Vitellogenin and Lipovitellin: Zinc Proteins of *Xenopus laevis* Oocytes

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ABSTRACT: *Xenopus laevis* vitellogenin is a plasma protein that contains a total of 5 mol of metal/440 kDa dimer, 2 mol of zinc, and 3 mol of calcium (Montorzi et al. (1994) *Biochem. Biophys. Res. Commun. 200,* 1407−1413]. There are no other group IIB or transition metals in the molecule. The zinc atoms are removed instantaneously by 1,10-phenanthroline (OP) (p*K* 4.8). Once internalized by receptor-mediated endocytosis, vitellogenin is cleaved into multiple polypeptides, i.e., the two lipovitellin subunits (1 and 2) plus phosvitin; these are then stored as microcrystals within yolk platelets. We here show by metal analysis of the individual proteins generated by vitellogenin processing that zinc and calcium occur in different domains of the vitellogenin polypeptide chain. All of the vitellogenin zinc is present in lipovitellin, in amounts equal to 1 mol of zinc/141 kDa. Calcium, in contrast, is detected exclusively in phosvitin which, in addition, contains 3 mol of magnesium/35 kDa, apparently acquired following vitellogenin entry into the oocyte. The zinc in lipovitellin is removed by OP in a concentration-dependent manner with a p*K* of 4.8, identical to that obtained for vitellogenin, and by exposure to acidic conditions (below pH 5). Following removal of zinc, the two lipovitellin subunits remain associated, suggesting that zinc is not involved in their interaction. On exposure to 1% SDS, lipovitellin does dissociate into 106 and 33 kDa subunits. The presence of stoichiometric quantities of zinc in both vitellogenin and lipovitellin calls for the study of the hitherto unrecognized biochemistry and functions of these proteins in zinc metabolism and development of the frog oocyte and embryo.

Xenopus laevis oocytes take up zinc throughout all six stages of maturation until by stage VI the concentration is 1 mM (Nomizu et al., 1993). The major fraction of this accumulation correlates with vitellogenin uptake during stages III−VI (Wallace & Jared, 1968; Wallace et al., 1983). An examination of zinc in vitellogenin biochemistry confirms the validity of this conclusion. Vitellogenin contains both zinc and calcium (Montorizi et al., 1994). It is synthesized in the liver under the influence of estrogen, secreted into plasma, and then taken up by oocytes through receptor-mediated endocytosis (Wallace, 1978; Wallace et al., 1983; Wallace & Jared, 1968; Banaszak et al., 1991; Hansen & Riebesell, 1991); thereafter it undergoes proteolytic processing within cellular organelles (Banaszak et al., 1991). The cleavage products that derive from the N- and C-terminal regions of vitellogenin are the two subunits of lipovitellin and phosvitin which are stored within yolk platelets (Cook, 1961, 1968; Jared et al., 1973; Opresko et al., 1980a; Wall & Meleka, 1985; Wall & Patel, 1987; Wiley & Wallace, 1981; Butler, 1983; Griffin et al., 1984; Griffin, 1985). Using methods that prevent metal contamination, on the one hand, and their removal, on the other, we have purified both lipovitellin and phosvitin to homogeneity and determined their metal content by atomic absorption spectrometry. We find that the zinc atoms of vitellogenin are associated with its lipovitellin domains while calcium is present in that of phosvitin. The latter, in addition, contains 3 mol of magnesium/mol which are acquired during uptake and storage of the vitellogenin cleavage products within yolk platelets. Examination of the characteristics of zinc binding to both vitellogenin and lipovitellin with chelating agents reveals that 1,10-phenanthroline (OP) removes the zinc atoms from vitellogenin and lipovitellin in a concentration-dependent manner and with a nearly identical p*K*. This is consistent with the postulate that the zinc binding sites in both proteins are the same. Dialysis against 1 mM OP readily generates apovitellogenin and -lipovitellin. Removal of zinc from lipovitellin does not cause its dissociation into two subunits.

These results now permit the identification of the ligands that are the zinc binding sites and their coordination chemistry as well as their functional/structural role(s). It is now also possible, moreover, to elucidate the roles and distribution of this metal in *X. laevis* embryogenesis and metamorphosis.

MATERIALS AND METHODS

To prevent adventitious metal contamination, all plastic and glassware used for storage of buffers and preparation and analysis of metals were soaked overnight in 3 N HNO_3 and washed extensively with ultrapure water before use. Buffers and other solutions were treated routinely with Chelex 100 resin to remove metal contaminants (Falchuk et al., 1988).

Vitellogenin Purification and Characterization. Vitellogenin was purified as previously described (Kato et al., 1986; Montorzi et al., 1994). Its synthesis was induced by two injections of 4 mg of estrogen (Sigma, St. Louis, MO), each administered 7 days apart into the dorsal lymph sac of several female *X. laevis* frogs, 6−7 cm long, obtained from Xenopus I (Ann Arbor, MI). One week later the animals were sacrificed, blood was collected by cardiac puncture, and serum was obtained by centrifugation at 3000*g* for 10 min at 4 °C. Aliquots of serum were adjusted to contain 35%

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saturated $(NH_4)_2SO_4$, followed by centrifugation at 50000*g*. The supernatant was dialyzed against 25 mM Hepes, 1 mM PMSF, and 500 μM leupeptin, pH 7.5, and 1 mL aliquots were applied to a Mono-Q column, HR 5/5 (Pharmacia, Uppsala, Sweden), equilibrated with 25 mM Hepes, pH 7.5 at 22 °C. The sample was eluted with a linear gradient ranging from 0 to 0.5 M NaCl in 25 mM Hepes buffer, pH 7.5. One-milliliter fractions were collected at a flow rate of 2 mL/min, and the eluate was monitored at 254 nm. Leupeptin was added to the eluate to achieve a final concentration of 1 mM. The total amino acid composition and N-terminal amino acid sequence of the peak that elutes with 0.43 M NaCl were determined as previously described (Montorzi et al., 1994). The zinc content of the purified vitellogenin was analyzed by atomic absorption spectrometry. Its molecular weight was determined by SDS−PAGE.

