The Protein Conformation and a Zinc-Binding Domain of an Autoantigen from Mouse Seminal Vesicle

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ABSTRACT The protein conformation of a mouse seminal vesicle autoantigen was studied by circular dichroism spectroscopy. At pH 7.4, the spectrum in the UV region appears as one negative band at 217 nm and one positive band at 200 nm. This together with the predicted secondary structures indicates no helices but a mixture of β form, β turn, and unordered form in the protein molecule. The conformation is stable even at pH 10.5 or 3.0. The spectrum in the near-UV region consists of fine structures that are disturbed in acidic or alkaline solution.

The environments around Trp^2 and Trp^{82} of this protein were studied by intrinsic fluorescence and solute quenching. They give an emission peak at 345 nm, and about 87% of them are accessible to quenching by acrylamide. Correlating the quenching effect of CsCl and KI on the protein fluorescence to the charged groups along the polypeptide chain suggests the difference in the "local charge" around the two tryptophan residues. The presence of $ZnCl_2$ in the protein solution effects no change in the circular dichroism but perturbs the fluorescence due to Trp^{82}. Analysis of the fluorescence data suggests a Zn^{2+}-binding site on the protein, which cannot coordinate with both Ca^{2+} and Mg^{2+}. The association constant for the complex formation is $1.35 \times 10^5 \pm 0.04 \times 10^5\ M^{-1}$ at pH 7.4.

INTRODUCTION

The different types of cells in the seminal vesicles of mammals may respond differently to androgenic action, which is considered to be mediated through a hormonal receptor complex (Chang et al., 1988; Zhou et al., 1994). Seminal vesicles in adult mammals secrete a group of protein components that constitute the main portion of seminal plasma. However, their biological significance remains obscure. Finding the structure and function of the androgen-stimulated protein components in the seminal vesicle secretion (SVS) becomes important to the elucidation of the transduction of the hormonal signal into their physiological effects. With this regard, the rodent is a good experimental animal. The primary structures for some of the major protein components in rat and mouse SVS have been established, but they show no correlation with the protein structures known thus far, despite the fact that their gene expression is shown to be androgen dependent (Ostrowski et al., 1979; Mansson et al., 1981; Chen et al., 1987). Much less progress has been made, however, in the study of the minor protein components in SVS, except for a Kazal-type trypsin inhibitor (Lai et al., 1991, 1994).

Recently, we have demonstrated an autoantigen (SVA) in mouse seminal vesicle, which exists exclusively in this accessory gland and is a minor protein component of SVS (Yu et al., 1993). It is an androgen-stimulated, 19-kDa glycoprotein with a core protein consisting of 131 amino acid residues. Its primary structure, which shows no significant similarity to protein sequences collected in the databank (Yu et al., 1993), suggests no clues to its function. Under these circumstances, more information obtained by other approaches would be valuable for establishing the protein structure, which may shed some light on its function. This work was carried out in this direction. Here, we report the conformation of SVA based on circular dichroism (CD) and its complex formation with Zn^{2+}, which is able to perturb the protein fluorescence. The protein has no detectable CD bands that are characteristic of α helix. Rather, its CD spectrum resembles that of a β form, suggesting that the protein contains a considerable amount of β forms and possibly β turns. The protein has a Zn^{2+}-binding site that cannot coordinate with other divalent metal ions such as Ca^{2+} and Mg^{2+}.

MATERIALS AND METHODS

Preparation of SVA

Outbred ICR mice were purchased from Charles River Laboratories (Wilmington, MA) and were maintained and bred in the animal center at the College of Medicine, National Taiwan University. Animals were treated following the institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14 h light, 10 h dark) at 21–22°C and were provided with water and National Institutes of Health 31 laboratory mouse chow ad libitum. According to our previous procedure (Yu et al., 1993), SVA I and SVA II were purified from mouse SVS, which was collected from mature male mice. SVA I was used throughout the study unless stated otherwise.

Measurement of CD and fluorescence spectra

The concentration of SVA was determined by Lowry's method (Lowry et al., 1951). The CD spectra were measured with a Jasco J-700 spectro-

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polarimeter under constant flushing with N_2 at room temperature. The mean residue elipticity, $[\theta]$, was estimated from the mean residue weight, which was calculated from the primary structure. The fluorescence intensity, expressed in arbitrary units, was measured at room temperature with a Hitachi F-4000 fluorescence spectrophotometer. Both the excitation and the emission slit width were 10 nm. Ramam emission due to the scattering of solvent was minimized by adjusting the intensity scale. The protein solution was freshly prepared. It took no more than 5 min to scan a spectrum to prevent protein precipitation. $F_{\lambda_1}{}^{\lambda_2}$ represented the fluorescence intensity at wavelength λ_2 (nm) when the fluorophore was excited at wavelength λ_1 (nm).

