Isolation of a novel protein from the outer layer of the vitelline membrane

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The outer layer of the vitelline membrane from hen egg yolk consists of ovomucin, vitelline membrane outer layer protein I (VMOI) and lysozyme. Here we report the occurrence of a further basic protein (pI 11.5) in the outer layer, which was designated as vitelline membrane outer layer protein II (VMOII). It was dissociated from the outer layer in a 10% (w/v) NaCl solution and purified to homogeneity by ion-exchange chromatography. VMOII is a simple protein with a molecular mass of 6000 Da, as determined by sedimentation equilibrium analysis. The amino acid composition of VMOII was characterized by the absence of Met and high contents of cystine (half) (14%) and basic amino acids (6% Arg, 6% Lys and 3% His). Analysis of carboxymethylated VMOII indicated that all cysteine residues were involved in disulphide bonding, which appears to facilitate the binding of SDS to the protein. Sequence comparison of the N-terminal 20 residues revealed no identity with other known proteins. VMOII contained a small amount of α -helix and was quite resistant to heat denaturation.

INTRODUCTION

The vitelline membrane of hen eggs, separating the yolk from the egg white, consists of two distinct layers of different compositions and structures, the inner layer (*lamina perivitellina*) and the outer layer (*lamina extravitellina*) (Bellairs *et al.*, 1963; Jensen, 1969). We have developed a simple method to separate intact the two layers, and have characterized their macromolecular components (Kido & Doi, 1988). The inner layer consists of a three-dimensional network of thick fibres which are made up of three major soluble glycoproteins, GP-I, GP-II and GP-III, as well as the insoluble glycoprotein GP-IV (Kido *et al.*, 1975, 1976, 1977; Kido & Doi, 1988). The outer layer, in contact with the albumen, comprises a number of lattice-work sublayers of fine fibrils which consist of ovomucin together with lysozyme and vitelline membrane outer layer protein I (VMOI) (Back *et al.*, 1982; Kido & Doi, 1988). Ovomucin in the outer layer has a different subunit structure from that of ovomucin in albumen, and is a carbohydrate-rich super macromolecule consisting of at least five subunits linked through disulphide bonds which are essential in maintaining the structural integrity of the membrane (Kido & Doi, 1988). Lysozyme and VMOI are both simple basic proteins and are easily dissociated from the ovomucin fibril in high-salt solutions (Back *et al.*, 1982; Back, 1984).

The two vitelline membrane layers are synthesized in different organs: the inner layer is formed in the ovary before ovulation, whereas the outer layer is formed in the upper oviduct after ovulation (Bain & Hall, 1969). They appear to have their own biological functions. For example, when ova recovered from the body cavity, thus lacking the outer layer, were incubated with fresh cock sperm, the network structure of the inner layer was preferentially broken down at the animal pole (Bakst & Howarth, 1977). The breakdown seemed to be due to hydrolysis of the inner layer components by acrosin (a trypsin-like enzyme) of sperm. In contrast, when ova taken from the oviduct, thus having both the inner and outer layers, were incubated, no breakdown occurred, i.e. the presence of the outer layer prevented hydrolysis. Therefore the outer layer may serve *in vivo* as a

barrier to prevent pathological polyspermy (Bakst & Howarth, 1977), but no biochemical investigations have been carried out in order to clarify the mechanism of inhibition of hydrolysis.

We further examined the macromolecular components of the vitelline membrane in order to investigate its functional properties. In this paper we describe the isolation and characterization of a new basic protein in the outer layer, named vitelline membrane outer layer protein II (VMOII). We found that VMOII was considerably resistant to tryptic digestion.

MATERIALS AND METHODS

Materials

Reagents and solvents used for gas-phase sequencing and for phenylthiohydantoin amino acid identification were obtained from Applied Biosystems. All other chemicals were of analytical grade and were used as supplied. CM-Toyopearl and Toyopearl HW-55F were purchased from Tosoh Co., Tokyo, Japan. Standard proteins for electrophoresis and gel filtration were obtained from Pharmacia LKB Biotechnology and Sigma respectively.

