Purification and Characterization of the Cell Surface Virulent A Protein from *Aeromonas salmonicida*†

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ABSTRACT: The predominant cell surface protein (A protein) of *Aeromonas salmonicida* has been purified in high yield from outer membranes by using a combination of detergent and chaotropic extraction methods as well as exclusion and ion-exchange chromatography. The A protein was primarily monomeric, M_r 50000, but readily formed oligomers at high protein or low salt concentrations. Several isoelectric forms were distinguishable with purified protein as well as in situ on the cell surface. Neither phosphate nor carbohydrate was detectable. The A protein was hydrophobic in composition and the N-terminal sequence highly hydrophobic. From CD spectra the A protein exhibited 14% α helix and 19–28% β structure and could also be readily crystallized. By fluorescent antibody staining the A protein was shown to cover the entire cell surface but was absent from A protein deficient mutants. This protein appears to have no apparent enzymatic activity but rather constitutes a macromolecular refractile protein barrier essential for virulence.

Numerous species of Gram-positive and Gram-negative bacteria possess superficial layers composed of regularly arrayed structures of tetragonal or hexagonal symmetry (Thorne, 1977; Sleytr, 1978; Stewart & Murray, 1982). Such layers are largely composed of protein subunits assembled with a high degree of structural regularity over the cell surface. These subunits are not covalently attached either to each other or to underlying cell wall components although they appear to be associated with them (Thorne et al., 1975; Chester & Murray, 1978; Hastie & Brinton, 1979a,b). In a few cases protein subunits of these layers have been solubilized and reassembled either in the absence or in the presence of template structures (Hastie & Brinton, 1979a,b; Sleytr, 1978; Masuda & Kawata, 1981). Cell surface proteins of these kinds are of considerable interest because they represent the predominant interface between the cell and the environment, have been functionally implicated in the protection against predators (Buckmire, 1971), lytic enzyme (Nermut & Murray, 1967; Wallinder & Neujahr, 1971), heavy metals (Beveridge, 1979; Beveridge & Murray, 1976), and bacteriophage (Ishiguro et al., 1981), and have also been suggested to have a permeability function as well (Stewart et al., 1980).

Aeromonas salmonicida, an important pathogen of fish, possesses a superficial coat called the A layer (Udey & Fryer, 1978; Trust et al., 1980; Hamilton et al., 1981). This layer is principally composed of a 50-kdalton tetragonally arrayed protein known as the A protein (Kay et al., 1981) which protects the cell from bacteriophage (Ishiguro et al., 1981) and serum complement (Munn et al., 1982) as well as several proteolytic enzymes (unpublished results). The A layer and A protein have also been shown to be essential for the virulence and the autoaggregating property of this organism (Kay et al., 1981; Ishiguro et al., 1981) and have even been suggested to be an essential component of vaccines (McCarthy et al., 1982).

Beyond purification, no detailed studies on the properties of bacterial surface layer protein subunits have been reported. In this paper we report on the large-scale purification and physical and chemical properties of the A protein of *A. salmonicida*.

Materials and Methods

Growth Conditions. Virulent *A. salmonicida* A450 was used in all studies. Cells were grown in complex media (SP broth) consisting of 2% glucose, 50 g of tryptone, 30 g of yeast extract, 7.5 g of NaCl, and 3.5 mL of 10 N NaOH per L. The pH was adjusted to 7.25 with concentrated H_2SO_4. Cells were grown for 48 h from 1% inoculum at 23 °C in a 10-L fermentor. The pH was controlled, and the glucose content was repeatedly adjusted to 2% immediately after exhaustion. Aeration was maintained at 12 L min^{-1}. Cells (160 g wet weight) were harvested by centrifugation and frozen at −20 °C.