Analysis of fluorescence data

The fluorescence data of SVA in the presence of quenchers were analyzed by a normal Stern-Volmer plot:

$$\frac{F_0}{F} = 1 + K_q[\phi] \quad (1)$$

where F_0 and F are the protein fluorescence collected from full emission scans in the absence and in the presence of quencher, respectively, $[\phi]$ is the quencher concentration, and K_q is the Stern-Volmer quenching constant. The slope of the limiting straight line from a plot of F_0/F versus $[\phi]$ defines the parameter of K_q (Lehrer and Leavis, 1978). The same data were analyzed by a modified Stern-Volmer plot (Lehrer, 1971):

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K_q[\phi]} \quad (2)$$

where f_a is the fraction of fluorescent groups accessible to quencher. A plot of $F_0/(F_0 - F)$ versus $1/[\phi]$ will yield a straight line whose slope is $(f_a K_q)^{-1}$ and intercept is $1/f_a$.

The modified Scatchard plot (Epstein et al., 1974) was constructed to analyze the fluorescence data of a complex formed by Zn^{2+} and SVA:

$$\frac{|\Delta F|}{[L]_{free}} = (K_L \cdot F_\infty) - K_L \cdot |\Delta F| \quad (3)$$

where ΔF is the change in protein fluorescence on adding metal ion, L, and F_∞ is the protein fluorescence in the absence of metal ion. K_L is the association constant of the complex concerned. Throughout the titration, $\Delta F/[L]_{total}$ was plotted against ΔF, because $[L]_{free}$ was close to $[L]_{total}$.

RESULTS

Gross conformation of SVA

We found that SVA was not reactive to Ellman's reagent (Ellman et al., 1961), indicative of the absence of a free thio group on the protein molecule. The protein also did not react with Ellman's reagent when the experiment was performed in the presence of 6.0 M urea. Apparently, there are no free cysteins that are partially or fully buried in the protein molecule. SVA I and SVA II have the same protein core (Yu et al., 1993), and they show no difference in their CD spectra. The CD spectrum of 16.5 μM SVA in 5 mM Tris-buffered saline (TBS) at pH 7.4 has at least five bands: bands I–III in the near-UV region arise from nonpeptide chromophores (Fig. 1, right side) and bands IV and V in the UV region are mainly due to peptide chromophores (Fig. 1, left side). Based on the CD spectra of protein conformation (Greenfield and Fasman, 1969; Chen et al., 1972, 1974;

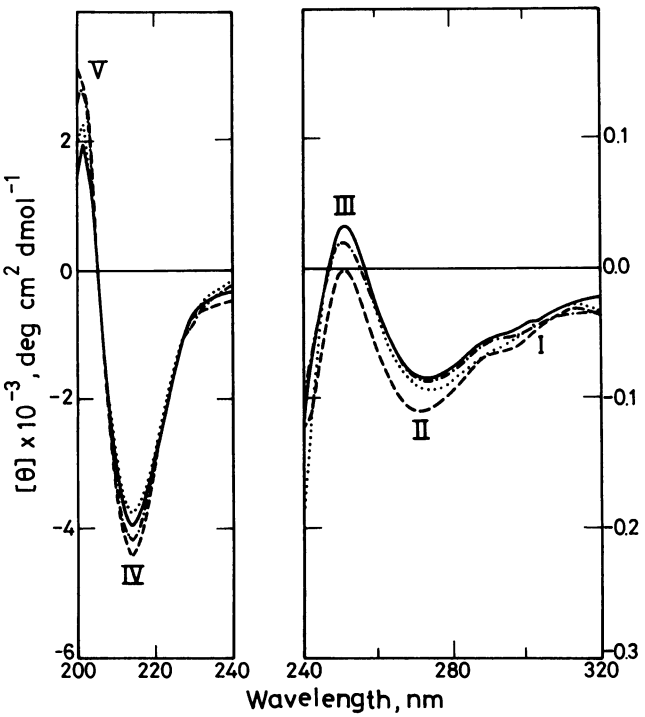

FIGURE 1 Circular dichroism of SVA. The protein was in 5 mM TBS at pH 7.4 (——) and in the presence of 1 mM $CaCl_2$ (· · ·), 1 mM $ZnCl_2$ (– – –) or 1 mM $MgCl_2$(– · –).

