Protein composition of rhabidosomes isolated from *Aquaspirillum itersonii*

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Rhabidosomes are tubular microstructures composed of proteins that are found in a variety of bacteria and algae. These structures, which are resistant to disruption by many denaturing agents, have potential application as a biomaterial and may serve as a new model for the study of self assembly. When rhabidosomes were purified and analysed by SDS-PAGE the presence of three proteins with molecular masses of 53, 29 and 18 kDa was revealed. However, lysozyme treatment of the rhabidosome preparations containing the three proteins resulted in the selective release of the 18 kDa protein from the rhabidosome complex. N-Terminal sequence analysis and amino acid composition analysis were performed on all three proteins. The amino acid composition of the 18 kDa protein closely matched the amino acid composition of protein H (a peptidoglycan-associated protein from *Pseudomonas aeruginosa*). These results suggest that the 18 kDa protein may be a contaminating peptidoglycan-associated protein and not part of the rhabidosome structure. Western blot analysis using antisera raised against rhabidosomes revealed that the 53 kDa component protein does not react with the antisera. We speculate that the 53 kDa protein may form the inner core of the rhabidosomes, whilst the 29 kDa protein forms the outer sheath of these structures.

Introduction

The self-assembly of protein monomers into higher order, tubular structures such as microtubules, flagella and pili occurs widely in nature. One type of tubular structure, called a rhabidosome, has been described in bacteria and algae (Correl & Lewin, 1964; Chang & Allen, 1974). Rhabidosomes contain a sheath that measures approximately 25 nm in diameter with a variable length and an inner core measuring approximately 8 nm in diameter. Although rhabidosomes are composed of protein subunits, once assembled they exhibit remarkable stability to a variety of extreme conditions such as treatment with acid, base, urea, guanidinium hydrochloride, and a range of organic solvents (Delk & Dekker, 1972). Rhabidosomes have been described in several bacteria including *Saprospira grandis* and *Aquaspirillum itersonii* but remain poorly characterized, and their origin and function, as well as the mechanism of self-assembly, are currently unknown. These structures have been compared to microtubules of eukaryotes and phage particles. Evers & Murray (1980) compared rhabidosomes and phage tail particles from

A. itersonii but found them to be composed of distinct protein components and not related.

Besides being of interest as a new model for studying protein self-assembly, rhabidosomes may have potential use as a novel biomaterial. We recently reported a procedure for the metallization of these structures that may render them magnetic and electrically conductive (Pazirandeh *et al.*, 1992). To gain insight into the function and mechanism of self-assembly of these structures we have begun a detailed study of rhabidosomes. Here we report our initial characterization of rhabidosomes from *A. itersonii*.

Methods

Materials. *A. itersonii* was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Precast polyacrylamide gels (4–20%) were from Novex. Molecular mass markers were obtained from Bio-Rad. All other chemicals were from Sigma and were of reagent grade or better.

Bacterial growth and purification of rhabidosomes. Bacterial cultures were grown in peptone succinate-salt broth (Hylemon *et al.*, 1973) for 72 h and then harvested by centrifugation at 6000 g. The pellet was washed once in 100 ml of 0.1 M-Tris/HCl pH 7.5 and centrifuged again. The rhabidosomes were purified using two protocols. Protocol 1, a modification of the method of Evers & Murray (1980), has been described previously (Pazirandeh *et al.*, 1992). Protocol 2, described

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below, differs from protocol 1 in that the bacteria were treated with lysozyme to hydrolyse the peptidoglycan layer. Washed bacterial cells were resuspended in 100 ml of 0.1 M-Tris/HCl, pH 7.5, 0.005 M-EDTA and lysozyme was added to a final concentration of 0.2 mg ml⁻¹. This suspension was kept on ice for 30 min and subsequently adjusted to 20 mM-MgCl₂. DNAase (2 mg) was added and this suspension was gently stirred for 30 min. SDS was added to a final concentration of 1% (w/v) and the sample was heated at 40 °C for 1 h. The SDS-treated sample was then centrifuged at 55000 *g* for 90 min and the supernate was discarded. The pellet was resuspended in 20 ml 0.05 M-Tris/HCl, pH 7.5, and centrifuged again as above. The pellet was resuspended as above and centrifuged at 15000 *g* for 20 min. The supernate was collected and recentrifuged at 55000 *g* for 90 min. The resulting pellet was resuspended in 0.5 ml 0.05 M-Tris/HCl, pH 7.5 and loaded onto a sucrose step gradient (30–50%, w/w). The gradient was centrifuged at 100000 *g* for 2 h and 0.5 ml fractions were collected and analysed by SDS-PAGE. The fractions rich in rhabdosomes were pooled and dialysed in 0.05 M-Tris/HCl, pH 7.5 for 8 h with one change of buffer.