Purification of A Protein. Frozen cells were thawed, resuspended in 500 mL of 20 mM Tris-HCl[1] (pH 7.3) (TB), and broken in the presence of RNase and DNase by a single pass through a French pressure cell at 16000 psi. Particulate matter and membranes were sedimented by centrifugation at 30000*g* for 1.5 h. Microscopic observation of the pellet indicated that breakage was complete. The pooled membrane pellets were resuspended by blending and extraction for 35 min at room temperature in 1 L of 1% sodium laurylsarcosinate (SLS) (Filip et al., 1973). The residual outer membranes (OM) containing A protein were collected by centrifugation at 30000*g* for 80 min. The SLS extraction was repeated on the membrane pellet to get rid of cytoplasmic membrane contamination. The SLS-insoluble material (OM) was resuspended in 370 mL of TB, sedimented once more at 135000*g* for 2.5 h, and kept at 0 °C. This procedure yielded 19.8 g of OM. The various fractions were monitored for their A protein content by $NaDodSO_4$ electrophoresis on 12% polyacrylamide gels. The OM fraction contained an abundance of the A protein, with relatively little loss. The OM pellet was then resuspended in 500 mL of 2% deoxycholate, 0.2 M NaCl, 5 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF), stirred for 1 h at room temperature, and centrifuged at 135000*g* for 3 h. This procedure was repeated on the pellet. The pellet was then resuspended in 340 mL of 6 M guanidine hydrochloride, 5 mM EDTA, and 20 mM Tris-HCl (pH 8.0), stirred for 1 h at room temperature, and centrifuged at 135000*g* for 3 h. Approximately 70% of the DOC pellet was solubilized. The supernatant containing the A protein (360 mL) was desalted by

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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Sephadex G-25 chromatography (bed volume 1800 mL, void volume 720 mL) in TB buffer. All the protein eluted broadly between 800 and 1200 mL. A large sharp peak also eluted just prior to the salt front which proved to be lipopolysaccharide (LPS). The pooled protein sample was loaded onto a DEAE-Sepharose CL-6B column (175 mL bed volume), equilibrated with 10 mM Tris-HCl (pH 8.0), washed with 4 bed volumes of the equilibration buffer, and eluted on a linear gradient of 2.5 L of 0–0.35 M NaCl in the same buffer. The major peak consisting of A protein eluting at 110 mM NaCl was pooled. This peak was concentrated by either membrane filtration or dialysis against poly(ethylene glycol) to 10–20 mg mL^{-1} and either stored on ice or frozen.

Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out either by the method of Ames (1974) or by Hancock & Carey's (1979) modification of the system of Neville (1971). Nondenaturing gels were run in the absence of $NaDodSO_4$. Nondenaturing isoelectrofocusing (IEF) gels were run according to the Bio-Rad manual procedure and were stained with Coomassie blue either by conventional methods or by the perchloric acid rapid stain (Reisner et al., 1975). Denaturing IEF gels in the presence of 8 M urea and 2% NP40 were run in the system of O'Farrell (1975).

Molecular Weights. Solution molecular weights were determined by analytical ultracentrifugation on a Beckman Model E ultracentrifuge equipped with photoelectric scanner and interference optics. Molecular weight estimations were also carried out by high-performance liquid chromatography (HPLC) in the presence and absence of 0.1% $NaDodSO_4$ on an Altex Spherogel-Tsk (7.5 mm × 60 cm) molecular sieving column by using a Beckman Model 100A HPLC. Columns were calibrated with molecular weight standards of 14 000–24 000 in the same buffer as the sample. $NaDodSO_4$–polyacrylamide gel electrophoresis on calibrated gels was used to estimate the monomeric molecular weight.

Circular Dichroism (CD) Measurements. CD spectra were recorded on a Cary 60 spectropolarimeter fitted with a Model 6001 CD accessory operating over a wavelength range of 310–190 nm employing technology described previously (Oikawa et al., 1968). The CD data are expressed in terms of molar ellipticities.

N-Terminal Sequence Analysis. The amino acid sequence was carried out on a Beckman Model 890C protein sequenator by using a 0.1 M quadral program. Phenylthiohydantoin derivatives were isocratically separated by the procedure of Tarr (1981) on octadecylsilane ultrasphere HPLC columns (Altex) fitted to a Beckman Model 332 HPLC equipped with a Hewlett-Packard 3390A integrator.