Chiang et al., 1978; Perczel et al., 1992), the lack of a double minimum at 222 and 208–210 nm, which is characteristic of α helical conformation, suggests no helices in the SVA molecule. Furthermore, the protein is not completely unordered, which would have shown a strong negative band near 197 nm. Band IV is a negative one with a minimum at 217 nm, which bears some resemblance to that of the β form around 217 nm. Band V is a weak positive one with a maximum around 200 nm, which is much smaller than that of the β form around 195 nm. Apparently, the SVA molecule contains some ordered structure other than the helix, probably a mixture of β form, β turn, and unordered form (see Discussion). Band II is a negative one with a minimum at 274 nm and band III a very weak positive one with a maximum at 252 nm. Band I may be a broad negative one that is concealed in the range 290–320 nm. According to the fine CD structures of aromatic groups of a protein in the near UV region, the wavelength range for tryptophan, tyrosine, and phenylalanine is usually located between 280 and 310, 265 and 290, and 250 and 270 nm, respectively (Chen et al., 1982). It is suggested that band II arises from tyrosine residue(s) and band I may be assigned to tryptophan residue(s). The CD spectrum of phenylalanine residues in a protein is usually weak, suggesting the contribution of phenylalanine residue(s) to band III. The description of bands I–III to specific residues may be fortuitous. The CD data from site-specific mutagenesis for the protein in the future may support the assignments. Addition of $CaCl_2$, $MgCl_2$, or $ZnCl_2$ to a final concentration of 1 mM in the

protein solution changes slightly the CD due to both peptide and nonpeptide chromophore (Fig. 1).

The secondary structures of SVA are stable over a wide range of pHs. Even at pH 3.0 or 10.5, the profiles of CD bands IV and V remain essentially the same as in neutral solution, although there is a slight change in magnitude (Fig. 2, left side). In contrast, the CD spectrum due to the nonpeptide chromophores is sensitive to pH (Fig. 2, right side). Band I does not alter at pH 10.5 but becomes prominent with a more negative extremum at pH 3.0, suggesting that the protonation of an acidic amino acid residue disturbs the configuration of the band I-associated aromatic residue. At pH 10.5 or 3.0, band II becomes a less negative band with a shift of extremum to longer wavelength, and band III changes to a large positive band.

Status of tryptophan residues of SVA

One SVA molecule contains nine tyrosine residues and two tryptophan residues, namely Trp^2 and Trp^{82}. Excitation of the protein in 5 mM TBS (pH 7.4) at 275 nm manifests two emission bands with maxima at 306 and 345 nm (Fig. 3 *a*). The peak at the shorter wavelength is contributed by tyrosine residues that do not transfer their resonance energy to tryptophan residues. The peak at the longer wavelength arises from tryptophan residues, which are excited directly with absorption energy or indirectly through resonance energy transferred from their proximate tyrosine residue(s). Apparently, the tyrosine residues responsible for the peak at