Purification of VMOII

The vitelline membrane was isolated according to the method described previously, using freshly laid hens' eggs obtained from a local poultry farm (Kido & Doi, 1988). The isolated membrane (150 mg dry weight) was incubated in 25 ml of 10% (w/v) NaCl for 1 h at 23 °C with slight agitation. This incubation allows all soluble macromolecular components of the outer layer to dissociate from the membrane (Kido & Doi, 1988). After removal of the membrane, the incubation medium was dialysed against sodium acetate buffer (0.2 M-acetic acid adjusted to pH 4.6 with sodium acetate) at 4 °C. The dialysed solution was centrifuged for 2 min at 10000 *g* to remove a small amount of precipitate produced during dialysis and then applied to a CM-Toyopearl (650 M) column (2.5 cm $\times$ 30 cm) previously equilibrated with the same acetate buffer. The column was washed with 200 mM-NaCl in acetate buffer and then eluted with a NaCl gradient from 250

Abbreviations used: RCM, reduced and carboxymethylated; VMOI, vitelline membrane outer layer protein I; VMOII, vitelline membrane outer layer protein II

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to 350 mM (320 ml each) in order to collect the major salt-soluble components of the outer layer, i.e. lysozyme and VMOI. After a further wash of the column with 0.5 M-NaCl (100 ml), VMOII was then eluted with 1 M-NaCl in 0.2 M-acetate buffer, pH 4.6, at a flow rate of 60 ml/h. The fractions containing VMOII were pooled, concentrated, dialysed against 10 mM-acetate buffer, pH 4.5, and freeze-dried for storage. Protein concentrations were determined by the method of Lowry *et al.* (1951), using BSA as standard (Sigma, lot no. 67F-9368).

Electrophoresis

SDS/PAGE was performed in a gradient gel as described elsewhere (Laemmli, 1970; Kido & Doi, 1988). In order to evaluate the effects of reduction and oxidation of VMOII on its mobility, VMOII was incubated in 0.1 M-Tris/HCl buffer (pH 6.8) containing 1% SDS and various amounts (0, 2, 10 or 100 mM) of 2-mercaptoethanol for 2 h at room temperature prior to electrophoresis. To re-oxidize the reduced VMOII, O₂ was bubbled into the protein solution for 1 h at 4 °C. Limited proteolysis of the outer layer proteins was performed by using *Staphylococcus aureus* V8 proteinase, trypsin and chymotrypsin in the presence of SDS (Cleveland *et al.*, 1976). Acidic-disc PAGE (Reisfeld *et al.*, 1962) was carried out in order to estimate the isoelectric point of VMOII. The separating gel consisted of 11% polyacrylamide in potassium acetate buffer (0.38 M-acetic acid adjusted pH to 4.3 with KOH), and the concentrating gel contained 3.8% polyacrylamide in potassium acetate buffer (0.06 M-acetic acid adjusted pH to 5.4 with KOH). The electrophoresis buffer was 338 mM- β -alanine solution adjusted to pH 4.5 with acetic acid. Urea (8 M) was included in a gel for acid/urea-disc electrophoresis.

Gel-filtration chromatography

Gel-filtration chromatography was performed on a Toyopearl HW-55F column (1 cm $\times$ 50 cm) at 23 °C at a flow rate of 12 ml/h. The column had been previously equilibrated either with 0.1 M-sodium acetate buffer, pH 4.6, containing 0.3 M-NaCl, or with 1% SDS. Samples of intact VMOII and RCM-VMOII (reduced and carboxymethylated VMOII), which had previously been dialysed against the corresponding column equilibration solutions, were applied. The standard proteins used for estimation of apparent molecular mass were ovalbumin (43 kDa), carbonic anhydrase (29 kDa), α -chymotrypsin (25.2 kDa), trypsin inhibitor (20.2 kDa), lysozyme (14.3 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa).

