Rhaphidosomes from *Aquaspirillum itersonii*: disruption and subsequent renaturation and characterization of the component proteins

Mehran Pazirandeh,¹ Jeffery Deschamps,² Subash Baral,³
Rachael Owlett,³ Angeli Aggerwal¹ and James R. Campbell¹

Author for correspondence: Mehran Pazirandeh. Tel: +1 202 404 6073. Fax: +1 202 767 9594.

¹ Center for Biomolecular Science and Engineering, Naval Research Laboratory, Washington, DC 20375, USA

² Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, DC 20375, USA

³ GeoCenters, Inc., Ft Washington, MD 20744, USA

Rhaphidosomes, tubular proteinaceous microstructures found in a variety of bacteria and algae, are resistant to disruption by many denaturing agents. They have potential application as a biomaterial and may also serve as a model for the study of self-assembly. Several reagents were tested for their ability to disrupt rhaphidosomes isolated from *Aquaspirillum itersonii* into their component proteins. Only treatments with 0.1 M hydrochloric acid and 6 M guanidinium hydrochloride were found to disrupt rhaphidosomes. A protocol was developed to renature the disrupted component proteins using rapid dilution and subsequent dialysis of the residual guanidinium hydrochloride. The renatured proteins were shown to have secondary structure as determined by circular dichroism. Furthermore, upon renaturation the two component proteins interacted with each other to form a complex of molecular mass > 2500 kDa. This complex, which may be a precursor in the pathway to rhaphidosome formation, is currently being used to study the self-assembly process of these unique structures *in vitro*.

Keywords: *Aquaspirillum itersonii*, rhaphidosomes, protein self-assembly, biomaterials

INTRODUCTION

A variety of bacteria and algae have been described which contain proteinaceous nanometre-scale tubular structures called rhaphidosomes (Correl & Lewin, 1964; Chang & Allen, 1974). Although rhaphidosomes were once thought to be derived from defective phage particles, Evers & Murray (1980) compared rhaphidosomes and phage tail particles from *Aquaspirillum itersonii* and found them to be composed of distinct protein components and not related. The function and mode of assembly of these structures are currently unknown. Although rhaphidosomes are composed of protein subunits, they exhibit a remarkable stability to a variety of extreme conditions such as treatment with HCl, urea, guanidinium hydrochloride (GHCl) (25 °C), and a range of organic solvents (Delk & Dekker, 1972). The only treatments currently known to disrupt these structures are boiling in SDS or, as reported by Delk & Dekker (1972), treatment with 10 M GHCl at 100 °C for 10 min or with 0.1 M NaOH at room temperature for 24 h. The remarkable stability of rhaphidosomes is unique and different from some of the other

self-assembling molecules (e.g. microtubules) which disassemble into their component proteins under milder conditions (Avila, 1990).

The extreme stability of rhaphidosomes makes them of potential use as a novel biomaterial (Pazirandeh *et al.*, 1992). They may also serve as a model for the study of self-assembly. We recently reported on the protein composition of rhaphidosomes from *A. itersonii* (Pazirandeh & Campbell, 1993). These structures were found to be composed of two proteins, of molecular mass 53 kDa and 29 kDa. As a prelude to studying the self-assembly process of these structures *in vitro* we report here on the disruption of rhaphidosomes with GHCl, and characterization of the renatured protein complex.

METHODS

Materials. *A. itersonii* was obtained from the ATCC (Rockville, MD, USA). Pre-cast polyacrylamide gels [4–20% (w/v) Tris/glycine and isoelectric focusing (IEF)] were from Novex. Molecular mass markers were obtained from Bio-Rad; they included phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. pI markers and all other chemicals were from Sigma and were of reagent grade or better. The pI markers used were amylo-

Abbreviations: GHCl, guanidinium hydrochloride; 2-ME, 2-mercapto-ethanol.

glucosidase, pI 3.6; trypsin inhibitor, pI 4.6; lactoglobulin, pI 5.1; carbonic anhydrase II, pI 5.9; carbonic anhydrase I, pI 6.6; myoglobin, pI 7.2; lactic dehydrogenase, pI 8.6; and trypsinogen, pI 9.3.

Bacterial growth and purification of rhabidosomes. The procedures for bacterial growth and the purification of rhabidosomes were as previously described (Pazirandeh & Campbell, 1993).

SDS-PAGE, native PAGE and IEF. SDS-PAGE was performed as described previously (Pazirandeh & Campbell, 1993) according to the method of Laemmli (1970). Native gels were electrophoresed as described for SDS-PAGE, with the exception that the running buffer and sample buffer did not contain SDS and the samples were not boiled. IEF electrophoresis was performed according to the manufacturer's instructions (Novex). The anode buffer was 10 mM phosphoric acid and the cathode buffer was 20 mM lysine and 20 mM arginine. Sample buffer contained 40 mM lysine, 40 mM arginine and 30% (w/v) glycerol. The gels were electrophoresed at 100 V for 1 h, 200 V for 1 h, and 500 V for 30 min.