OP removes the zinc of vitellogenin. The concentrations as well as the time of incubation required for its complete removal were determined by incubating aliquots of purified vitellogenin with solutions ranging from 10^{-6} to 10^{-3} M OP for 4 h. Each preparation was then rechromatographed on the Mono-Q column to separate OP from the protein. OP concentrations of 10^{-5}, 10^{-4}, and 10^{-3} M were selected to determine the time required for the removal of zinc from vitellogenin. Aliquots of the protein were incubated with the OP for periods ranging from less than 1 to 240 min. After incubation, the protein was applied once again to the Mono-Q column to separate the chelating agent from the protein. The protein−OP mixture is introduced into the column in less than 1 min, which defines the shortest period of exposure to the agent. In all cases, the eluted vitellogenin was analyzed for zinc and protein content.

Purification and Characterization of Lipovitellin and Phosvitin. Ovulation of stage VI eggs was induced by an initial injection of 50 IU of human chorionic gonadotropin (Sigma, St. Louis, MO) into the dorsal lymph sac, followed by a second injection of 500 IU of the hormone 60 h later. Ovulation normally occurred 12−14 h after the second administration of hormone. Eggs were either used immediately or stored in aliquots of approximately 300−500 eggs at 70 °C. The oocytes were suspended in 2% L-cysteine and 0.16 M NaOH for 2−3 min to dissolve their gelatin coat. They were washed extensively in 20 mM Hepes, pH 7.5, and then homogenized gently in 20 mM Hepes, 0.25 M sucrose, and 1 mM PMSF, pH 7.5. The homogenate was layered onto a 0.15 mL sucrose cushion (density 1.27 g/mL) and centrifuged at 750*g* for 5 min to obtain yolk platelets. The green yolk platelet layer was separated from the dark supernatant and resuspended in 5 volumes of homogenization buffer. Remaining cytosolic contaminants as well as other cellular components were removed from the yolk platelet preparation by repeated washing and centrifugation (3−4 times) until the supernatant layer was entirely clear after centrifugation. Aliquots of the yolk platelets were used to prepare fractions of their proteins, lipids, and pigment in order to determine the amount of zinc in each of the fractions. The isolated yolk platelets were dissolved in 2 volumes of 1.0 M NaCl and centrifuged at 10000*g* for 10 min to remove any insoluble material. Aliquots of solubilized platelets were delipidated by mixing the sample with chloroform/methanol (0.8:1:2) as described (Bligh & Dyer, 1959). The mixture was vortexed for 5 min after which time 1 part each of chloroform and water was added. Once the organic and aqueous phases separated, the protein in the aqueous phase was collected by centrifugation at 3000*g* for 10 min. Spectra of the organic phase material was obtained in a UV spectrophotometer in the absence and presence of mercaptoethanol. The latter was added to achieve a final concentration of 1%. The protein layer, easily removed from the organic/aqueous interphase, was dissolved in 1 mL of 15 N HNO_3 and hydrolyzed by boiling for 4 min. The zinc content of platelets and the protein hydrolysate as well as that of the chloroform and methanol phases was determined.

The major yolk platelet proteins, lipovitellin and phosvitin, were purified from stage VI eggs to identify the location of the zinc and calcium of the parent vitellogenin molecule. The platelets were purified as described above and were dissolved in 2 volumes of 1.0 M NaCl and 25 mM Hepes, pH 7.5, and centrifuged at 10000*g* for 10 min to remove any insoluble material. To separate the dissolved yolk platelet proteins, lipovitellin from phosvitin, 2 volumes of saturated $(NH_4)_2SO_4$ solution at 0 °C was added to the preparation. After centrifugation at 50000*g* in a Beckman SW 40 rotor for 60 min at 2 °C, the ammonium sulfate supernatant, containing phosvitin, was dialyzed overnight against metal-free 25 mM Hepes, pH 7.5. The lipovitellin pellet was resuspended in 5 volumes of 66% saturated $(NH_4)_2SO_4$ and recentrifuged. The process was repeated three times. Thereafter the pellet was resuspended in 3 volumes of 1.0 M NaCl and dialyzed against 1.0 M NaCl and 25 mM Hepes, pH 7.5. The solubilized product was then chromatographed on Sepharose 6B (120 × 0.7 cm) equilibrated with 1 M NaCl and 25 mM Hepes, pH 7.5. The absorbance of the eluate was recorded at 280 nm and the amino acid and metal contents of purified lipovitellin and phosvitin were determined. The lipovitellin subunits, 1 and 2, were separated by standard methods (Wallace 1963a,b, 1965; Hegenenauer et al., 1977; Ohlendorf et al., 1977; Wallace et al., 1990). Briefly, the protein was incubated in 1% SDS, boiled for 10 min, cooled, and then chromatographed on Sepharose 6B (120 × 1 cm). This procedure separates the individual subunits which can be identified on the basis of their N-terminal sequences.

The effect of chelating agents on the metal content of lipovitellin was determined. Aliquots of purified lipovitellin were precipitated by addition of 25 mM Hepes, pH 7.5, to reduce the NaCl concentration to 50 mM. The precipitates were incubated overnight with each of the following chelating agents at concentrations ranging from 10^{-6} to 10^{-2} M: 1,10-phenanthroline, ethylenediaminetetraacetic acid, dipicolinic acid, and pyridylazoresorcinol. The lipovitellin suspensions were then centrifuged at 10000*g* for 10 min, the supernatants collected, and the pellets hydrolyzed by boiling for 3 min in 1 mL of metal-free 15 N HNO_3. Samples were taken for zinc and protein analyses.

Apolipovitellin was obtained by overnight dialysis of the native protein against 1 mM 1,10-phenanthroline in 1 M NaCl and 25 mM Hepes, pH 7.5. The protein was then dialyzed for 48 h against 1 M NaCl and 25 mM Hepes, pH 7.5. Lipovitellin was reconstituted with zinc by dialyzing the apoprotein against 1 μM $ZnCl_2$, 1 M NaCl, and 25 mM Hepes, pH 7.5. The reconstituted protein was redialyzed against 1 M NaCl and 25 mM Hepes, pH 7.5. Again aliquots were taken for metal and protein analyses.