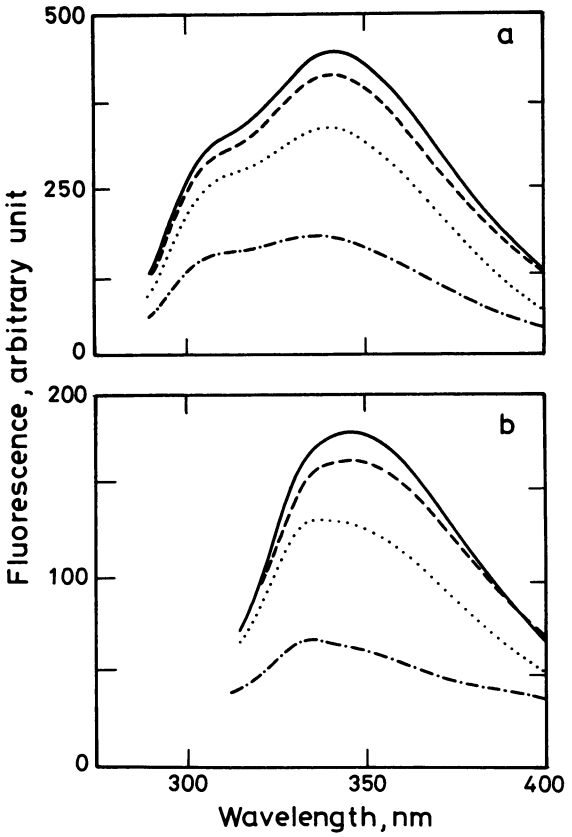

FIGURE 3 Fluorescence emission spectra of SVA. The emission spectra were scanned with excitation wavelength at 275 nm (*a*) or 295 nm (*b*). The protein was at 5 μM in 5 mM TBS at pH 7.4 (——) and in the presence of 0.1 M CsCl (– – –), 0.1 M KI (· · · ·) or 0.1 M acrylamide (– · – ·).

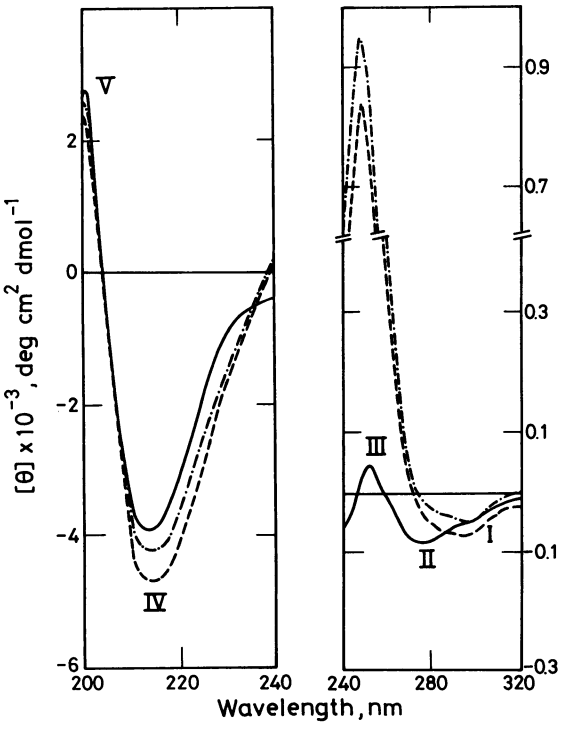

FIGURE 2 pH dependence of CD of SVA. Curves for pHs: (——), 7.4; (– – –), 3.0; (– · – ·), 10.5.

306 nm may be on the domains of protein, which are separable from Trp^2 or/and Trp^{82} and their neighboring tyrosine residues for the peak at 345 nm. Excitation of the protein at 295 nm ensures the protein fluorescence due to the two tryptophan residues. There emerges only one emission band with a maximum at 345 nm (Fig. 3 *b*). Addition of CsCl, KI, or acrylamide to the protein solution diminishes the protein fluorescence (Fig. 3). A blue shift occurs in the tryptophyl spectrum during the course of a quenching experiment performed for each of the three quenchers. Acrylamide as well as KI at a concentration higher than 0.2 M in the protein solution shifts the maximum of the emission band to 332 nm. CsCl at 0.4 M in the protein solution shifts the maximum to 335 nm. Fig. 4 displays a normal Stern-Volmer type of graph that was plotted according to Eq. 1 by fitting the data collected from the full emission scan as the protein was excited at 295 nm in the presence of quenchers. The plot from the quenching effect of acrylamide shows significant downward curvature, revealing the presence of fluorophor heterogeneity regarding the two tryptophan residues. The downward curvature is also caused by either KI or CsCl, although it is not as prominent as that caused by acrylamide. On the basis of f_a determined by fitting the data from quenching effect to Eq. 2 (Fig. 4, inset), about