Immunological Methods. Antisera against the A protein were raised in rabbits by an injection of 50 μg of protein in Freund's complete adjuvant, followed by two successive injections of 50 μg in Freund's incomplete adjuvant at 2-week intervals. Fresh normal serum from the same rabbit was obtained prior to inoculation. The IgG fraction was prepared by $(NH_4)_2SO_4$ precipitation and DEAE-Sepharose chromatography (Garvey et al., 1977). Immunotransblots of $NaDodSO_4$–polyacrylamide gel electrophoresis membrane preparations were prepared by electrophoresing the gels onto nitrocellulose sheets by a Bio-Rad Trans-Blot Cell equipped with a Model 160/1.6 power supply. Blots were stained by the immunoperoxidase method (Anderson & Hickman, 1981). Cells stained indirectly with fluorescent antibody were observed and photographed with a Zeiss fluorescence microscope.

Crystallizations. Crystallizations of the A protein were carried out by vapor diffusion. Hanging 50-μL drops of pure

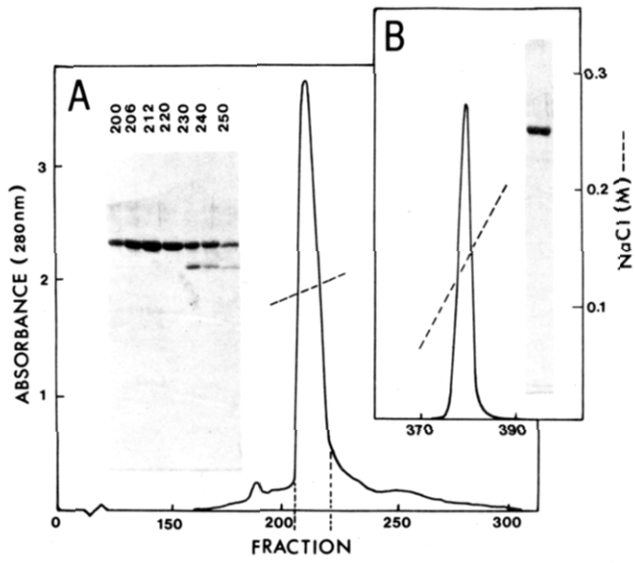

FIGURE 1: Purification of A protein from *A. salmonicida* outer membranes. Desalted guanidine hydrochloride extracted proteins were chromatographed on a 1.3 cm × 33 cm (175 mL bed volume) DEAE-Sepharose column. The column was developed with a linear gradient of 0–0.35 M NaCl in 10 mM Tris-HCl (pH. 7.0), and 7-mL fractions were collected. (A) The initial separation, from which the fractions indicated under the peak were chromatographed once more (B). (Inset) $NaDodSO_4$–polyacrylamide gel electrophoresis profiles of selected peak fractions.

A protein in 10 mM Tris-HCl (pH 8.0) and 0.1 M NaCl were exposed to 1.5-mL aliquots of a variety of solvents, salts, or desiccants in plastic multichamber wells at 4 °C.

Results

The purification of the A protein from membrane preparations of *A. salmonicida* involves the removal of inner-membrane material by solubilization in SLS, followed by the selective removal of most of the contaminating OM proteins by solubilization with DOC. This procedure resulted in a relatively clean membrane preparation composed of the two principle OM associated proteins (49 and 42 kdaltons) (Kay et al., 1981). From this fraction the A protein was solubilized in 6 M guanidine hydrochloride in a relatively pure state, but this procedure also released some 42-kdalton material. The degree of this contamination appears to vary with the preparation. The vast majority of the LPS appears to also be liberated at this stage and was removed and recovered during desalting. The A protein was finally eluted as a sharp peak on DEAE-Sepharose (Figure 1). The total yield of A protein by this method was 523 mg/160 g wet weight of cells and was estimated to be greater than 99% pure by $NaDodSO_4$–polyacrylamide gel electrophoresis and staining with either Coomassie blue or silver (Oakley et al., 1980) (Figure 1B). No LPS was detected by either silver staining or Schiff's staining methods, and no carbohydrate was detectable by GLC analyses of the alditol acetates of acid-hydrolyzed A protein. However, a trace of anti-LPS antibody was still detectable in immune sera after immunization with this preparation (Trust et al., 1982), suggesting that a small amount of LPS still remained tightly adsorbed to the protein. Nevertheless the preparation appeared to be pure by immunodiffusion, immunoelectrophoresis, and two-dimensional gel electrophoresis followed by electrophoretic transblotting against anti-A protein antisera, which indicated that only the several isoelectric forms of A protein were cross-reactive and that no other immunologically detectable proteins were present by this sensitive method (Figure 2). Purity was also confirmed by N-terminal amino