SDS-PAGE and Western blotting. SDS-PAGE was performed essentially as described by Laemmli (1970). Purified rhabdosome samples were dissolved in solubilization buffer containing 50 mM-Tris/HCl, pH 7.5, 3% (w/v) SDS, 1.7 M-2-mercaptoethanol and 20% (w/v) glycerol, and boiled for 2–5 min. Samples were then loaded on precast 4–20% (w/v) gels and electrophoresed at 30 mA (constant current). Following electrophoresis, samples were stained with 0.1% Coomassie blue in 50% (v/v) methanol for several hours. The gels were destained with several changes in 50% methanol, 10% (v/v) acetic acid. Western blotting was performed according to Towbin *et al.* (1979). Antisera was raised in rabbits using rhabdosomes prepared by protocol 1 as a source of antigen. Approximately 200 µg rhabdosomes in a volume of 50 µl was mixed with an equal volume of adjuvant (Hunter's Titer Max) and injected into the rabbit subcutaneously. At day 41 the rabbit was bled by arterial puncture and the serum recovered.

N-Terminal sequence and amino acid composition analysis. Rhabdosomes were subjected to SDS-PAGE and the proteins were transferred from the gel to polyvinylidene difluoride membranes according to Matsudaira (1987), and stained and destained as described above. The individual protein bands were excised from the membrane and were submitted to the Baylor College of Medicine Amino Acid Sequencing Facility for N-terminal amino acid analysis and amino acid composition analysis.

Transmission electron microscopy. Five microlitres of the rhabdosome preparation was placed onto a Formvar-coated electron microscope grid for one min. Excess sample was removed with filter paper and the grid was stained with a 1% (w/v) solution of uranyl acetate for 1 min. Samples were viewed on a Zeiss model EM 10CR transmission electron microscope.

Results and Discussion

Isolation and SDS-PAGE analysis of rhabdosomes

During our initial work, rhabdosomes were isolated using a modification of the method of Evers & Murray (1980) (protocol 1). This method involves the isolation of rhabdosomes from a fraction rich in murein sacculi. Whereas Evers & Murray used sonication to release rhabdosomes from the murein sacculi, we used the detergent Sarkosyl to avoid extensive breakage of rhabdosomes that may occur during sonication.

Fig. 1. Transmission electron microscopy of rhabdosomes. Rhabdosome preparation stained with 1% uranyl acetate. Bar, 100 nm.

Electron micrographs of these preparations show rhabdosome sheaths with a diameter of 17 nm and variable lengths ranging up to 1300 nm (Fig. 1). These preparations, when analysed by SDS-PAGE, reveal the presence of three proteins with molecular masses 53, 29 and 18 kDa (Fig. 2, lane 1). These are the same proteins that were reported as 55, 29 and 16 kDa proteins, respectively, in our previous publication (Pazirandeh *et al.* 1992). The omission of the sulphhydryl reagent 2-mercaptoethanol had no effect on the migration of these proteins (Fig. 2, lane 2), indicating these proteins are not held together by sulphhydryl groups. However, with the exception of the 18 kDa protein the rhabdosome component proteins appear to be in a stable complex and not disrupted unless boiled in solubilization buffer prior to SDS-PAGE (Fig. 2, lanes 3 and 4).

Fig. 2. SDS-PAGE analysis of rhapidosomes. Rhapidosomes purified using protocol 1 were added to an equal volume of solubilization buffer and treated as follows prior to SDS-PAGE analysis: lanes 1, boiled for 2 min; 2, boiled in solubilization buffer lacking 2-mercaptoethanol for 2 min; 3, heated to 60 °C for 5 min; 4, held at 25 °C for 5 min. M, molecular mass markers.

We had observed that when viewing rhapidosome preparations by the electron microscope, some fields were contaminated with what appeared to be cellular debris (membrane and/or cell wall components). In order to minimize this problem, rhapidosomes were subjected to an alternative purification protocol that involved treatment of the bacteria with lysozyme to hydrolyse the peptidoglycan layer, followed by further purification by sucrose gradient centrifugation (see protocol 2 in Methods). When these preparations were analysed by SDS-PAGE, it was observed that the rhapidosomes contained only the 53 and 29 kDa proteins and lacked the 18 kDa protein (Fig. 3). We reasoned, therefore, that the low molecular mass protein may be a contaminating cell-wall-associated protein and not part of the rhapidosomes.