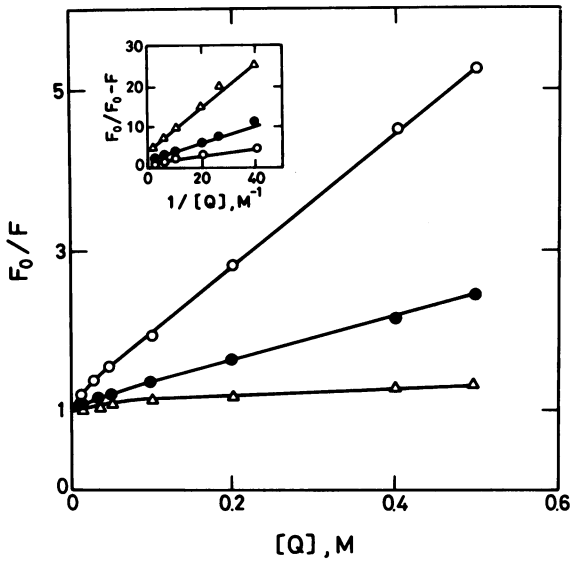

FIGURE 4 Susceptibility of the two tryptophan residues of SVA toward quenchers. The fluorescence data of SVA in the presence of quencher were fitted with Eq. 1 described in the text. The modified Stern-Volmer plot based on Eq. 2 is given in the inset. △, in CsCl solution; ●, in KI solution; ○, in acrylamide solution. The data collected from the full emission scan as the protein was excited at 295 nm were used for the analysis. The correlation coefficient was calculated to be larger than 0.98 for each linear curve in the inset.

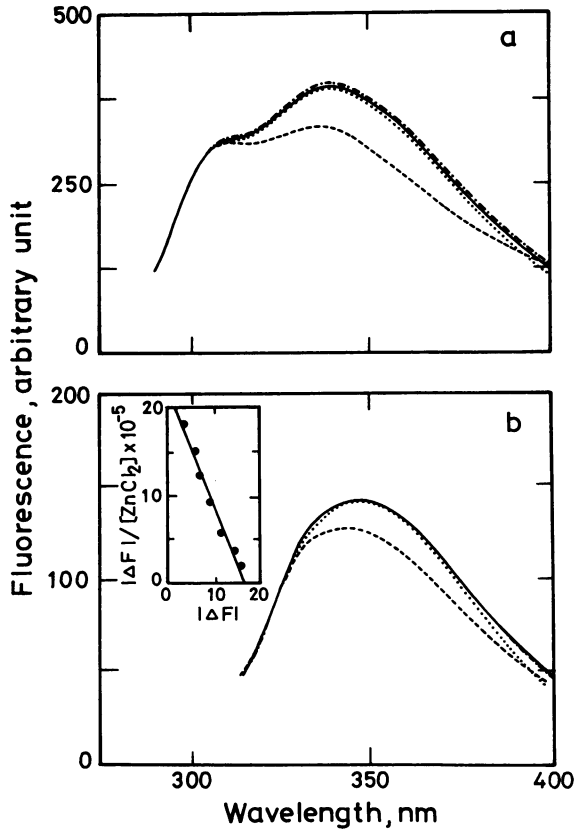

FIGURE 5 Effect of divalent metal ion on the fluorescence of SVA in 5 mM TBS at pH 7.4. The emission spectra were scanned with excitation wavelength at 275 nm (*a*) or 295 nm (*b*). The SVA protein was at 6.5 μM alone (——) and in the presence of 0.1 mM $CaCl_2$(– · – ·), 0.1 mM $MgCl_2$ (· · · ·) or 0.1 mM $ZnCl_2$ (– – –). The modified Scatchard plot for the binding of Zn^{2+} to SVA is given in the inset. The data of from adding $ZnCl_2$ to the protein solution were analyzed by using Eq. 3 with linear-regression fitting. The correlation coefficient was calculated to be more than 0.98.

33 ± 2.0%, 65 ± 2.0% and 87 ± 4.0% of the two tryptophan residues are likely to be accessible to CsCl, KI, and acrylamide, respectively (Fig. 4). These data suggest the two tryptophans on the protein surface. One may be around an acidic amino acid residue and the other may be around a basic amino acid residue.

Characterization of the Zn^{2+}-binding site on SVA

Fig. 5 gives the fluorescence emission spectrum of SVA in the presence of 0.1 mM $ZnCl_2$, $MgCl_2$, or $CaCl_2$ in the protein solution at pH 7.4. Neither $CaCl_2$ nor $MgCl_2$ in the protein solution changes the protein fluorescence with respect to both spectral profile and emission intensity. In contrast, $ZnCl_2$ in the protein solution shifts the tryptophan-associated peak to 335 nm and diminishes F_{275}^{345} as well as F_{295}^{345} to a considerable extent, although it effects only a slight change in F_{275}^{306}. Fitting the data of F_{295}^{345} obtained from adding Zn^{2+} to the protein solution with Eq. 3 shows a linear curve in the modified Scatchard plot (Fig. 5 *b*, inset). This manifests the existence of a singular type of Zn^{2+}-binding site on the protein molecule. The association constant of this affinity site is $1.35 \times 10^5 \pm 0.04 \times 10^5$ M^{-1}. Fig. 6 displays the resolution of an incubated solution containing 0.16 mM SVA and 0.83 mM $ZnCl_2$ in TBS at pH 7.4 by a PD-10 column. A peak of Zn^{2+} emerges in the elution profile of protein before the appearance of free Zn^{2+}. These data together indicate the ability of Zn^{2+} to bind to SVA.