To examine the effect of zinc removal on the subunit interaction of lipovitellin, the intact lipovitellin was incubated

in 1 mM dipicolinic acid in 1 M NaCl and 10 mM Hepes, pH 7.5, and chromatographed on Sepharose 6B as above, followed by determination of the zinc content of each eluted fraction. The elution pattern of the holoprotein was compared to that of the individual lipovitellin 1 and 2 subunits, first separated as described above and then chromatographed on the same Sepharose 6B column. The fractions were also subjected to SDS−PAGE to determine the purity and molecular weight of the polypeptides. The N-terminal sequences of selected polypeptides identified their identities.

Additionally, the spontaneous dissociation of zinc from lipovitellin was examined by incubating the purified protein in buffers, selected on the basis of their pK_a values, adjusted to pHs ranging from 10.5 to 2.5. The buffers (pH) were phosphate (2.54), formate (3.75), succinate (4.21), citrate (4.76), succinate (5.64), MES (6.10), Hepes (7.50), and CAPS (10.4). In each case, the buffer was 50 mM and contained 1 M NaCl. After overnight incubation, the protein solutions were chromatographed on Sepharose 6B as described above. The eluted protein was collected and its metal and protein contents were determined.

Metal Analyses. Methods for metal analysis and the steps used to avoid spurious metal contamination during sample preparation have been described (Nomizu et al., 1993). Briefly, the samples were analyzed for zinc, iron, copper, manganese, nickel, magnesium, and calcium by atomic absorption spectrometry with a Perkin-Elmer 4100 ZL instrument equipped with a transverse heated graphite furnace. Since matrix modifiers were not added, the ashing (pyrolysis) and atomizing temperatures and ramp and hold speeds as well as sample volumes injected were optimized in each case to operate at maximum signals. The furnace was preheated at 80 °C to reduce drying time. Standards and samples were read twice and accepted when the relative difference between the two readings was less than 10% at a concentration higher than 2 ng/mL. Only standard curves with a correlation coefficient >0.99 were used in metal analysis.

Amino Acid Analyses and N-Terminal Sequences. Amino acid analyses were performed by HPLC separation and fluorescence detection of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate-derivatized amino acids generated from purified vitellogenin, lipovitellin, and phosvitin fractions after the samples were hydrolyzed with 6 N HCl for 20 h. Unhydrolyzed samples of purified vitellogenin and of the separated lipovitellin subunits 1 and 2 were applied to a DITC membrane and then subjected to N-terminal sequence analysis on a ProSequencer (Millipore, Bedford, MA).

Gel Electrophoresis. The molecular weights of purified protein subunits were estimated by SDS−PAGE and carried out at 40 mA. For vitellogenin preparations, a 6% stacking gel and a 9% resolving gel were used. For lipovitellin and phosvitin, 9% and 12% gels, respectively, were utilized. Gels were stained with 0.1% Coomassie blue, 50% methanol (v/v), 10% acetic acid (v/v), and 0.1 M $AlCl_3$.

RESULTS

Frog vitellogenin is purified in a two-step procedure by first separating it from serum proteins precipitated by 35% saturated $(NH_4)_2SO_4$ and then from the remainder by Mono-Q chromatography (Montorzi et al., 1994). The protein that elutes as a single peak from this column with 0.43 M NaCl is identified initially as vitellogenin on the basis of its characteristically higher serine content which is about 30% phosphoserine (Table 1). Vitellogenin is composed of two polypeptides that separate into two distinct bands migrating on SDS−PAGE as molecules of about 220 and 212 kDa, respectively. They are recognized as vitellogenin A and B on the basis of their N-terminal sequences (Table 2). The N-terminal sequences of the two isoforms differ by five amino acids. Vitellogenin contains one zinc per 220 kDa monomer but no other group IIIB or transition metal (Table 3) and 1.5 mol of calcium per monomer. Native plasma vitellogenin is a dimer which, hence, contains a total of 5 mol of metal, 2 mol of zinc, and 3 mol of calcium per molecule.

Table 1: Amino Acid Composition of *X. laevis* Vitellogenin, Lipovitellin, and Phosvitin

amino acid	vitellogenin (mol %)	lipovitellin (mol %)	phosvitin (mol %)
Asp	8.4	8.6	6.5
Glu	12.6	13.2	12.9
Ser	10.3 (3.2)[a]	7.2 (0.15)[a]	37.3 (25.5)[a]
Gly	5.0	4.8	2.8
His	2.9	2.9	4.8
Arg	5.1	5.4	6.6
Thr	5.4	5.4	1.0
Ala	9.0	9.5	3.4
Pro	4.8	5.1	6.6
Tyr	3.0	3.2	1.2
Val	5.9	6.2	0.8
Met	2.7	2.9	1.3
Ile	5.0	5.5	0.8
Leu	8.1	8.7	2.0
Phe	4.2	4.3	1.8
Lys	7.6	7.0	10.0

[a] Phosphoserine.

Table 2: N-Terminal Sequences of Vitellogenin and of Lipovitellin Isoforms

	vitellogenin			lipovitellin		
cycle	found	A[a]	B[b]	found	α[c]	β[d]
2	Glu	Glu	Glu	Glu	Glu	Glu
3	Arg, Lys	Arg	Lys	Arg, Lys	Arg	Lys
4	Thr, Ser	Thr	Ser	Thr, Ser	Thr	Ser
5	His, Gln	His	Gln	His, Gln	His	Gln
6	Ile, Tyr	Ile	Tyr	Ileu, Tyr	Ileu	Tyr
7	Glu	Glu	Glu	Glu	Glu	Glu
8	Pro	Pro	Pro	Pro	Pro	Pro
9	Val, Phe	Val	Phe	Val, Phe	Val	Phe
10	Phe	Phe	Phe	Phe	Phe	Phe
11	Ser	Ser	Ser	Ser	Ser	Ser

[a] Gerber-Huber et al., 1987. [b] Germond et al., 1984. [c] Ohlendorf et al., 1977. [d] Wiley & Wallace, 1981.

Table 3: Metal Content of Vitellogenin, Lipovitellin, and Phosvitin

metal	vitellogenin (mol/220 kDa)	lipovitellin (mol/141 kDa)	phosvitin (mol/30 kDa)
Zn	**1.02**	**1.06**	**0.20**
Ca	**1.50**	**ND[a]**	**2.10**
Mg	0.15	0.10	3.00
Cd	ND	ND	ND
Mn	0.06	ND	0.05
Fe	0.15	0.10	0.50
Co	ND	ND	ND
Ni	ND	ND	ND
Cu	0.09	0.03	ND

[a] Not detected.