To further define the origin of the 18 kDa protein and determine whether treatment with lysozyme causes the solubilization of any or all of the rhapidosome component proteins, partially purified rhapidosomes were prepared by protocol 1 and subjected to lysozyme treatment. Following treatment with lysozyme, the samples were centrifuged at 55000 *g* for 90 min to pellet the rhapidosomes, and both the pellet and the soluble fraction were subjected to SDS-PAGE analysis. As shown in Fig. 4, without lysozyme treatment the rhapidosome component proteins (53 and 29 kDa), as

Fig. 3. SDS-PAGE analysis of rhapidosomes isolated by lysozyme treatment of bacteria (see Methods, protocol 2). Lanes: R, rhapidosomes; M, molecular mass markers.

well as the 18 kDa protein were pelleted at 55000 *g* (Fig. 4, lane 1), whereas the supernate contained only a contaminating 42 kDa protein (Fig. 4, lane 2). However after treatment with lysozyme some of the 18 kDa protein was solubilized and appeared in the supernatant fraction (Fig. 4, lane 4, see arrow), whereas neither the 53 kDa nor the 29 kDa proteins were solubilized and remained in the pellet fraction (Fig. 4, lane 3). The lowest molecular mass band present in Fig. 4, lane 4 is lysozyme. Although it is possible that the 18 kDa protein is part of the rhapidosome structure, its absence from preparations treated with lysozyme suggests that it may be a contaminating cell-wall- or peptidoglycan-associated protein. Several peptidoglycan-associated proteins have been isolated and characterized (Rosenbusch, 1974; Mizuno, 1987; Mizuno & Kageyama, 1979). A common feature of these proteins that is also shared by the 18 kDa protein described here is that they remain insoluble and associated with cell wall components in the presence of up to 2% SDS below 60 °C, but become solubilized above that temperature (see Fig. 2, lanes 3 and 4). We speculate that the presence of the 18 kDa protein in rhapidosomes prepared by protocol 1, as well as in rhapidosomes prepared by the Evers & Murray (1980) method, and its absence in rhapidosomes prepared by protocol 2 is due to the fact that in protocol 2 the

Fig. 4. Solubilization and release of the 18 kDa protein by treatment of rhabdosomes with lysozyme. Purified rhabdosomes obtained by protocol 1 (containing all three proteins) were treated with lysozyme ($20 \mu\text{g ml}^{-1}$) for 1 h at 4°C . The sample was then centrifuged at $55000 g$ for 90 min to pellet the rhabdosomes. The pellet, as well as the supernate were analysed by SDS-PAGE. Lanes: 1, pellet from control sample not treated with lysozyme; 2, supernate from control sample not treated with lysozyme; 3, pellet from lysozyme-treated sample; 4, supernate from lysozyme-treated sample. M, molecular mass markers. The arrow indicates the band in lane 4 representing the solubilized 18 kDa protein in the supernate.

Table 1. *N*-terminal amino acid sequence of rhabdosome component proteins and 18 kDa protein

Protein (kDa)	Sequence
53	P L I D K P E N A L L V V E K
29	T A A F V D N F Q H R L G K I
18	A T G N T A P T T A G K R G P

bacteria are first treated with lysozyme to digest the peptidoglycan layer. The lysozyme treatment thus solubilizes and releases the 18 kDa protein and therefore it does not appear in the rhabdosome fraction. However, neither the Sarkosyl treatment (protocol 1) nor sonication (Evers & Murray) release the 18 kDa protein from the peptidoglycan layer, and during the $55000 g$ centrifugation used to pellet rhabdosomes, some peptidoglycan material with its associated 18 kDa protein is also pelleted and is present in these rhabdosome preparations.

Amino acid sequence and composition analysis

N-Terminal amino acid sequence and amino acid composition analysis were performed for the rhabdosome component proteins as well as the 18 kDa

Table 2. Amino acid composition of the rhabdosome component proteins and 18 kDa protein

The values for Trp, Asn and Gln were not determined.