Disruption of rhabidosomes into component proteins. Purified rhabidosomes were treated with the following reagents: 0.1 M NaOH, 0.1 M HCl, 8 M urea, 6 M GHCl, chymotrypsin (1/20, w/w) and *n*-butanol. Incubation was performed at a protein concentration of 1 mg ml⁻¹ for 2 h at 25 °C except for 6 M GHCl, which was incubated at 80 °C for 2 h. Following incubation, a portion of the sample was examined by transmission electron microscopy as described below. To the remainder an equal volume of solubilization buffer was added and the sample was divided into two fractions. One fraction was boiled and the other kept at room temperature before analysis by SDS-PAGE as described above.

Renaturation of GHCl-treated rhabidosome component proteins. Rhabidosomes were incubated with 6 M GHCl at 80 °C for 2 h at a final concentration of 1 mg ml⁻¹. Following treatment the rhabidosomes were diluted 50-fold in a buffer containing 0.05 M Tris/HCl, 0.005 M EDTA and 0.5 M GHCl for 2 h. This sample was then dialysed twice against 0.05 M Tris/HCl, 0.005 M EDTA. The precipitate that formed during this procedure was sedimented by centrifugation and the supernatant was concentrated by Centricon-30 membrane filters to a final concentration of 1 mg ml⁻¹.

Transmission electron microscopy. This was performed as described previously (Pazirandeh *et al.*, 1992).

Circular dichroism. Circular dichroism of the protein samples was measured over the wavelength range 250–190 nm using a Jasco J-600 spectropolarimeter. The cell used had an optical path length of 0.2 mm. The experimental solutions contained 0.1–0.3 mg protein ml⁻¹ in 0.05 M Tris/HCl buffer pH 7.5. Blank measurement using the buffer solution did not show detectable circular dichroism.

The amount of different conformers present was estimated using ellipticity values of myoglobin as standard. The amounts of different conformers were estimated with an accuracy of $\pm 10\%$.

Size-exclusion HPLC. This was performed using a Beckman System Gold HPLC equipped with a model 168 diode array detector and a TSK 4000SW (Toya Soda Inc.) column. Samples and calibration standards were analysed in 50 mM Tris buffer (pH 7.5) containing 5 mM EDTA at a flow rate of 0.5 ml min⁻¹ while monitoring at 280 nm. Calibration standards used were thyroglobulin, alcohol dehydrogenase, ovalbumin, myoglobin and vitamin B₁₂ (Bio-Rad), and apoferritin, β -amylase, bovine

serum albumin and carbonic anhydrase (Sigma). The column void corresponded to a molecular mass of over 2500 kDa.

RESULTS AND DISCUSSION

Disruption of rhabidosomes into component proteins

Rhabidosomes from *Saprospira grandis* were reported by Delk & Dekker (1972) to be extremely stable structures and were not dissociated under a variety of conditions including treatment with 1 M HCl, 6 M GHCl (at 25 °C), 8 M urea or a range of organic solvents. The stability of rhabidosomes to a variety of harsh conditions is desirable for the use of these structures as a biomaterial. However, we were interested in finding conditions that would disrupt rhabidosomes, for subsequent *in vitro* self-assembly experiments. A number of reagents (Table 1) were tested for their ability to disrupt the rhabidosomes of *A. itersonii*. Rhabidosome preparations were treated with the various reagents and were subsequently analysed by electron microscopy and SDS-PAGE for the presence of intact rhabidosomes. The SDS-PAGE analysis utilizes the property of intact rhabidosomes (Pazirandeh & Campbell, 1993) in which the component proteins do not dissociate and therefore do not enter the gel matrix unless boiled in solubilization buffer. As shown in Table 1 and Figs 1 and 2, the only reagents that disrupted rhabidosomes into their component proteins were 0.1 M HCl and 6 M GHCl (at 80 °C for 2 h): no rhabidosomes were observed by electron microscopy after treatment with these reagents (Fig. 1b, c) and only under these conditions did the rhabidosome component proteins enter the gel without boiling the samples in SDS solubilization buffer (Fig. 2, lanes 1 and 5).