FIGURE 2: Two-dimensional gel electrophoresis of pure A protein from *A. salmonicida*. A sample of purified A protein was separated by the procedure of O'Farrell (1975) and stained with siliver (Oakley et al., 1980). (B) A duplicate gel was electrophoretically blotted onto nitrocellulose and stained by the immunoperoxidase method after reaction with anti-A protein antiserum.

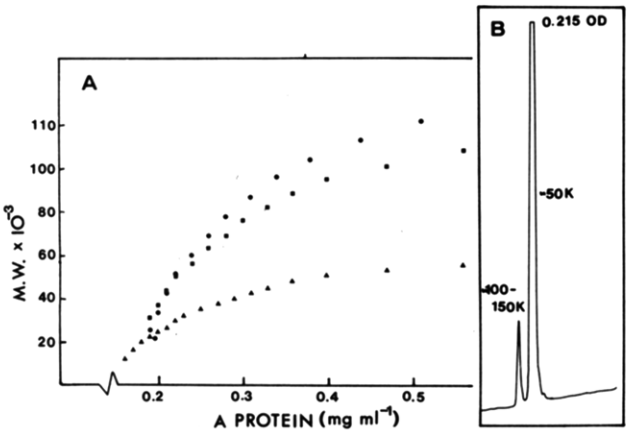

FIGURE 3: Molecular weight characterization of the A protein from *A. salmonicida*. (A) Samples of A protein (10 mM Tris-HCl, pH 7.0) analyzed by ultracentrifugation in an analytical ultracentrifuge at different speeds: (●, ■) spins at 10000 rpm spins and (▲) at 14000 rpm. (B) Separation of A protein monomers and oligomers by HPLC molecular sieving chromatography. The large peak corresponds to the 50-kdalton monomer and the small peak of 110–150-kdalton aggregates.