Amino acid	Composition (mol %)		
	53 kDa	29 kDa	18 kDa
Ala	12.10	13.93	13.71
Arg	5.28	1.83	5.14
Asp	11.20	2.17	10.22
Cys	0.19	ND	ND
Glu	9.05	9.94	13.11
Gly	7.84	18.55	13.99
His	2.13	0.41	0.98
Ile	2.91	10.50	0.69
Leu	11.25	11.99	6.62
Lys	4.39	2.31	5.52
Met	0.17	0.33	ND
Phe	4.36	0.59	2.99
Pro	5.15	0.51	2.86
Ser	5.81	2.28	6.40
Thr	7.78	24.21	6.80
Tyr	3.80	0.24	1.83
Val	6.56	0.32	9.40

ND, Not detected.

protein. These data (Tables 1 and 2) reveal that these proteins are unique and not multimers of each other. Multimers of self-assembling proteins that are stable in SDS have been reported for other self-assembling structures such as pili (Parge *et al.* 1990) and the S-layer protein (Faraldo *et al.*, 1991). A search of the NBRF-PIR protein data bank for similar amino acid sequences did not reveal any homology to any of the three proteins.

The amino acid composition of the 53 kDa protein did not reveal an abundance or lack of any particular amino acid. However, examination of the amino acid composition of the 29 kDa protein revealed that threonine, glycine and alanine account for over 50% of the total amino acids of this protein. The significance of the abundance of these amino acids, particularly threonine is currently unknown. Further work aimed at cloning the genes encoding these proteins may reveal a new, and as yet unidentified, self-assembly motif present in this protein.

Examination of the amino acid composition of the 18 kDa protein revealed a striking resemblance to the amino acid composition of one of the peptidoglycan-associated proteins (protein H isolated from *Pseudomonas aeruginosa*). Fig. 5 shows the comparison of the amino acid composition of the 53, 29 and 18 kDa proteins with protein H. As shown in Fig. 5(a) the 18 kDa protein and protein H compare very well in their amino acid composition whereas a comparison of the 29 and 53 kDa proteins with protein H reveals significant differences in amino acid composition (Fig. 5b, and c, respectively). This observation further supports the

Fig. 5. Comparison of the amino acid composition of protein H from *Pseudomonas aeruginosa* (◆) with (a) 18 kDa protein (□), (b) 29 kDa protein (□) and (c) 53 kDa protein (□). Amino acid designations are: 1, Ala; 2, Arg; 3, Asp; 4, Glu; 5, Gly; 6, His; 7, Ile; 8, Leu; 9, Lys; 10, Met; 11, Phe; 12, Pro; 13, Ser; 14, Thr; 15, Tyr; 16, Val.

hypothesis that the 18 kDa protein may be a peptidoglycan-associated protein.

Western blot analysis of rhapidosomes

Antisera against rhapidosomes was raised in rabbits using rhapidosomes prepared by protocol 1 (containing all three proteins, see Fig. 2) as a source of antigen. The

Fig. 6. Western blot analysis of rhapidosomes. Rhapidosomes isolated by either protocol 1 (lane 1) or protocol 2 (lane 2) were subjected to SDS-PAGE and analysed by Western blotting using antisera raised against native rhapidosomes. Lane M, prestained molecular mass markers.

rhapidosome preparation used to produce the antisera, as well as the rhapidosome preparation that was purified using protocol 2 (with lysozyme) were subjected to SDS-PAGE and Western blot analysis. Western blots revealed that the 18 and 29 kDa proteins, but not the 53 kDa protein, react with the antisera (Fig. 6). One possible explanation for the lack of reactivity of the 53 kDa protein is that this protein may be located on the inside of intact rhapidosomes and is thus inaccessible to the immune system. Rhapidosomes from *Saprospira grandis* and *Spirulina* have been reported to contain an outer sheath and an inner core. The above results suggest that the 29 kDa protein may form the sheath and the 53 kDa protein may form the inner core of the molecule.

Although rhapidosomes were originally described almost 20 years ago, a comprehensive study regarding

their function or the mechanism of assembly of these structures has yet to be completed. The potential use of rhabidosomes as a biomaterial derives from their ability to be metallized, their durability under extreme conditions, and their small size and anisotropic shape. Data are not currently available on the mechanism of self-assembly of rhabidosomes. However, with the availability of individual component proteins it should be possible to define the conditions under which rhabidosomes can be made to self-assemble. Whether rhabidosomes form spontaneously from their component proteins, or require additional factors (ATP, GTP) or proteins (rhabidosome-associated proteins) for self-assembly remains to be established. Experiments aimed at forming these structures *in vitro* from their component proteins are currently in progress in our laboratory.

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