
Arg	15	11
Cys	22	21
	total 380	total 350

[a] Compositional analysis based upon a molecular weight of 38K for the deglycosylated 43-kDa glycoprotein. [b] Compositional analysis based upon a molecular weight of 35K for the deglycosylated 41-kDa glycoprotein. [c] ND = not determined.

binding curve for each envelope type. It should be emphasized that the K_d and n values determined from this analysis (Table I) were only apparent values observed under the defined experimental conditions. Polycomponent systems such as the egg envelope undoubtedly contained a heterogeneous population of ANS binding sites. In the absence of a defined model for the number of sites and their interaction, our data simply represent an average of all classes of binding sites. The CE appeared to be more hydrophobic than the VE or FE as it possessed a higher affinity for ANS than the VE or FE; however, the VE and FE contained a greater number of ANS binding sites than the CE.

Ferritin Binding Studies. There were pronounced differences in the abilities of the CE, VE, and FE to bind ferritin. After incubation with 10 mg/mL ferritin, the CE appeared reddish brown in contrast to the other envelope types. The CE bound 275 μg of ferritin/mg of envelope protein, in comparison to the VE which bound 59 μg of ferritin/mg of envelope protein and the FE which bound 41 μg of ferritin/mg of envelope protein.

Table III: Hexosamine Composition of the 43- and 41-kDa Glycoproteins following Acid Hydrolysis[a]

	43 kDa		41 kDa	
	untreated	TFMS treated	untreated	TFMS treated
glucosamine	4.8	0.8	5.7	1.1
galactosamine	9.9	4.5	10.8	0.4

[a] Values expressed as moles of hexosamine per mole of protein.

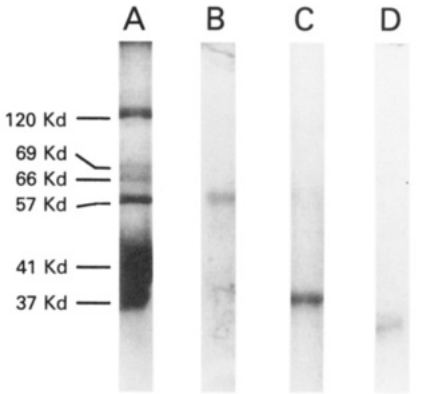

FIGURE 6: SDS–PAGE of TFMS deglycosylated components. Purified envelope components were treated with TFMS as described under Materials and Methods. SDS–PAGE was performed in separating gels of 8% polyacrylamide under reducing conditions. Envelope components were visualized by Coomassie Blue staining. (A) VE; (B) TFMS-treated 57 kDa; (C) TFMS-treated 43 kDa; (D) TFMS-treated 41 kDa.

Compositional Analysis of the 43- and 41-kDa Glycoproteins. The 43-kDa glycoprotein from the CE and the 41-kDa glycoprotein from the VE were chemically characterized in order to identify the nature of the 43- to 41-kDa conversion. Table II presents the amino acid compositions of the 43- and 41-kDa components. A notable feature associated with their compositions was the high percentages (12–13%) of proline and half-cystine (6%), which were, respectively, 3-fold and 2-fold higher than the average values calculated for globular proteins (Dayhoff & Hunt, 1972). The percentage of lysine (3–4%) which occurred in the 43- and 41-kDa components was half the amount typically found in globular proteins.

Table III summarizes the results of hexosamine analysis of the glycosylated (untreated) and deglycosylated (TFMS-treated) forms of the 43- and 41-kDa components. The hexosamine compositions of the glycosylated forms were nearly identical. Edge et al. (1981) reported that asparagine-linked *N*-acetylglucosamine is retained in TFMS-treated glycoproteins. The 43- and 41-kDa components accordingly each possess 1 N-linked saccharide unit, as each retains approximately one glucosamine residue following TFMS treatment and acid hydrolysis. A difference, however, appears among the galactosamine content of the TFMS-treated components. Edge et al. (1981) reported that TFMS treatment of glycoproteins results in the loss of both peripheral and serine/threonine-linked *N*-acetylgalactosamine residues, although the former are lost at a much faster rate than the latter. The association of 4.5 mol of galactosamine/mol of TFMS-treated 43-kDa glycoprotein suggests that, for perhaps steric reasons, the TFMS-catalyzed acidolysis of *N*-acetylgalactosamine residues in the 43-kDa glycoprotein proceeded at a slower rate than in the 41-kDa glycoprotein.