Sedimentation equilibrium analysis

Purified VMOII (0.5 mg/ml) dissolved in 0.1 M-sodium acetate buffer (pH 4.5) containing 0.2 M-NaCl, with or without 2-mercaptoethanol, was thoroughly dialysed against the same buffer and used for analysis, which was performed with a Hitachi DA-7 analytical ultracentrifuge. Molecular mass was calculated using a value for the partial specific volume of VMOII estimated from its amino acid composition (James & Timasheff, 1974).

Determination of amino acid composition

RCM-VMOII was obtained as follows (Crestfield *et al.*, 1963). Lyophilized VMOII (500 μ g) was dissolved in a 8 M-urea solution (1 ml) containing 25 mM-EDTA, pH 8.0. To the solution was added 2-mercaptoethanol to a final concentration of 6 mM, and the mixture was incubated for 8 h at 23 °C. For carboxymethylation, monoiodoacetic acid (1.5 M in 1 M-NaOH) was added to the solution to a concentration of 12 mM under dim light, while maintaining the pH at 8.5 with 6 M-NaOH. After a 1 h incubation, the solution was exhaustively dialysed against 8 M-urea and then against water for amino acid analysis. We observed that under

these conditions all cysteinyl residues were carboxymethylated. Lyophilized RCM-VMOII was hydrolysed at 150 °C for 0.5, 1, 2 or 3 h in a saturated 6 M-HCl atmosphere. The contents of acid-labile amino acids were estimated by extrapolation to zero-hydrolysis time. To detect free cysteine residues, VMOII was carboxymethylated as above without prior reduction by 2-mercaptoethanol.

N-Terminal sequence analysis

VMOII (6–18 μ g) in 0.1% SDS was directly applied to the glass fibre sheet in the reaction chamber of a gas-phase Sequenator (Applied Biosystems, model 470 A) equipped with on-line h.p.l.c. (TSK-GEL ODS-80 TM) for analysing phenylthiohydantoin derivatives of amino acids. Analysis was performed three times and an identical sequence was deduced each time. Searches for sequence identities were carried out by using the program Homolog (Nishikawa *et al.*, 1987) with the NBRF-PIR Database (National Biomedical Research Federation, release 26).

Spectrophotometric analysis

C.d. measurement was performed with a JASCO J-600 spectrophotometer as described elsewhere (Doi *et al.*, 1990). To estimate the tryptophan content, the u.v. absorption spectrum of VMOII was measured in 6 M-guanidine hydrochloride (Edelhoch, 1967).

Scanning calorimetry

Molar heat capacities as a function of temperature were measured with an ultrasensitive scanning microcalorimeter (MC-2; MicroCal Inc., MA, U.S.A.). The concentration of VMOII was 1.4 mg/ml in 0.1 M-sodium acetate buffer, pH 4.6. A scan rate of 45 °C/h was used for the measurements.

RESULTS

Purification of VMOII

It was shown previously that nearly all of the macromolecular components of the outer layer of the vitelline membrane, except ovomucin, are preferentially dissociated from the membrane in a high-salt solution (Back *et al.*, 1982; Back, 1984; Kido & Doi, 1988). When the extract of the membrane in 10% (w/v) NaCl was subjected to CM-Toyopearl cation-exchange chromatography, all proteins were adsorbed to the column in the initial buffer (Fig. 1). Two major peaks were eluted by a NaCl gradient (250–350 mM), corresponding to VMOI and lysozyme judging

Fig. 1. Ion-exchange chromatography of the salt-soluble proteins from the vitelline membrane

The high-salt extract of the vitelline membrane was dialysed against 0.2 M-sodium acetate buffer, pH 4.6, and applied to a CM-Toyopearl (650 M) column (2.2 cm $\times$ 30 cm) which was previously equilibrated with the same buffer. The elution was carried out with a gradient and stepwise rises in salt concentration, as indicated. The two major peaks eluted with the salt gradient at 470 and 580 ml corresponded to VMOI and lysozyme respectively (see Fig. 2b). The sharp peak eluted at 920 ml was found to be VMOII.