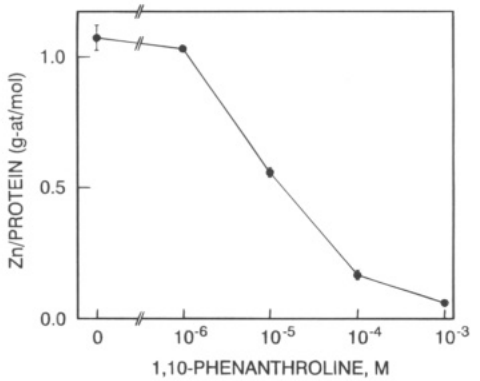

FIGURE 1: Removal of zinc from vitellogenin incubated with 1,-10-phenenthroline (OP) at concentrations between 10^{-6} and 10^{-3} M. Four nanomoles of vitellogenin/mL was preincubated with OP for 4 h and the protein separated from the chelating agent by chromatography on a Mono-Q column, HR 5/5. The amount of zinc remaining with the protein was determined as described in the text.

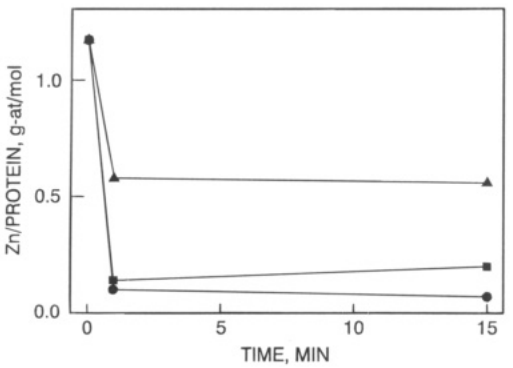

FIGURE 2: Effect of varying the time of preincubation with OP on zinc removal from vitellogenin. Five to fifteen nanomoles of vitellogelin/mL was preincubated for varying time periods for up to 240 min with OP at 10^{-5} (●), 10^{-4} (■), and 10^{-3} (▲) M. Aliquots were chromatographed on a Mono-Q column to separate the protein from the chelating agent. The results shown here are for incubation for less than 1 min and for 15 min. The data are identical for time periods up to 240 min.

The capacity of OP to remove zinc from vitellogenin has been examined in detail. Zinc removal is concentration dependent (Figure 1). OP concentrations of 10^{-6} M are not effective, though at higher concentrations, it progressively removes the metal. OP, 10^{-3} M, removes all zinc from vitellogenin which is recovered as an apoprotein by chromatography on Mono-Q. The p*K* for removal is 4.8. Figure 2 illustrates the time course of zinc removal from the protein by different OP concentrations and is instantaneous at all of them. Within less than 1 min of injecting the mixture, the Mono-Q column separates OP from the protein.

Lipovitellin and Phosvitin. The yolk platelets contain zinc bound to the yolk protein component. Thus, treatment of solubilized yolk platelet protein with chloroform/methanol separates the protein which aggregates at the chloroform/methanol interface from lipids and a green pigment; both of the latter remain in the chloroform phase. On the basis of its characteristic spectrum with a major band at 383 nm, a shoulder at 457 nm, and a smaller band at 670 nm, the pigment is biliverdin (Redshaw et al., 1971). In the presence of mercaptoethanol, both the major 383 nm and minor 670 nm bands are reduced while there is an increase in the 452 nm one, all characteristic of bilirubin. The protein fraction

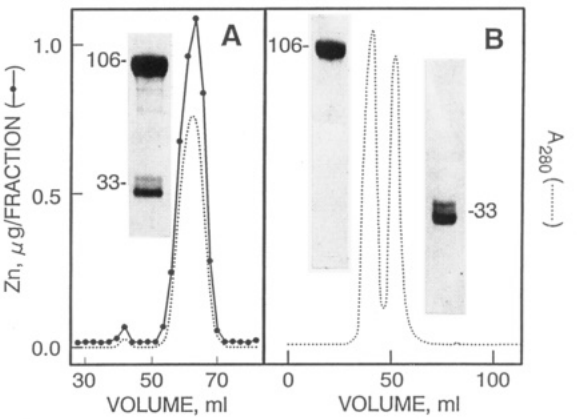

FIGURE 3: (A) Chromatography of lipovitellin on Sepharose 6B. Aliquots of lipovitellin in 1.0 M NaCl were loaded onto a Sepharose 6B column (120 × 1 cm) as described in the text. The zinc and OD_{280} were analyzed. The polypeptides present in the peak fraction were resolved by SDS−PAGE. (B) An aliquot of the peak fractions was incubated in 1% SDS, heated to 100 °C for 2 min, and applied to the Sepharose 6B column equilibrated with 0.1% SDS and 15 mM Tris-HCl, pH 8.0. The polypeptides in the peak fractions were analyzed by SDS−PAGE.

retains >96% of the zinc, and only traces are in the organic phase.

The yolk platelet protein that contains the zinc has been identified by purifying both lipovitellin and phosvitin and determining their metal contents. When yolk platelets are incubated in 1 M NaCl, their constituent proteins are solubilized. They are resolved by precipitation with 66% saturated $(NH_4)_2SO_4$. The protein fraction soluble under these conditions is phosvitin, on the basis of its molecular weight, amino acid composition (Table 1), high serine content (>68% is phosphoserine), and characteristic staining properties. It only stains with Coomassie blue when $AlCl_3$ is present as well (not shown). The protein that is precipitated by $(NH_4)_2SO_4$ is lipovitellin. It is solubilized by 1.0 M NaCl, eluting as a single peak which corresponds to a molecular weight of 141 kDa when chromatographed on Sepharose 6B (Figure 3A). The amino acid composition is that reported for lipovitellin (Table 1). The single Sepharose 6B peak is composed of two major polypeptides which appear on SDS−PAGE as broad bands of approximately 100−120 and 30 kDa. Two minor polypeptides migrating as molecules of about 70 and 35 kDa are present also. Boiling in the presence of 1% SDS dissociates these polypeptides, which can then be separated into two peaks by chromatography on Sepharose 6B (Figure 3B). The first and second peaks which elute contain both the 100−120 kDa and the 35 and 32 kDa bands, respectively. Amino acid analysis of the two fractions identifies them as lipovitellin subunit 1 (first peak) and lipovitellin subunit 2 (second peak), on the basis of data reported (Burley & Cook, 1961; Ohlendorf et al., 1977; Wiley & Wallace, 1981) and confirmed by N-terminal sequence analysis of each of the polypeptides (Table 2). For peak 1, at least two sequences are found corresponding to those of lipovitellins 1α and 1β, both with molecular weights of about 100 000 (Wiley & Wallace, 1981; Wahli et al., 1981). These N-terminal sequences are identical to those obtained from vitellogenins A and B (Table 2), establishing that the latter are the precursors for the two forms of lipovitellin 1 subunits. The N-terminal sequence data for the proteins in peak 2 (not shown) correspond to those of lipovitellins 2α, 2β, and 2τ