Effect of Chemical and Enzymatic Deglycosylation on the 43- and 41-kDa Glycoproteins. Figure 6 represents an SDS–PAGE analysis of the TFMS-treated components. The

43-kDa component had a deglycosylated molecular weight of 38K, while the 41-kDa component had a deglycosylated molecular weight of 35K. This fact indicated that the 43- and 41-kDa components contained polypeptides of differing lengths. Both glycoproteins contained approximately 15% carbohydrate by weight as determined by molecular weight differences before and after deglycosylation. Figure 6 also shows that the 57-kDa protein which was added to the CE in the oviduct was unaffected by TFMS treatment, and therefore was considered to be nonglycosylated.

The deglycosylation effects of endoglycosidase F were similar to those obtained by TFMS treatment. The 43- and 41-kDa components were reduced to molecular weights of 39K and 37K, respectively, suggesting that N-glycosidic linkages were present on both components.

Carboxypeptidase Digestion of the 43- and 41-kDa Components. The C-terminal sequence of each component was resolved following combined carboxypeptidase P and Y digestion as follows: 43-kDa component, -/(Ser, Leu)-Val-COOH; 41-kDa component, -/Leu-(Ser, Arg)-COOH.

Discussion

The physicochemical properties of the three egg envelopes, CE, VE, and FE, reported here clearly reflect their structural differences. The CE, VE, and FE were differentially affected by increases in temperature. The higher T_m's for the CE and FE relative to the VE indicated that more thermal energy was required to solubilize these envelopes in comparison to the VE. The inter- and intramolecular attractions between the CE and FE components must therefore be stronger and/or more numerous than those between the VE components.

Envelope dissolution was found to be markedly influenced by the addition of a neutral salt, such as NaCl, to the melting buffer. NaCl stabilized the native conformation of the envelope as its presence in the solubilization buffer increased the T_m's of the envelopes. The T_m's of the CE, VE, and FE increased to differing extents with respect to ionic strength, again illustrating differences in their macromolecular organizations.

The observed effects of salt on the suppression of envelope solubilization were in agreement with known mechanisms of protein stabilization and salting-out (Brandts, 1969; von Hippel & Schleich, 1969; Arakawa & Timasheff, 1982). The presence of salt inhibits solubilization by strengthening hydrophobic interactions, suppressing normal ionization, and driving the macromolecular conformation toward a minimum surface area. Since the FE was affected to a greater extent than the CE or VE by salt, one may posulate that it possesses either more hydrophobic bonds, more dissociable groups, and/or a greater surface area relative to the CE and VE. The fact that the FE possessed the highest T_m of all the envelope types further attested to the increased strength and number of inter- and intracomponent interactions in this envelope type.

Curves profiling the extent of VE solubilization in solutions of varying pH possessed inflection points at pH 8.0, indicating the involvement of an ionizable group(s) with a pK_a of about 8.0 in the melting process. In accordance with this observation, the following equation may be written:

$$\underset{\text{insoluble}}{\text{VE-XH}} \underset{pKa = 8.0}{\rightleftharpoons} \text{VE-X}^- + \text{H}^+ \xrightarrow{\text{thermal energy}} \underset{\text{soluble}}{\text{VE-X}^-}$$

At pH >8.0, the dissociation reaction was favored, as was VE dissolution. The functional group(s) ("XH") which dissociate(s) could be an imidazole, amino, or sulfhydryl entity. The effect of NaCl on envelope solubilization was in accordance with its effects on the ionization of dissociable groups. Solutions of high ionic strength are known to increase the pK_a of acids and decrease the pK_a of bases (Tanford, 1962). According to the above reaction, NaCl-induced suppression of ionization would also result in a suppression of solubilization.

The envelope deformation experiments showed that the deformability of the egg surface changed following exposure to oviductal fluid and again following fertilization. Resistance of the envelopes to deformation paralleled their resistance to dissolution upon heating. The ranking of envelopes according to both stiffness and T_m was VE < CE < FE. Since the VE is the only class of envelope penetrable by sperm, it is reasonable that they are also the least physically resistant envelope as the mechanical aspects of sperm penetration are greatly influenced by the viscoelastic character of the envelope (Green & Purves, 1984).