Renaturation and characterization of the rhabidosome component proteins

Although treatment with either HCl or GHCl resulted in the disruption of rhabidosomes into their component proteins, treatment with the former reagent resulted in the immediate formation of a precipitate, indicating irreversible aggregation of the denatured proteins. Therefore all subsequent denaturation experiments were performed with GHCl. Dialysis of the denatured protein in aqueous buffer following GHCl treatment of rhabidosomes resulted in over 90% of the protein forming aggregates and less than 10% of the protein remaining in the soluble phase. A protocol was developed (see Methods) where the disrupted proteins were first rapidly diluted in 0.5 M GHCl and subsequently dialysed against an aqueous buffer. Using this method only about 30% of the protein was found to be in the precipitated form (data not shown). The two factors, rapid dilution, and the presence of low concentrations of GHCl, may allow intramolecular folding interactions to take precedence over intermolecular interactions as well as partially refold the proteins and destabilize 'wrong' aggregates or 'wrong' intermediates on the folding pathway (Jaenicke & Rudolph, 1990).

When these proteins were concentrated and analysed by SDS-PAGE and electron microscopy there was no

Table 1. Disruption of rhapidosomes into component proteins

Condition	Disruption into component proteins*	
	SDS-PAGE	EM
HCl (0.1 M)	+	+
NaOH (0.1 M)	—	—
Chymotrypsin (1/20, w/w)	—	—
Urea (8 M)	—	—
GHCl (6 M, 80 °C)	+	+
Butanol (100 %)	—	—

*Disruption was monitored by SDS-PAGE as well as by electron microscopy (EM) (see Methods).

Fig. 1. Transmission electron microscopy of rhapidosomes after treatment with HCl and GHCl. Rhapidosomes were treated with various reagents (see Methods and Table 1) and were then examined by electron microscopy for the presence of intact rhapidosomes. (a) Untreated rhapidosomes; (b) rhapidosomes treated with HCl; (c) rhapidosomes treated with GHCl. Not shown in this figure are rhapidosomes which were treated with NaOH, n-butanol, 8 M urea, and chymotrypsin. Electron microscopy revealed the presence of intact rhapidosomes (similar to those seen in panel a) after treatment with these reagents. A minimum of 10 viewing fields were examined for samples treated with HCl and GHCl before concluding that there were no intact rhapidosomes present. Bars, 200 nm.

evidence of rhapidosomes in these samples. However, circular dichroism spectra of the renatured rhapidosome proteins showed the presence of the following secondary

Fig. 2. SDS-PAGE of rhapidosomes after treatment with various reagents. Rhapidosomes (1 mg ml^{-1}) were treated with the reagents listed below (see Methods) and subsequently analysed by SDS-PAGE. The samples were kept at room temperature (as opposed to being boiled) after the addition of the SDS-PAGE sample buffer. Lanes: M, molecular mass markers; 1, 0.1 M HCl; 2, 0.1 M NaOH; 3, n-butanol; 4, 8 M urea; 5, 6 M GHCl; 6, chymotrypsin; 7, untreated rhapidosomes.

structures: 22.6 % helix, 38.4 % sheet, 16.5 % turns and 23.4 % random coil structures (Fig. 3). Further characterization revealed that the renatured proteins formed a complex of molecular mass $> 100 \text{ kDa}$ as opposed to the proteins disrupted with GHCl, which remained as monomers of molecular masses 53 and 29 kDa. This indication of complex formation was based on experiments where rhapidosomes denatured in the presence of GHCl as well as rhapidosomes which were denatured and subsequently renatured (by the rapid dilution method) were run through Centricon-100 filter membranes and both the upper and the lower fluids were then subjected to SDS-PAGE. As shown in Fig. 4 (lanes 1 and 2) in samples which had been denatured and subsequently renatured both the 29 and the 53 kDa proteins remained exclusively in the top portion of the filter. This is in contrast to samples which had been denatured by GHCl (Fig. 4, lanes 3 and 4), in which the 29 and 53 kDa protein bands are present in both the upper and lower portions of the Centricon membrane, indicating that both these proteins pass through the membrane (which has a cutoff of about

Fig. 3. Circular dichroism spectra of rhabidosome component proteins. Rhabidosomes were denatured with GHCl and subsequently renatured (see Methods). The sample was adjusted to a protein concentration of 0.1 mg ml^{-1} in 0.05 M Tris/HCl pH 7.5 and subjected to circular dichroism analysis. Myoglobin was used as the standard. —, Observed; ----, calculated.

Fig. 4. SDS-PAGE analysis of denatured and renatured rhabidosomes after Centricon-100 filtration. Rhabidosomes were denatured with GHCl and a portion was subsequently renatured (see Methods). Both samples were run through Centricon-100 filters and then subjected to SDS-PAGE. Lanes: M, molecular mass markers; 1, top portion of Centricon from renatured sample; 2, bottom portion of Centricon from renatured sample; 3, top portion of Centricon from denatured sample; 4, bottom portion of Centricon from denatured sample.