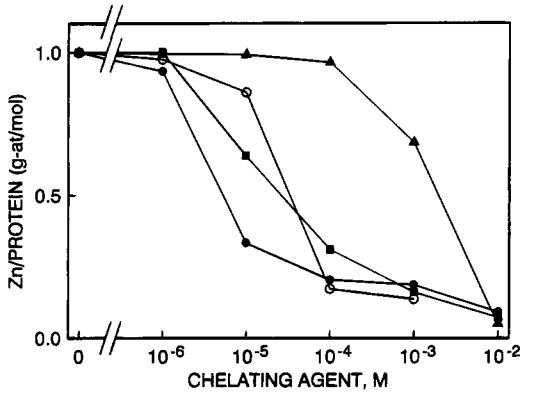

FIGURE 4: Removal of zinc from lipovitellin by various chelating agents. Approximately 7 nmol of lipovitellin was incubated overnight with each chelating agent at concentrations between 10^{-6} and 10^{-2}. The chelating agents include ethylenediaminetetraacetic acid (●), 1,10-phenanthroline (■), dipicolinic acid (○), and pyridylazoresorcinol (▲). Samples were then dialyzed against metal-free buffer without chelating agent, and the amount of zinc remaining with the protein was determined.

(Ohlendorf et al., 1977; Wiley & Wallace, 1981). Chromatography in the absence of SDS at different temperatures (4 or 22 °C), or in the presence of 25% ethylene glycol, or diverse salt concentrations from as high as 1 M to as low as 0.3 M, or incubating the protein with dithiothreitol or mercaptoethanol does not affect subunit structure; a single peak elutes in each case. The protein fraction is not soluble below 0.3 M NaCl. Hence, the subunit interaction could not be examined in the absence of salt.

The yolk platelet zinc is associated entirely with lipovitellin. In fact, zinc is the only metal associated stoichiometrically with lipovitellin; all other metals present are found in much lesser amounts (Table 3). The average amount of zinc associated with lipovitellin obtained after Sepharose 6B chromatography (Figure 3A) is 7.8 nmol/mg of protein, corresponding to 1 mol/141 000 Da, the reported molecular mass of frog lipovitellin and the value corresponding to its elution from Sepharose 6B. The metal content cited is based on analyses of ten separate preparations from oocytes of different frogs whose protein content varied over a 100-fold concentration range.

In contrast, the calcium of vitellogenin is associated exclusively with phosvitin. Moreover, at some point during the uptake and processing by the egg of vitellogenin and its cleavage products and their assembly in the yolk platelets, phosvitin acquires magnesium (Table 3) while its zinc content remains only 0.2 mol/30 kDa.

The role of the zinc in lipovitellin is unknown. On the one hand, it could be functional, and on the other, it could be structural and might maintain the interaction of lipovitellin subunits 1 and 2. In fact, we have examined the chromatographic behavior of native and apolipovitellin. Zinc was removed from lipovitellin by using two different approaches. For one, the protein was incubated with a number of chelating agents. Among those, EDTA turned out to remove zinc most effectively: at 10 μM it removes more than 50% of the zinc during overnight dialysis; 10-fold larger amounts of 1,10-phenanthroline and dipicolinic acid are required to achieve the same effect. Pyridylazoresorcinol was least effective in removing the metal (Figure 4). In addition, we have examined the effect of pH on zinc binding. Lipovitellin retains zinc between pH 5 and pH 10.5 but loses it below

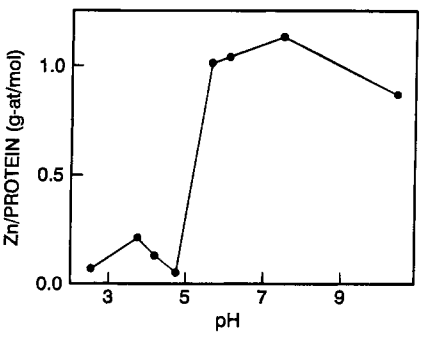

FIGURE 5: Removal of zinc from lipovitellin by exposure to acidic conditions.

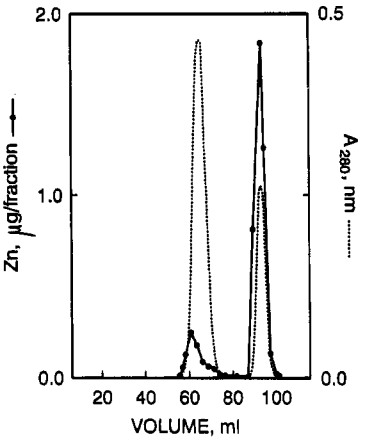

FIGURE 6: Effect of zinc removal on the interaction of lipovitellin 1 and 2 subunits.

Table 4: Reconstitution of Lipovitellin

lipovitellin	protein (nmol/mL)	zinc (nmol/mL)	Zn/protein (mol/mol)
original	53.7	52.24	0.97
		53.40	0.99
		56.39	1.05
washed	9.71	8.98	0.92
apo	14.28	0.96	0.07
reconst	2.86	2.93	1.02
		2.72	0.95

pH 5. At pH 4.9 nearly all of the metal is displaced (Figure 5) though the removal of zinc does not dissociate the subunits.