Fig. 2. PAGE of VMOII and other outer layer proteins

(a) SDS/PAGE (lanes a–e) was carried out in a 16% gel. Samples applied were molecular mass markers (lane a), and VMOII in the absence (lane e) or the presence of 100 mM- (lane b), 10 mM- (lane c), or 2 mM- (lane d) 2-mercaptoethanol. Acid/urea disc electrophoresis (lane f) of VMOII was performed in a 12% gel. The amount of VMOII loaded on the gels was 2 μ g (lanes b–e) or 10 μ g (lane f). The marker polypeptides (6 μ g of each) were myoglobin and its fragments having molecular masses as follows (from the top): 17.2, 14.6, 8.24, 6.38 and 2.56 kDa. (b) SDS/PAGE was carried out in a 5–30% gradient gel without reduction of the samples; the whole high-salt extract of the vitelline membrane (lane a), VMOI (lane b), lysozyme (lane c) and purified VMOII (lane d) were applied. VMOI and lysozyme were obtained from the two peak fractions eluted at 470 ml and 580 ml in CM-Toyopearl column chromatography as depicted in Fig. 1. Samples containing 10 μ g were applied.

from their electrophoretic mobilities (see Fig. 2b) and their amino acid compositions (Back *et al.*, 1982; Kido & Doi, 1988). A sharp peak was then obtained by eluting the column with 1 M-NaCl (Fig. 1). The fractions comprising this peak were pooled, concentrated and examined by electrophoresis.

Electrophoresis

SDS/PAGE gave a single band with an apparent molecular mass of 9 kDa after protein staining by Coomassie Brilliant Blue (Fig. 2a, lane b). The band was not visualized by periodic acid–Schiff staining (Fairbanks *et al.*, 1971), suggesting the absence of a carbohydrate moiety. A single band was also observed by acid/urea disc gel electrophoresis (Fig. 2a, lane f), confirming the homogeneity of the purified protein. This new protein was called VMOII, by analogy with VMOI.

The mobility of VMOII on SDS/PAGE was greatly affected by the redox state of the protein (Fig. 2a, lanes b–e). As described above, reduced VMOII gave a single band of 9 kDa (Fig. 2a, lane b). In the absence of reductant, a band of 15 kDa was obtained (Fig. 2a, lane e), while in the intermediate range of reductant concentrations both of these bands were observed, in addition to a third band between them (Fig. 2a, lanes c and d). The shift of the band position was reversible and solely depended on the redox state of the molecule; when reduced VMOII was re-oxidized, the same 15 kDa band was observed. The possibility that the 15 kDa band represented a dimer was ruled out, as VMOII in the absence of SDS showed a constant mobility in acid/urea disc electrophoresis regardless of the redox state. Moreover, unfolding of VMOII by 8 M-urea in the absence of a reducing agent and subsequent urea/SDS electrophoresis also gave an apparent molecular mass of 15 kDa, while that of reduced VMOII was 9 kDa. The results indicated the existence of

Fig. 3. Proteolytic digestion patterns of the outer layer salt-soluble proteins

VMOII (lanes a, d, g and j), lysozyme (lanes b, e, h and k) and VMOI (lanes c, f, i and l) were incubated in Tris/HCl, pH 7.0, containing 0.5% SDS at 100 °C for 2 min and treated with *Staphylococcus aureus* V8 proteinase (lanes d–f), trypsin (lanes g–i) or chymotrypsin (lanes j–l) at 37 °C. The ratio of substrate to enzyme was 10:1 (w/w) in each case. The bands corresponding to enzymes observed in the upper part of gel are not shown. Samples containing 15 μ g were applied.

intramolecular disulphide bonds in the molecule which have a critical role in the interaction of VMOII with SDS (see below). It should be noted that VMOII co-migrates with lysozyme in the absence of a reducing agent (Fig. 2b, lane a), which may explain, in conjunction with its small quantity, why the previous studies did not recognize its presence (Back *et al.*, 1982; Kido & Doi, 1988).