DISCUSSION

CD spectroscopy is one of the most sensitive physical methods for studying protein conformation in a solution. The α helix, β form, β turn, and unordered form all display their characteristic CD bands in the UV region. The content of various types of secondary structures in a protein molecule has been determined by quantitative analysis of its CD spectra (Greefield and Fasman, 1969; Chen et al., 1972, 1974; Chiang et al., 1978; Perczel et al., 1992; Sreerama and Woody, 1994). This usually gives a good to excellent estimate of the helicity, but the estimation for other conformations remains equivocal. The CD spectrum of the β turn bears some resemblance to that of the β form, and the estimation of the β turn does not seem to be significantly related to the x-ray results of protein of known three-dimensional structure (Chiang et al., 1978). Nevertheless, the CD spectrum of a protein remains valuable in the justification of its secondary structures predicted by the methods that may locate the helices, β forms, β turns, and coil regions along a polypeptide chain. In this regard, the

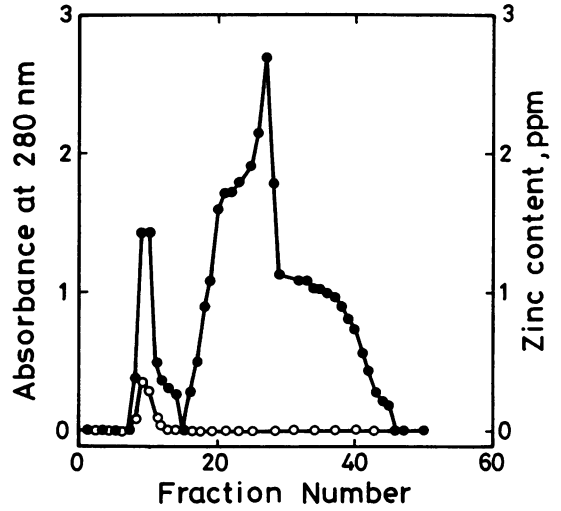

FIGURE 6 Demonstration of the ability of SVA to bind to Zn^{2+}. A solution of 0.83 mM $ZnCl_2$ and 0.16 mM SVA in 5 mM TBS at pH 7.4 was preincubated at room temperature for 2 h. The solution was subjected to chromatography on a PD-10 column. Each 0.45-ml effluent was collected. The absorbance of protein solution was measured at 280 nm (○), and the Zn^{2+} content was determined from atomic absorption analysis (●).

Chou and Fasman algorithm (Chou and Fasman, 1974a,b, 1978) and the GOR algorithm (Garneir et al., 1978), using different criteria, are popular for predicting the secondary structures based on protein sequences. Either method succeeds with a certain degree of accuracy for the prediction. For the time being, we applied the former to predict the potentials for the formation of secondary structures in SVA (Fig. 7). The segments of residues 15–20, 33–38, and 45–59 contain the overlapping α-potential residues that form the helix and β-potential residues that form the β form. The helical formation of the three peptide segments may be overpredicted in view of our CD results, which indicate the absence of helical conformation in the SVA molecule.

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        10         20         30         40         50
         :          :          :          :          :
FWYHGLHNEPRNYTLTLNMKITPSTSITNKKGNEFRVQLTVKNNVKQCQE
               aaaaaa               aaaaaa      aaaaaa
bbbbbbbb  bbbbbbbbb     bbbbb     bbbbbbb bbbbbbb
          tttt         tttt  tttt         tttt

        60         70         80         90        100
         :          :          :          :          :
VEINTEHERYIKFLSGPSTFETCICTISDFFWDIYVSESTYLTLSASILP
aaaaaaaaa
bbbbb    bbbbbb    bbbbbbbbb  bbbbbbb  bbbbbb
      tttt   tttttt                     tttt      tt

       110        120        130
         :          :          :
YKNKCPDSDEPLLYGDSYQMYNITDKINVTP

                bbbbbbbb bbbbb
ttttttttttt     tttt
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FIGURE 7 The predicted secondary structures of SVA. The secondary structures were predicted by Chou and Fasman algorithm. a, helix former; b, β form former; t, β turn former.