Thus, preincubation with dipicolinic acid removes 90% of the zinc from lipovitellin, but chromatography of the zinc-depleted protein on Sepharose 6B results in a single peak (Figure 6) whose elution volume is the same as that of the holoprotein (Figure 3A). Zinc elutes in the volume corresponding to that of dipicolinic acid, but neither proteins nor amino acids were detected in this fraction. The resultant apoprotein can be reconstituted completely by incubation with zinc-containing buffers (Table 4). The behavior of metal-depleted lipovitellin toward SDS and boiling is identical to that of the native protein. It dissociates into two peaks corresponding to the lipovitellin 1 and 2 subunits, respectively, as detected by chromatography on Sepharose 6B (Figure 3B).

DISCUSSION

Vitellogenin is a glycophospholipoprotein found in insects, worms, fish, reptiles, amphibians, and birds [reviewed in

Banaszak et al. (1991)]. It is synthesized in the liver of amphibians and birds in response to estrogen. In the frog, the hormone activates up to four vitellogenin genes that give rise to multiple forms of the protein (Wiley & Wallace, 1978, 1981; Wahli et al., 1979, 1980; Germond et al., 1983). Once synthesized, vitellogenin is secreted into plasma as a dimer where in about 6 days it reaches a peak plasma value, proportional to the amount of hormone administered. When induced by the administration of doses of hormone larger than that normally secreted by the frog, e.g., two doses of 4 mg of estrogen given over a 2-week interval, vitellogenin accounts for over 50% of the protein in plasma (Wallace & Jared, 1978; Kato et al., 1986; Montorzi et al., 1994). Vitellogenin is so abundant that such plasma turns green due to the presence of biliverdin complexed to the protein (Redshaw et al., 1971).

While *X. laevis* vitellogenin has long been known to contain calcium (Urist et al., 1958), we have reported that, in addition, it is a zinc protein (Tables 1−3) which contains 1 g at zinc and 1.5 g at Ca/220 kDa monomer (Montorzi et al., 1994). Plasma vitellogenin is a dimer of two polypeptides, each of which has a molecular mass of about 220 kDa. Therefore, the protein contains a total of 5 mol of metals, 2 mol of zinc, and 3 mol of calcium (Table 3).

The recognition that both zinc and calcium are intrinsic components of frog vitellogenin raises questions regarding the strength and physical−chemical characteristics of the metal binding sites, their location within the polypeptide chain of vitellogenin, their roles in its structure, and possible function, and in its receptor binding, its processing within the oocyte and/or storage in yolk platelets, and their ultimate fate in the embryo and tadpole. All five metal atoms are bound tightly since their binding survives the isolation procedure which includes extensive dialysis steps. In the case of the zinc, the metal atoms are removed by OP in a concentration-dependent manner so that dialyzing the zinc protein against 10^{-3} M OP renders it zinc free as confirmed by direct analysis of the apoprotein by atomic absorption spectrometry (Figure 1). The removal of Zn is instantaneous at all concentrations studied, and preincubation for up to 240 min does not alter the extent of removal (Figure 2). The time dependence of metal removal by OP represents preliminary information with which to address the possible mechanisms of inhibition of the activities if they are metalloenzymes. In this regard, two possible modes of interaction are known. In many zinc enzymes, inhibition is the result of the formation of a reversible mixed complex between a metalloenzyme and OP while in others it is due to the complete removal of zinc by the chelating agent. In the former, the formation of the complex is reversed on dilution or by addition of another metal that competes with the intrinsic zinc in binding to the agent. Examples of the former include the instantaneous and reversible inhibition by OP of liver zinc alcohol dehydrogenase (Vallee et al., 1959; Vallee & Wacker, 1970) and *Euglena gracilis* RNA polymerase II (Falchuk et al., 1976). In the present case, the interaction between OP and the zinc in vitellogenin removes the metal completely within less than 1 min. The implications of this mode of interaction reflect the possible nature of the ligands that comprise the zinc binding site and the strength of the interaction, but the identification of the residues involved requires additional experimental effort.

To initiate this process, we have determined the location of the metals in the polypeptide chain of vitellogenin by taking advantage of its processing into its well-characterized domains once it is internalized by the oocyte. The availability of each of the vitellogenin cleavage products in pure form together with direct analysis of their metal content is a first step in identifying the location of each metal in the polypeptide sequence of vitellogenin itself when comparing the amino acid ligands that bind metals in both the parent and the cleavage molecules.

Thus, the vitellogenin polypeptides are composed of multiple domains that are processed into distinct proteins once vitellogenin is taken up by the oocyte. Prior to this event, vitellogenin binds to a 115 kDa receptor protein expressed on the membranes of maturing oocytes (Yusko & Roth, 1976; Stifani et al., 1990). The receptor−vitellogenin complex is internalized by endocytosis and can be detected within the cytosol in vesicles. These fuse both with one another and with lysozomes to form multivesicular bodies (Wall & Meleka, 1985; Wall & Patel, 1987). The fusion step is essential for the subsequent hydrolytic processing since it is in the multivesicular bodies that vitellogenin dissociates from its receptor to become exposed to specific proteases (Wallace et al., 1983; Wallace & Jared, 1968; Wall & Meleka, 1985; Wall & Patel, 1987; Opresko et al., 1980b). Cleavage of vitellogenin gives rise to phosvitin and lipovitellin (Cook, 1961; Wiley & Wallace, 1981; Griffin, 1985), which crystalize and constitute the principal proteins of the yolk platelet (Bergink & Wallace, 1974). Lipovitellin and phosvitin account for almost all of the vitellogenin precursor; a 200 amino acid segment of the parent molecule appears to undergo complete hydrolysis (Banaszak et al., 1991). Cleavage of the four vitellogenin isoforms expressed by the frog liver and secreted into the frog plasma results in multiple isolipovitelin and -phosvitin forms (Ohlendorf et al., 1977; Wiley & Wallace, 1978, 1981; Wahli et al., 1979, 1980; Germond et al., 1983; Gerber-Huber et al., 1987; Banaszak et al., 1991). The solubility properties of the vitellogenin differ from those of its cleavage products. Vitellogenin is transported from liver to oocyte in the plasma, and it must, therefore, be a soluble protein. In the egg, the products of vitellogenin hydrolysis are stored in yolk platelets. Clearly, these products are less soluble than the parent vitellogenin molecule since they are deposited in the platelets in the form of microcrystals (Roth & Porter, 1964; Ohlendorf et al., 1977; Banaszak et al., 1991).