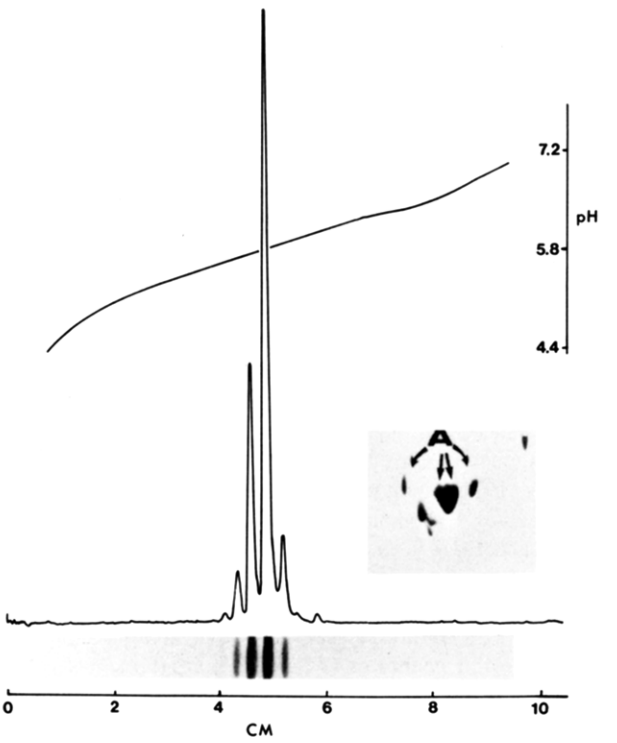

FIGURE 4: Isoelectric focusing of the A protein of *A. salmonicida*. A sample (50 μg) of pure A protein was electrofocused for 24 h in a pH 3–10 ampholine gradient containing 0.2% NP40 and 8 M urea. A duplicate gel was used for pH determination. The sample gel was stained with Coomassie brilliant blue, destained, and scanned at 600 nm on a Gilford spectrophotometer equipped with a gel scanner. (Inset) Two-dimensional gel region of whole cells of *A. salmonicida* which had been added directly to 0.1% $NaDodSO_4$ containing 0.1 mM PMSF and boiled prior to electrophoresis. The A protein is indicated by the arrow.

acid analysis (Asp). Curiously, the A protein was completely soluble after this purification, but it was still found to precipitate in low ionic strength buffer (<10 mM Tris-HCl).

The A protein had a molecular weight of 50100 ± 600 (10 determinations) by $NaDodSO_4$–polyacrylamide gel electrophoresis and by molecular sieving in 0.1% $NaDodSO_4$ by HPLC, but anomalous behavior was observed during both sedimentation analysis (Figure 3A) and molecular sievin g without $NaDodSO_4$ (Figure 3B). The solution sample was run both in the photoelectric scanner and also with interference optics. In either case the sample showed evidence of both aggregation and some dissociation. Molecular weight determinations at high protein levels revealed a major M_r 50000 component but only after a preliminary high-speed run to sediment aggregates (~30–40% of material). These aggregates appeared to have a molecular weight of at least 110000. These results are in good agreement with $NaDodSO_4$–polyacrylamide gel electrophoresis results of a monomeric molecular weight of approximately 50000 for the A protein (Figure 1). At low protein concentrations, however, little tendency to form multimers was indicated by HPLC molecular sieving (Figure 3B), and the small peak represented aggregate molecular weight of 100000–150000.

Under either nondenaturing or denaturing (2% NP40 plus 8 M urea) conditions the A protein focused into several discrete bands, having p*I*s of 5.7, 5.75, 5.85, and 5.9, respectively (Figure 4). Polyacrylamide gel electrophoresis in nondenaturing gels indicated three diffuse bands, also suggesting some tendency to form multimers (data not shown). Solubilization at room temperature for 30 min in 0.1% $NaDodSO_4$ resulted in complete conversion to the subunit form. However, multiple forms on IEF gels indicate small but definitive shifts in p*I*s. These remained unchanged after precipitation in acetone and ether washing to remove any traces of contaminating LPS. These are presumed to be deamidated forms of the protein as no phosphate was detected after $NaDodSO_4$ gel electrophoresis and autoradiography of cells grown in $[^{32}P]PO_4$, and no carbohydrate was detectable by GLC analysis of an acid-hydrolyzed sample converted to alditol acetates. These isoelectric forms were only partially generated during purification since they were also present on whole cells lysed directly in $NaDodSO_4$ in the presence of 0.1 mM PMSF and analyzed immediately by two-dimensional gel electrophoresis (Figure 4, inset), although the proportion of different isoelectric forms appeared to change during purification.

Analysis of the CD spectra of pure A protein (Figure 5) indicated that in solution A protein had a higher degree of secondary structure than the powder form with 14% α helix and ~19–28% β structure. The addition of neither 1 mM dithiothreitol (DTT) nor EDTA altered this proportion. The addition of 0.12% $NaDodSO_4$ resulted in an ~15% increase in α helix and a corresponding ~10% decrease in β structure. Both α helix and β form were increased by ~8% and 6.5–9.8%, respectively, in the presence of 30 mM octyl glucoside.