The predicted secondary structure, together with the position and sign of band IV of SVA (Fig. 1), strongly supports the existence of a considerable amount of β form in the protein molecule, although the presence of β turn cannot be ruled out.

There are four cysteins, namely Cys^{48}, Cys^{73}, Cys^{75}, and Cys^{105}, in the SVA molecules. Lack of a free thio group in the protein reveals the formation of two disulfide bridges from these cysteins. Disulfide bonds between Cys^{73} and Cys^{75} are unlikely because these would result in increasing structural strain along the backbone of Cys^{73}-Ile-Cys^{75}. Therefore, either the cross-linkages of Cys^{48} to Cys^{73} and Cys^{105} to Cys^{75} or the cross-linkages of Cys^{48} to Cys^{75} and Cys^{105} to Cys^{73} remain to account for the formation of two disulfide bonds that bring about the peptide regions around Cys^{48} and Cys^{105} near the polypeptide region around Cys^{73}-Ile-Cys^{75}. The positions of the two disulfide bonds divide the polypeptide chain of SVA into three regions: an N-terminal region of 47 residues, a central region of 58 residues, and a C-terminal region of 26 residues. The central region constitutes two loops based on the two disulfide bonds. Loop 1 is formed by cyclization of Cys^{48} to Cys^{73} or Cys^{75} and loop 2 by cyclization of Cys^{105} to Cys^{73} or Cys^{75}. If residues 69–77 form a β segment (Fig. 7), Cys^{73} and Cys^{75} should be at the same side of pleated sheet. This restricts the steric arrangement of the amino acid residues proximate to the four cysteins. The charged groups are distributed unevenly over the SVA molecules. At physiological pH values, the net charges are $+6e$ in the N-terminal region, $-2e$ in loop 1, $-e$ in loop 2, and $-5e$ in the C-terminal region. It is likely that there are positive charges clustered around Trp^2 and negative charges around Trp^{82}, which is near Asp^{83}.

It is well known that acrylamide, KI, or CsCl exerts dynamic quenching that is predominantly collisional. Our study about their quenching effect can yield information regarding the status of two tryptophan residues of SVA. According to the empirical rule of Kronman and Holmes (1971), the characteristics of λ_{max} of fluorescence suggest the two tryptophans on the protein surface. This is supported by their accessibility to acrylamide. The results from the study of ionic quenchers (Fig. 4) suggest the difference in the "local charge" around them. This is congruent to the charged groups around Trp^2 and Trp^{82}.

The chromatographic characteristics shown in Fig. 6 together with the results summarized from the effect of metal ions on the SVA fluorescence support a specific binding of Zn^{2+} to the protein. The diminution of F_{275}^{345} or F_{295}^{345} in the Zn^{2+}-SVA complex formation together with the consideration of the charged groups around the two tryptophan residues suggests that Trp^{82} is at or near the Zn^{2+}-binding site. The configuration around Trp^{82}, which is predicted in the β form, may not change drastically in the Zn^{2+}-SVA complex formation, as suggested by the observation that the CD fine structures are not altered by the addition of $ZnCl_2$ to the protein solution. A blue shift of the tryptophyl spectrum by the presence of acrylamide, KI, or CsCl in the

protein solution may be due to selective quenching of solvated tryptophyls, which emit at longer wavelength. It is likely that the coordination with Zn^{2+} may exclude water in the complex and thereby decrease the polarity of solvent around Trp^{82}. This causes a shift of λ_{max} of the tryptophan fluorescence to a shorter wavelength (Fig. 5). The inability of both Mg^{2+} and Ca^{2+} to diminish F_{275}^{345} or F_{295}^{345} implies the inflexibility of the Zn^{2+}-binding site, which is apparently not coordinated with Ca^{2+} or Mg^{2+}. It has been known for a long time that there are high concentrations of zinc in the male reproductive tract and semen of mammals. The zinc requirement of cells of the reproductive system, sperm, oocytes, as well as embryos has received particular attention. Despite the many reports relevant to the effect of zinc deficiency on the proliferation and differentiation of these cell types (Vallee and Falchuk, 1993), the function of this metal remains obscure. The biological significance of Zn^{2+}-SVA complex formation invites future investigation.

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