Limited proteolysis

Fig. 3 shows the proteolytic digestion patterns of three soluble proteins existing in the outer layer. VMOII is extremely resistant to *Staphylococcus aureus* V8 proteinase (lane d) and trypsin (lane g), and only slightly susceptible to chymotrypsin (lane j). Neither lysozyme nor VMOI gave any peptide fragments having an identical molecular mass to that of VMOII, indicating that VMOII is not a degradation product of the other proteins of the outer layer.

Amino acid composition

Amino acid analysis of RCM-VMOII showed that the most abundant residue was CM-cysteine (Table 1). However, these residues all exist as cystine in the intact protein, since CM-VMOII without prior reduction did not give any CM-cysteine residues on analysis. VMOII is also characterized by the absence of Met and by large quantities of basic and hydrophobic residues.

N-Terminal sequence

The first 20 amino terminal residues of VMOII were as follows: Leu-Pro-Arg-Asp-Thr-Ser-Arg-Xaa-Val-Gly-Tyr-His-Gly-Tyr-Xaa-Ile-Arg-Ser-Lys-Val. No proteins registered in the NBRF-PIR Database have identity with this stretch of sequence.

Isoelectric point determination

Because of the inordinate basicity of VMOII, isoelectric focusing using basic ampholyte failed to determine the isoelectric point accurately. Therefore acid disc gel electrophoresis was used to estimate the isoelectric point, and revealed that VMOII had a pI of 11.5, higher than that of lysozyme (Fig. 4).

Ultracentrifugal analysis

Sedimentation analysis of VMOII with and without 2-mercaptoethanol gave an identical sedimentation coefficient value, i.e. 1.56 S. This indicated not only the absence of intermolecular disulphide bonds, but also the absence of a gross change in hydrodynamic properties upon reduction of intramolecular S–S bonds. The molecular mass of VMOII determined by equilibrium ultracentrifugation in acetate buffer was calcu-

Table 1. Amino acid composition of VMOII

RCM-VMOII was hydrolysed in gaseous HCl for different periods of time and the hydrolysates were applied to an ion-exchange column of an automatic amino acid analyser equipped with a ninhydrin detection system. The contents of acid-labile residues were obtained by extrapolating to zero-hydrolysis time. The tryptophan content was determined by a spectroscopic method. The averages of three determinations are given together with the range of values. Analysis of CM-VMOII without prior reduction indicated the absence of cysteine residues. The numbers of residues were estimated by assuming that VMOII contained a single tyrosine residue. The minimum molecular mass calculated from the estimated numbers was 5900 Da, which was comparable with that (6000 Da) determined by sedimentation equilibrium analysis.

Amino acid	Content (mol %)	Estimated no. of residues
Asp	7.11 (7.1–7.6)	3.8
Thr	6.71 (6.0–6.7)	3.6
Ser	6.71 (5.6–6.9)	3.6
Glu	8.39 (8.3–9.1)	4.5
Gly	9.38 (9.3–9.6)	5.0
Ala	4.34 (4.3–4.8)	2.3
Cys (half)	13.62 (11.6–14.0)	7.3
Val	4.54 (4.0–4.8)	2.4
Met	0	0
Ile	3.46 (3.0–3.6)	1.8
Leu	6.42 (6.3–6.7)	3.4
Tyr	1.88 (1.5–2.0)	1.0
Phe	3.06 (3.0–3.3)	1.6
His	2.96 (2.9–3.2)	1.6
Lys	6.12 (5.9–6.5)	3.3
Arg	6.42 (6.2–6.7)	3.4
Pro	5.43 (5.3–5.6)	2.9
Trp	3.46	1.8

Fig. 4. Isoelectric point determination of VMOII

Acid-disc electrophoresis of standard proteins was carried out and their mobilities relative to that of Methyl Green were measured. From the linear relationship obtained between the pI and the mobility of standard proteins, the isoelectric point of VMOII was estimated (arrow). The standard proteins used for determination of pI were as follows, with their pI values in parentheses: egg white lysozyme (11.0), cytochrome *c* (10.5), trypsinogen (9.3), basic lentil lectin (8.7), neutral lentil lectin (8.5), acidic lentil lectin (8.2), horse myoglobin (7.3).

lated to be 6000 Da (Fig. 5). No difference was observed in the presence and the absence of 2-mercaptoethanol. The linearity of this plot confirmed the homogeneity of the purified fraction.