From the amino acid composition (Kay et al., 1981) the A protein was found to be substantially hydrophobic (~45% including Pro). This was also suggested by N-terminal sequence analysis (Figure 6). The N-terminal sequence is

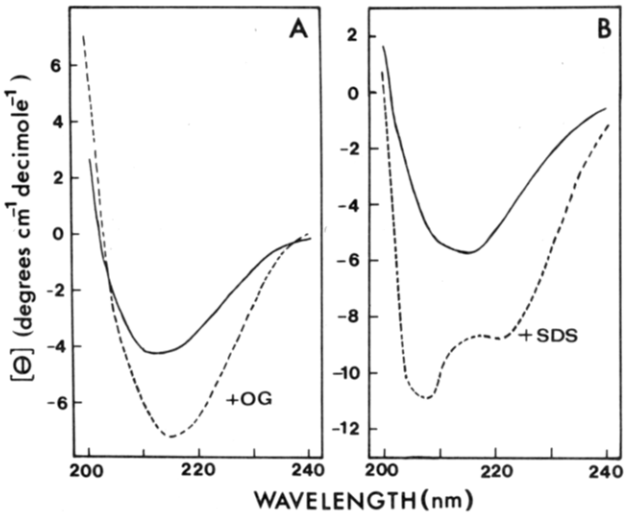

FIGURE 5: Circular dichroism spectra of pure A protein from *A. salmonicida*. CD are expressed in terms of molar ellipticities. (A) The powder form spectra: the broken line represents the spectrum in the presence of the nonionic detergent 0.1% octyl glucoside (OG). (B) The solution spectra: the broken line is in the presence of 0.1% sodium dodecyl sulfate ($NaDodSO_4$).

Residue #	1	2	3	4	5
Residue	asp	val	val	ile	gly
	6	7	8	9	10
	pro	asn	asp	asn	thr
	11	12	13	14	15
	phe	val	thr	asn	ser
	16	17	18	19	20
	leu	ala	ser	val	thr
	21	22	23	24	25
	lys	gln	pro	val	leu
	26	27			
	ser/cys	phe			

FIGURE 6: N-Terminal amino acid sequence of the *A. salmonicida* A protein.

exceptionally hydrophobic, and one of the two charged residues in this series was the N-terminal Asp which would be zwitterionic.

In a study on the crystallization properties of the A protein we were readily able to form small needlelike crystals (<1-mm diameter) (not shown) by hanging drop vapor diffusion particularly against saturated solutions of Na_2HPO_4, $MgSO_4$, or poly(ethylene glycol) (M_r 6000), the latter giving reproducibly the largest crystals. Crystallizations were performed at 4 °C, but the crystals were found not to be stable at room temperature, dissolving within 2–3 h.

Fluorescein-conjugated goat anti-rabbit antiserum stained anti-A protein IgG coated whole cells of strain A450 uniformly, indicative of a complete surface layer; however, an A protein deficient mutant, A450-2 (Ishiguro et al., 1981; Kay et al., 1981) was devoid of detectable fluorescence (not shown). The A protein dependent autoaggregation properties of this strain were also evident (Kay et al., 1981). These data confirm the cell surface localization of the A protein and also demonstrate that purified A protein has at least some native conformation (Figure 7).

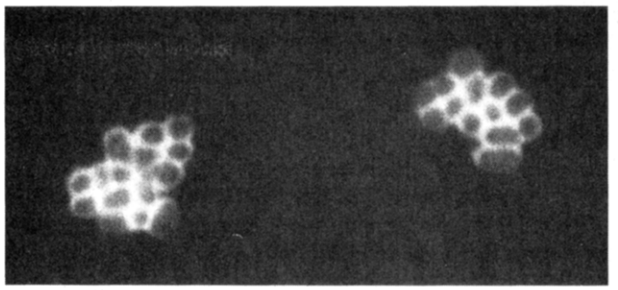

FIGURE 7: Fluorescent staining of whole cells of *A. salmonicida* A450. Formalin fixed cells were stained indirectly by using rabbit anti-A protein IgG followed by fluorescein-conjugated goat anti-rabbit antisera. Cells were visualized and photographed by epifluorescence with a Ziess fluorescent microscope. Fluorescence was enhanced in 0.1 N *n*-propyl gallate (Giloh & Sedat, 1982).