The conditions for purification of the vitellogenin cleavage products, as with those applied to vitellogenin itself, were designed to avoid metal contamination and in particular to omit chelating agents, such as the commonly used EDTA, from all buffers to preclude removal of metal from any of the proteins. The latter precaution is indicated by the finding that 1 mM EDTA removes zinc from lipovitellin (Figure 4). This observation may account, in part, for the failure to identify the presence of calcium, zinc, or any other metal in the lamprey yolk lipovitellin−phosvitin crystals used for X-ray crystalographic analysis (Raag et al., 1988; Banaszak et al., 1991) and which were isolated in the presence of EDTA.

The lipovitellin and phosvitin molecules purified here have been identified in terms of their characteristic solubility properties, electrophoretic mobilities, amino acid composition with their high phosphoserine contents (Table 1), N-terminal

sequences (Table 2), and biliverdin content (Begink & Wallace, 1974; Wiley & Wallace, 1981; Germond et al., 1983; Gerber-Huber et al., 1987; Banaszak et al., 1991). Zinc and calcium are not localized in the same region of vitellogenin. The lipovitellin portion of vitellogenin retains zinc after proteolytic processing. Solubilized, purified lipovitellin (Figure 3) contains approximately 1 mol of zinc/141 kDa (Table 3). The metal can be removed by a number of chelating agents (Figure 4) or by acidic conditions (Figure 5). The near identity of the pK for removal of the zinc by OP from both the precursor and the lipovitellin suggests that the binding site for zinc may be the same in vitellogenin and in lipovitellin, but this remains to be established by more direct analysis of the pertinent amino acid ligands. Such information is now being obtained by a number of approaches. Preliminary analysis of both lipovitellin and vitellogenin by EXAFS has defined the ligands that bind zinc as nitrogen and/or oxygen atoms, and the spectra generated appear to be identical for both proteins. Moreover, reconstitution of the lipovitellin apoprotein (Figure 6) with zinc generates a spectrum that is identical to that of the native holoprotein, suggesting the zinc has bound to the original site (Auld et al., in preparation). In the future, spectroscopically active atoms, particularly cobalt, will be used to reconstitute both proteins to address the problems of metal coordination geometry and to further examine binding sites using diverse spectroscopic techniques, including MCD and EPR (Vallee & Galdes, 1984).

Lipovitellin from *X. laevis* oocytes and embryos have been shown by others to bind nickel under *in vitro* conditions, though direct analysis of the metal content of the purified protein was not carried out (Grbac-Ivankovic et al., 1994). The present data demonstrate, however, that in fact lipovitellin contains only zinc; no other metals are associated with it in the oocyte (Table 3).

The calcium of vitellogenin is associated exclusively with phosvitin, which derives from the phosphoserine-rich domain of the precursor. Moreover, once in the yolk platelet, phosvitin apparently acquires magnesium in addition (Table 3). The source of the magnesium and the specific timing in the processing of vitellogenin that underlie the magnesium acquisition are unknown.

The role of the two metals in vitellogenin, of zinc in lipovitellin, and of calcium and magnesium in phosvitin remains to be determined. EDTA inhibits vitellogenin uptake (Wallace et al., 1973; Wall & Patel, 1987) presumably by preventing interaction of the protein with its receptor. This has been thought to point to a role for calcium in receptor binding (Wallace et al., 1973; Opresko & Wiley, 1987). However, this conclusion requires reexamination since we now know that vitellogenin also contains zinc, a metal that is known to play an important role in receptor–ligand interactions involving other molecules, e.g., the estrogen receptor (Schwabe et al., 1990). Since it can be removed by chelating agents (Figure 1), it is possible that it or calcium or both may be required for receptor binding. Data reported here on lipovitellin suggest that its zinc is not required for subunit interaction (Figure 6).

The recognition of vitellogenin as a zinc protein is of fundamental importance to the biochemistry of zinc during maturation of the frog oocyte. Moreover, the finding of zinc in the vitellogenin cleavage product lipovitellin, a molecule that accounts for over half of the oocyte protein and early embryo, suggests a critical role for the metal in the biology and biochemistry of the developing embryo and/or tadpole. Work in progress extends these conclusions to the oogenesis and embryogenesis of other species. Thus, chicken vitellogenin and lipovitellin have been purified, and both have been shown to be zinc metalloproteins. In addition to the corresponding lipovitellin 1 and 2 subunits described for the frog (Figure 3, Table 2), the chicken generates additional lipovitellin subunits that result from different cleavage patterns in processing of its vitellogenin isoforms. This permits comparative analysis of the zinc binding domains in these proteins that should aid in the identification of domains of the lipovitellin subunits that contain the zinc binding sites (Rashovetsky et al., unpublished).

A number of possible functions of these two proteins that relate to zinc metabolism can be considered at this time. Frog plasma vitellogenin is clearly a zinc carrier protein, and it mediates zinc uptake by *X. laevis* oocytes during the course of their maturation as will be reported (Falchuk et al., in preparation). Lipovitellin has been thought to serve as a reservoir of amino acids for future embryonic growth. These and other speculations regarding its function require reevaluation in the light of the present data. The large body of available information regarding the diverse functions of zinc in other proteins and enzymes will serve as background for the delineation of its role in lipovitellin (Vallee & Auld, 1993; Vallee & Falchuk, 1993). It may well be that the zinc in lipovitellin may have functions in catalysis and/or structure stabilization analogous to those already identified in many other proteins and enzymes (Vallee & Falchuk, 1993). Among these, the possibility that lipovitellin participates in its own proteolytic breakdown must clearly be considered. Alternatively, this molecule may be the source for metal to be transferred to a set of newly synthesized apoproteins of the embryo and/or tadpole different from that supplied by the cytosolic zinc described. Whatever the actual function may turn out to be, it will likely prove critical to the development, differentiation, and growth of the embryo and/or tadpole.

REFERENCES

Banaszak, L., Sharrock, W., & Timmins, P. (1991) *Annu. Rev. Biophys. Chem. 20*, 221–246.

Bergink, K. E., & Wallace, R. A. (1974) *J. Biol. Chem. 249*, 2897–2903.

Bligh, E. G., & Dwyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Burley, R. W., & Cook, W. H. (1961) *Can. J. Biochem. Physiol. 39*, 2897–2903.