Gel-filtration chromatography

To examine the effect of intramolecular disulphide bonds on

Fig. 5. Sedimentation equilibrium of VMOII

VMOII (0.2 mg/ml) in 100 mM-sodium acetate buffer (pH 4.5) containing 0.2 M-NaCl was loaded on to the cells and centrifuged at 20000 rev./min at 20 °C. The concentration of protein as a fraction of the radial distance from the centre of rotation was determined by scanning at 280 nm. The molecular mass was calculated to be 6000 Da using a partial specific volume of VMOII of 0.714, estimated from the amino acid composition.

Fig. 6. C.d. spectra of VMOII

VMOII (0.5 mg/ml) was dissolved in 100 mM-sodium acetate buffer, pH 4.6, (—), in the same acetate buffer containing 100 mM-2-mercaptoethanol (○) or in 6 M-guanidine hydrochloride (----). The mean residue weight of VMOII (111) estimated from the amino acid composition was used to calculate molecular ellipticity. Spectra were measured at 25 °C in a quartz cell with a light path of 0.1 mm.

binding to SDS, gel-filtration chromatography was carried out for intact VMOII and RCM-VMOII with and without SDS. In the absence of SDS, the apparent molecular masses of intact VMOII and RCM-VMOII were 6.3 kDa and 10 kDa respectively, while those in the presence of SDS were 15 kDa and 9 kDa. The > 2-fold increase in the apparent molecular mass of VMOII in the presence of SDS suggested that the presence of internal disulphide bonds enhances binding of SDS to VMOII.

Circular dichroism

C.d. spectra of native VMOII showed a broad negative at 205 nm, an inflection point at 195 nm, and a positive band around 190 nm (Fig. 6). These features, together with the absence of strong negative bands at 208 nm and 222 nm, suggested that VMOII contains a small amount of α -helix; 21 % helix content was estimated from the band at 208 nm (Greenfield & Fasman, 1969). Upon treatment of VMOII with 2-mercaptoethanol, the spectrum did not differ considerably from that measured without the treatment. The c.d. spectra of denatured VMOII in 6 M-guanidine hydrochloride showed a featureless pattern of ordinary random coil structure.

Thermal analysis

Measurement of the heat capacity change by differential scanning calorimetry indicated that VMOII was quite resistant to heat denaturation; the plot of a heat capacity per mol of the protein against temperature between 20 and 85 °C gave a slight steady increase without the positive peak usually indicative of a conformational change with the liberation of heat (results not shown). The heat-resistance of VMOII was also confirmed by the c.d. measurements, in that almost identical spectra were observed at 20, 45, 55 and 85 °C (Fig. 6).

DISCUSSION

The present study reveals the existence of a new basic protein, VMOII, in the outer layer of the hen egg vitelline membrane. The content of VMOII in the outer layer is estimated to be about 5%, taking account of the yield in ion-exchange chromatography (75%). Thus the composition of macromolecular components comprising the outer layer (Kido & Doi, 1988) should be revised as follows: 43% ovomucin, 32% lysozyme, 20% VMOI and 5% VMOII. Although all soluble proteins of the outer layer have high isoelectric points, being tightly associated with ovomucin fibrils which contain a considerable amount of sialic acid (Kido & Doi, 1988), VMOII is distinguished by high cystine content. The content of disulphide bonds, i.e. four in a molecule composed of 53 amino acid residues (estimated from the amino acid composition), is comparable with that of bovine trypsin inhibitor (three disulphide bonds in 58 residues; Dayhoff, 1972). Thus it is readily understandable that VMOII is highly resistant to proteinase digestion. It is interesting to see if the ability of the outer layer to protect the inner layer from hydrolysis by sperm acrosin is related to the concentration of VMOII in the layer. We observed that the outer layer solubilized in SDS was relatively resistant to tryptic digestion. It is possible that VMOII belongs to a new family of proteinase inhibitors rich in disulphide bonds, since the *N*-terminal primary structure had no sequence similarity with other known proteinase inhibitors such as chicken egg white cystatin, which is a cysteine peptidase inhibitor having two intramolecular disulphide bonds (Barrett, 1987). The determination of the complete amino acid sequence will be required to settle this issue.