Discussion

The production of large quantities of A protein required particular attention be paid to the growth conditions. The media described here are relatively complex but free of sera and other expensive nutrients used by others to grow such fastidious organisms. The most critical factor was the temperature which must be kept relatively low to prevent the rise of A protein deficient strains (Ishiguro et al., 1981). The purification procedure outlined here takes advantage of the strong association of the A protein with the OM, one which could be disrupted only with chaotropic agents, especially guanidine hydrochloride. Solubilization by chaotropic agents is not an uncommon property of such proteins (Sleytr, 1978; Masuda & Kawata, 1980). From the recovery and from two-dimensional gels of whole cell protein it is clear that the A protein is an unusually abundant protein ($\sim 5 \times 10^5$ copies/cell) which is approximately equivalent to the quantity of OM lipoprotein of other Gram-negative strains. The cell surface exposure of the A protein has been confirmed by both direct and indirect fluorescent staining, whereas mutants specifically deficient in A protein were unreactive.

The A protein has a monomeric molecular weight of 50 000 which corresponds to an estimated volume of 650–820 Å^3, based on the two estimates available for bacterial cell surface proteins (Henderson & Unwin, 1975; Miller, 1982). The A protein appears to form multimers readily as evidenced by sedimentation and HPLC molecular sieving. The appearance of multiple isoelectric forms is not unusual, particularly for cell surface localized proteins, and several other membrane proteins have also been shown to be present in multiple isoelectric forms. What is important is that these forms are also present on the cell surface in vivo and are not due solely to the purification procedure. A similar conclusion has recently been reached with the surface protein of *Micrococcus* (Baumeister et al., 1982), the pili of *Neisseria* (Lambden, 1982), and the variable surface antigen of trypanosomes (Pearson et al., 1982). Whether these minor variations are of physiological significance remains to be determined; however, even extensive deamidations generally lead to only minimal perturbations of overall protein structure (Hunter & Ludwig, 1972; Wofsy & Singer, 1963) and do not necessarily influence crystallization

properties. The appearance of several bands on IEF gels of the surface protein from two *Clostridium* species has been interpreted as due to a tendency to reassemble into larger structures (Sleytr & Thorne, 1976). We have discounted this possibility on the basis of the near identical appearance of multiple isoelectric forms even under strong denaturing conditions.

From the unusually hydrophobic amino acid composition (Kay et al., 1981) the A protein would likely precipitate in aqueous buffers but does so only at relatively low ionic strength. That this protein appears to form multimers as well as crystals, albeit unstable, indicates that it is not likely in a denatured form even after guanidine hydrochloride solubilization. Furthermore retention of antigenic properties even after $NaDodSO_4$ solubilization and electrophoresis argues for its unusual stability. The A protein has a defined secondary structure by CD analysis, although it is somewhat high in aperiodic structure when compared to other membrane proteins. The high glycine and proline content ($\sim$20%) would likely lead to a low helix content. A small enhancement of secondary structure by detergents is not unusual but does suggest that the association with membrane lipid or other hydrophobic protein may also enhance the degree of secondary structure. These properties are very similar to the outer membrane porins of *Escherichia coli* (Rosenbusch, 1974) and the surface spinae of a marine pseudomonad (Coombs et al., 1976). Other membrane-imbedded proteins and peptides normally exhibit increased α-helical content upon association with phospholipids (Hammer & Schullery, 1970; Cockle et al., 1978; Shirahama & Yang, 1979), but these are generally characterized by long spans of α helix (Wallace, 1982). The lack of effect by either DTT or EDTA suggests that neither sulfhydryl nor inorganic ionic linkages strongly influence secondary structure. The latter is particularly important since the A protein is relatively acidic. It is possible that the A protein in solution is not completely in its native cell surface form and association with either lipid, LPS, or protein influences its in vivo conformation. However, since fluorescent antibody to purified A protein reacts strongly with whole cells, at least that part of the molecule normally exposed at the cell surface appears to be in its native conformation.