100 kDa). These data indicate that upon renaturation of the rhabidosomes the individual proteins have assembled into a higher molecular mass form (over 100 kDa) which prevents them from passing through the Centricon membranes. Furthermore when the renatured protein was subjected to either native PAGE or IEF it migrated as a single species (results not shown), a further indication of interaction between the two monomeric proteins. A pI of 4.9 was calculated for the complex.

To determine more precisely the molecular mass of this complex, the sample was subjected to size-exclusion

Fig. 5. Size-exclusion HPLC analysis of renatured rhabidosome component proteins. Rhabidosomes were denatured with GHCl and subsequently renatured (see Methods) and subjected to HPLC. The two major peaks correspond to approximate molecular masses of 2500 kDa and 590 kDa. The SDS-PAGE profile of each peak is also shown (inset). Lanes: 1, the 2500 kDa peak; 2, the 590 kDa peak; M, molecular mass markers.

HPLC. Two high-molecular-mass complexes were observed, with apparent molecular masses of 590 kDa and over 2500 kDa (Fig. 5). These roughly correspond to aggregates consisting of six dimers and about 36 dimers (six of the smaller complex). SDS-PAGE analysis of the complex proteins revealed approximately equal ratios of the 29 and 53 kDa proteins based on staining intensity (Fig. 5, inset).

Other self-assembling proteins, including the S-layer of bacteria and the secretory proteins of *Chironomus tentans* larvae, have been proposed to assemble in a stepwise fashion whereby an intermediate complex is formed prior to the assembly of the final product (Jaenicke *et al.*, 1985; Sleytr *et al.*, 1992; Takumi *et al.*, 1991; Wellman & Case, 1989). Whether the rhabidosome component proteins form a complex which is an intermediate in the pathway to rhabidosome formation remains to be established. Also to be established is the relationship of the two complexes (590 kDa and > 2500 kDa) and whether any other factors (ATP, GTP) or other self-assembly proteins need to be added to these intermediates for the production of rhabidosomes *in vitro*. We are currently conducting experiments to address these questions.

REFERENCES

- Avila, J. (1990). Microtubule dynamics. *FASEB J* **4**, 3284–3290.
- Chang, H. Y. & Allen, M. M. (1974). The isolation of rhabidosomes from the blue-green alga, *Spirulina*. *J Gen Microbiol* **81**, 121–130.
- Correll, D. L. & Lewin, R. A. (1964). Rod shaped ribonucleoprotein particles from *Saprospira*. *Can J Microbiol* **10**, 63–74.

- Delk, A. S. & Dekker, C. A. (1972).** Characterization of rhapidosomes from *Saprospira grandis*. *J Mol Biol* **64**, 287–295.
- Evers, M. & Murray, R. (1980).** The comparison of rhapidosomes and defective bacteriophage particles from *Aquaspirillum itersonii*. *Can J Microbiol* **26**, 1312–1319.
- Jaenicke, R. & Rudolph, R. (1990).** Folding proteins. in *Protein Structure: a Practical Approach*, pp. 191–223. Edited by T. E. Creighton. New York: Oxford University Press.
- Jaenicke, R., Welsch, R., Sara, M. & Sleytr, U. B. (1985).** Stability and self-assembly of the S-layer protein of the cell wall of *Bacillus stearothermophilus*. *Biol Chem Hoppe-Seyler* **366**, 663–669.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Pazirandeh, M. & Campbell, J. (1993).** Protein composition of rhapidosomes isolated from *Aquaspirillum itersonii*. *J Gen Microbiol* **139**, 859–864.
- Pazirandeh, M., Baral, S. & Campbell, J. (1992).** Metallized nanotubules derived from bacteria. *Biomimetics* **1**, 39–48.
- Sleytr, U. B., Pum, D., Sara, M. & Messner, P. (1992).** Two dimensional protein crystals as patterning elements in molecular nanotechnology. In *AIP Conference Proceedings 262, Molecular Electronics – Science and Technology*. Edited by A. Aviram. New York: American Institute of Physics.
- Takumi, K., Koga, T., Oka, T. & Endo, Y. (1991).** Self-assembly, adhesion, and chemical properties of tetragonally arrayed S-layer proteins of *Clostridium*. *J Gen Appl Microbiol* **37**, 455–465.
- Wellman, S. E. & Case, S. T. (1989).** Disassembly and reassembly *in vitro* of complexes of secretory proteins from *Chironomus tentans* salivary glands. *J Biol Chem* **264**, 10878–10883.
-
- Received 25 November 1994; revised 27 February 1995; accepted 1 March 1995.