VMOII has a remarkable resistance to heat. The stability of the protein may be partially attributed to large quantities of disulphide bonds. However, preliminary measurements by scanning calorimetry indicated that VMOII was also stable in the presence of reductants, suggesting that other stabilizing forces, such as hydrophobic interactions, play a significant role in maintaining the structure of the protein. Although the reduction of the intramolecular disulphide bonds of VMOII does not greatly affect the secondary structure, it appears to have a large effect on the affinity of VMOII for SDS. On SDS/PAGE, proteins migrate depending on their chain length because SDS-polypeptide complexes have similar hydrodynamic shapes and charge densities provided that SDS binds to the peptide chains at a constant ratio, i.e. 1.4 g/g of protein (Reynolds & Tanford, 1970). Furthermore, the mobility of a reduced protein in the presence of SDS is expected to be lower than that of the non-reduced counterpart, since the reduced protein, having an unfolded and extended conformation upon complex formation with SDS, should give a large hydrodynamic volume. This is indeed the case for most proteins with disulphide bonds (Fish *et al.*, 1970). However, VMOII when reduced gave a considerably higher mobility on SDS gel electrophoresis, i.e. the reduction of internal disulphide bonds decreased the apparent molecular mass (Fig. 2a, lanes b and e). This implies that the structural integrity

maintained by the internal disulphide bonds enhances the binding of SDS, leading to a VMOII-SDS complex with a larger apparent molecular mass than that of the reduced counterpart. This notion is consistent with the results of gel-filtration chromatography, where protein-SDS complexes move according to their hydrodynamic volume regardless of their net charges. It will be of interest to identify such a structural element furnished by disulphide bonds. It should be noted that carboxymethylation of reduced VMOII introduces negative charges and may affect its ability to bind SDS. However, when the disulphide bonds of VMOII are cleaved and protected by the presence of a reducing reagent on chromatography, its elution volume is similar to that of RCM-VMOII, indicating that the highly basic nature of VMOII only slightly influences the binding of SDS. It is also interesting to note that partially reduced VMOII appears to form a stable complex with SDS, as shown by the minor band migrating between the two bands corresponding to oxidized and reduced VMOII (Fig. 2, lanes c and d). Whether this form of VMOII merely represents a partially reduced isoform, or is a product of disulphide exchange not observed without SDS, remains to be resolved.

In this study VMOII was isolated from an extract of the whole vitelline membrane in a high-salt (10% NaCl) solution, since it was shown previously that the high-salt extract contained the outer layer components only (Kido & Doi, 1988). Indeed, VMOII was not found in the inner layer when the SDS-soluble components of the two separated layers were examined. Furthermore, VMOII appears not to be present in the albumen of freshly laid eggs, as CM-Toyopearl chromatography of the high-salt extract of egg white did not separate any protein corresponding to VMOII. However, when unfertilized eggs were stored at 30 °C for several days, VMOII was completely dissociated from ovomucin into the albumen, probably because of the raised pH (9.2–9.3) which was due to the decreased pressure of CO₂ escaping from the egg shell. In fact, when VMOII was added back to the VMOII-free vitelline membrane kept in buffers of various pHs, all VMOII was associated with the membrane at pH values lower than 8.0, 50% was associated at pH 9.0 and none was associated at pH 10.

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