Postfertilization hardening of egg envelopes is a phenomenon occurring throughout the animal kingdom (Schmell et al., 1983). Hardening of the *Xenopus laevis* envelope has previously been defined on the basis of resistance to enzymatic and chemical dissolution (Wolf et al., 1976; Urch & Hedrick, 1981). The extreme stiffness of the FE reported in this paper is the first physical evidence for hardening of an amphibian egg envelope following fertilization.

Conversion of the envelope from one form to another did not result in a significant change in the total number of sulfhydryl groups. However, the CE did exhibit a significantly lower percentage of solvent-accessible sulfhydryl groups. Quantitation of the oxidation state of sulfhydryl groups in the CE, VE, and FE was undertaken in order to test the hypothesis that differences in the solubility and deformability of the three envelope classes are accountable on the basis of differences in the number of disulfide linkages. The CE, VE, and FE possessed equivalent numbers of reduced sulfhydryl groups (only 2–4% of available sulfhydryl groups were in the reduced state); therefore, other types of interactions, such as the various noncovalent forces discussed in relation to envelope solubilization, must account for differences in the physical behavior of the CE, VE, and FE.

The relative hydrophobicities of the CE, VE, and FE were ascertained by measuring ANS binding. If the envelopes are ranked according to their K_d for ANS, then CE < VE = FE. However, if they are ranked according to the number of binding sites for ANS, then CE < VE < FE. It is difficult to ascribe one envelope type as being more hydrophobic than another. The CE had a greater affinity for ANS by 1 order of magnitude when compared to the VE or FE, but had a lower number of binding sites. The strongest conclusion which may be drawn from these experiments was that all three envelope classes exhibited small differences in their interaction with ANS, a reflection of differences in the conformations of their solvent-accessible hydrophobic domains. However, the overall weak affinity of the envelopes for ANS indicated that they were generally hydrophilic in nature.

Quantitative analysis of ferritin binding to the three classes of envelopes also provided evidence for their structural uniqueness. Gerton and Hedrick (1986a) have shown that the p*I* of the 43-kDa component of the CE is shifted to a more acidic value as a result of its hydrolysis to 41 kDa in the CE to VE conversion. It is attractive to postulate that the basic domain which was lost from the 43-kDa glycoprotein during the conversion process was the portion of the CE responsible for binding ferritin. Our analysis of the 43- and 41-kDa conversion indicated that the domain which was lost originated from the C-terminal end of the polypeptide. As the N-ter-

minals of both components are blocked (Gerton, 1980), the hydrolytic event converting the 43-kDa component to 41 kDa likely involves cleavage of the C-terminal portion of the polypeptide. Support for this hypothesis relates to the fact that the C-terminals of both components are different.

The hexosamine compositions of the 43- and 41-kDa components were similar, indicating that significant amounts of carbohydrate were not lost from the 43-kDa component as a result of its conversion to 41 kDa. SDS–PAGE analysis of the TFMS-treated components indicated that they were composed of core polypeptides of differing lengths. The 43-kDa component was reduced to 38 kDa and the 41-kDa component to 35 kDa. Similar results were obtained following endoglycosidase F treatment. The 43-kDa component was reduced to 39 kDa and the 41-kDa component to 37 kDa.

In conclusion, taken together, these results provide evidence that the physicochemical characteristics of the *Xenopus laevis* egg envelope change upon exposure to oviductal fluid and again at fertilization and that these changes correlate with the morphological and macromolecular changes which also occur. It is remarkable that the limited molecular alteration of selected envelope components serves to modify the physicochemical behavior of the entire envelope. These alterations as they occur in the CE to VE conversion within the oviduct entail the addition of 57-kDa protein with the concomitant loss of a 2-kDa C-terminal polypeptide from the 43-kDa component and result in the modification of the CE to a form (VE) which exhibits increased solubility, deformability, and accessibility to disulfide bond reduction and hydrophobic dye binding. Modification of the envelope within the oviduct is a necessary prerequisite to fertilization and is brought about by an arginine-specific serine protease secreted by the pars recta portion of the oviduct (Hardy & Hedrick, 1989). The VE to FE conversion following fertilization also results in a major modification in the physicochemical characteristics of the envelope. The combined effects of adding cortical granule lectin and fertilization layer components to the envelope and altering the 69-kDa component to 66 kDa and the 64-kDa component to 61 kDa create a new envelope form (FE) which is extremely rigid and resistant to solubilization. These changes are well suited to the new role of the envelope as a block to polyspermy and as a protective shell for the developing embryo.

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