Butler, E. J. (1983) in *Physiology and Biochemistry of the Domestic Fowl* (Freeman, B. M., Ed.) Academic Press, London, U.K.

Cook, W. H. (1961) *Nature 4782*, 1173–1175.

Cook, W. H. (1968) in *Egg quality: A Study of the Hen is Egg* (Carter, T. C., Ed.) pp 109–132, Oliver Boyd, Edinburg, U.K.

Falchuk, K. H., Mazus, B., Ulpino, L., & Vallee, B. L. (1976) *Biochemistry 15*, 4468–4475.

Falchuk, K. H., Hilt, K. L., & Vallee, B. L. (1988) *Methods Enzymol. 158*, 422–434.

Gerber-Huber, S., Nardelli, D., Haefliger, J.-A., Cooper, D. N., Givel, N., et al. (1987) *Nucleic Acids Res. 15*, 4737–4760.

Germond, J. E., ten Heggeler, B., Schubiger, J. L., Walher, P., Westley, B., & Wahli, W. (1983) *Nucleic Acids Res. 11*, 2979–2997.

Grbac-Ivankovic, S., Antonijczuk, K., Varghese, A. H., Plowman, M. C., Antonijczuk, A., Korza, G., Ozols, J., & Sunderman, F. W., Jr. (1994) *Mol. Reprod. Dev. 38*, 256–263.

Griffin, H. D. (1985) *Comp. Biochem. Physiol. 85B*, 469–472.

Griffin, H. D., Perry, M. M., & Gilbert, A. B. (1984) in *Physiology and Biochemistry of the Domestic Fowl* (Freeman, B. H., Ed.) pp 345−380, Academic Press, London, U.K.
Hansen, P., & Riebesell, M. (1991) in *The Early Development of Xenopus laevis*, pp 1−48, Springer-Verlag, Berlin.
Hegenenauer, J., Ripley, L., & Nace, G. (1977) *Anal. Biochem. 78*, 308−311.
Jared, D. W., Dumont, J. N., & Wallace, R. A. (1973) *Dev. Biol. 35*, 19−28.
Kato, Y., Imai, K., & Hanaoka, Y. (1986) *Endocrinol. Jpn. 33*, 883−889.
Montorzi, M., Falchuk, K. H., & Vallee, B. L. (1994) *Biochem. Biophys. Res. Commun. 200*, 1407−1413.
Nomizu, T., Falchuk, K. H., & Vallee, B. L. (1993) *Mol. Reprod. Dev. 36*, 419−423.
Ohlendorf, D. H., Barbarash, G. R., Trout, A., Kent, C., & Banaszak, L. J. (1977) *J. Biol. Chem. 252*, 7922−8001.
Opresko, L. K., & Wiley, H. S. (1987) *J. Biol. Chem. 262*, 4109−4115.
Opresko, L., Wiley, H. S., & Wallace, R. A. (1980a) *Cell 22*, 47−57.
Opresko, L., Wiley, H. S., & Wallace, R. A. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1556−1560.
Raag, R., Appett, K., Xnong, N.-H., & Banaszak, L. (1988) *J. Mol. Biol. 200*, 553−569.
Redshaw, M. R., Follett, B. K., & Lawes, G. J. (1971) *Int. J. Biochem. 2*, 80−83.
Roth, T. F., & Porter, K. R. (1964) *J. Cell Biol. 20*, 313−332.
Schwabe, J. W., Neukkhauks, R. D., & Rhodes, D. (1990) *Nature (London) 348*, 458−461.
Stifani, S., Nimpf, J., & Schneider, W. J. (1990) *J. Biol. Chem. 265*, 882−888.
Urist, M. R., Schjeide, O. A., & McLean, F. C. (1958) *Endocrinology 63*, 570−585.
Vallee, B. L., & Wacker, W. E. C. (1970) in *The Proteins* (Neurath, H., Ed.) 2nd ed., Vol. 5, pp 1−192, Academic Press, New York.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. 56*, 283−430.
Vallee, B. L., & Auld, D. S. (1993) *Acc. Chem. Res. 26*, 543−551.
Vallee, B. L., & Falchuk, K. H. (1993) *Physiol. Rev. 73*, 79−118.
Vallee, B. L., Williams, R. J. P., & Hoch, F. L. (1959) *J. Biol. Chem. 234*, 2621−2626.
Wahli, W., Dawid, J. B., Wyler, T., Jaggi, R. B., Weber, R., & Ryfell, G. U. (1979) *Cell 16*, 523−549.
Wahli, W., Dawid, J. B., Wyler, T., Weber, R., & Ryfell, G. U. (1980) *Cell 20*, 107−117.
Wahli, W., Dawid, I. B., Ryffel, G. U., & Weber, R. (1981) *Science 212*, 298−304.
Wall, D. A., & Meleka, I. (1985) *J. Cell Biol. 101*, 1651−1664.
Wall, D. A., & Patel, S. (1987) *J. Biol. Chem. 262*, 14779−14789.
Wallace, R. A. (1963a) *Biochim. Biophys. Acta 74*, 495−504.
Wallace, R. A. (1963b) *Biochim. Biophys. Acta 74*, 505−518.
Wallace, R. A. (1965) *Anal. Biochem. 11*, 297−311.
Wallace, R. A. (1978) in *The Vertebrate Ovary* (Jones, R. E., Ed.) pp 469−502, Plenum, New York.
Wallace, R. A., & Jared, D. W. (1968) *Science 160*, 91−92.
Wallace, R. A., Nickel, J. M., Ho, T., & Jared, O. W. (1972) *Dev. Biol. 29*, 255−272.
Wallace, R. A., Opresko, L., Wiley, H. S., & Selman, K. (1983) *Ciba Found. Symp. 98*, 228−248.
Wallace, R. A., Carnevali, O., & Hollinger, T. (1990) *J. Chromatogr. 519*, 75−86.
Wiley, H. S., & Wallace, R. A. (1978) *Biochem. Biophys. Res. Commun. 85*, 153−159.
Wiley, H. S., & Wallace, R. A. (1981) *J. Biol. Chem. 256*, 8626−8634.
Yusko, S. C., & Roth, T. F. (1976) *J. Supramol. Struct. 4*, 89−97.

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