The sequence data indicate that the N-terminal region is particularly hydrophobic with approximately 50% hydrophobic amino acids and 30% polar but not charged. Recently, a N-terminal tetrapeptide sequence of a similar protein from an atypical strain was reported (Evenberg & Lugtenberg, 1982) and differs by only a single residue (Leu for Val) from that reported here. We have also recently determined the sequence of a 30-residue N-terminal segment from two other so-called "atypical" strains of *A. salmonicida*, and they are nearly identical with that reported here. Thus there appears to be a high degree of conservation in the primary sequence of this protein from various species (W. W. Kay et al., unpublished results).

The A protein of *A. salmonicida* has been suggested to be involved in cellular adhesion and virulence (Udey & Fryer, 1978; Trust et al., 1980; Evenberg et al., 1982). However, the only evidence regarding its role has been the absolute requirement for virulence (Key et al., 1981; Ishiguro et al., 1981) which appears to be at least partially due to the protection of the cell against serum killing (Munn et al., 1982). It is possible that other mechanisms of virulence attributed to other surface proteins could be ascribed to the A protein such as iron sequestration (Crosa, 1980) or enzymatic activity such as phospholipase A (Thorne et al., 1976). We have so far been unable to demonstrate any ion binding, phospholipase, or protease activity of the A protein, and so in the absence of other evidence the notion persists that the A protein represents a chemical, enzyme, complement, and bacteriophage refractile cell barrier, permeable only to essential solutes.

The A protein of *A. salmonicida* promises to be an excellent candidate for further structural analyses. Very few membrane proteins have been shown to form true three-dimensional crystals (Methews et al., 1979; Garavito & Rosenbusch, 1980; Michel & Oesterhelt, 1980; Leonard et al., 1981), and to our knowledge no other surface proteins of this kind have been crystallized. Hence little is known of the detailed molecular structure of such proteins. The development of stable crystals of the A protein suitable to X-ray diffraction analysis is currently in progress.

Acknowledgments

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Differences in the Interaction of the Polyene Antibiotic Amphotericin B with Cholesterol- or Ergosterol-Containing Phospholipid Vesicles. A Circular Dichroism and Permeability Study†

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ABSTRACT: The selective toxicity of the polyene antibiotic amphotericin B between pathogenic eukaryotic organisms and animal cells has often been said to originate in the presence of ergosterol in fungal membranes instead of cholesterol, found in membranes of animal cells. We have tested this hypothesis by measuring the proton efflux induced by amphotericin B in egg yolk phosphatidylcholine small unilamellar vesicles. By measuring circular dichroism under the same conditions, we monitored the interaction of the antibiotic and its conformational changes. Sterol-free vesicles are sensitive to amphotericin B, but the sensitivity of sterol-containing vesicles is always greater and increasingly so with increasing sterol concentration. Ergosterol-containing vesicles are more sensitive than cholesterol-containing vesicles. On the other hand, numerous amphotericin B conformers can be detected in sterol-containing vesicles, depending upon both the concentration of sterol and the amphotericin B sterol ratio. It appears that one conformer, or maybe two at high amphotericin B concentration, is responsible for the induced permeability. From their circular dichroism spectra, these two conformers are the same in the presence of ergosterol or cholesterol. The concentration of amphotericin B necessary to obtain the two conformers is higher with cholesterol than with ergosterol, which agrees with the permeability results.

The molecular basis of amphotericin B action on membrane ionic permeability is thought to be due to pore formation by amphotericin B–sterol complexes (Finkelstein & Holtz, 1973; Andreoli, 1974; De Kruijff & Demel 1974). However, alternative hypotheses have been proposed relating the sterol requirement of amphotericin B action to more general physicochemical properties of the membrane (Hsu Chen & Feingold, 1973). Numerous studies carried out on both biological and model membranes using either structural (spectroscopic) or functional (permeability) approaches have revealed that the interactions between amphotericin B and sterol-containing membranes are complex. Recently, Bolard et al. (1980) have shown by circular dichroism spectroscopy that numerous types

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