Biochemical characterization of the proteins of Paramecium secretory granules

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Summary

The proteins of trichocysts (secretory granules) from Paramecium tetraurelia have been biochemically characterized. Two-dimensional electrophoresis revealed 34 major components and at least 120 minor components, most with molecular weights ranging from 14,000 to 21,000 and isoelectric points ranging from 4.8 to 5.2. Comparison of two-dimensional electrophoretic patterns of trichocysts before and after exocytosis revealed only minor changes in these patterns, although the protein matrix undergoes a striking change in morphology. To clarify the interrelationships among trichocyst proteins, two proteins from extruded trichocyst matrix were purified to homogeneity and sequenced at their N termini. Their sequences are distinct, but they share limited homology.

Key words: secretion, exocytosis, trichocyst, Paramecium.

Introduction

Trichocysts, the secretory granules found in the unicellular eukaryote Paramecium tetraurelia, were first described by Ellis (1769). Approximately 1000 membrane-enclosed condensed trichocysts are attached to the inner surface of the cell membrane in an orderly array. (We refer to the whole organelle, with the body and tip sheaths, and the enclosing membrane, as the trichocyst; and to the secretory protein contents of the trichocyst as the matrix. Trichocyst matrices are referred to as condensed matrices before exocytosis and as extruded matrices after exocytosis.) Many other protozoans contain similar secretory organelles (Hausmann, 1978). The carrot-shaped condensed trichocyst matrix averages 1 µm × 3–7 µm and is divided into two domains, the tip and the body (Bannister, 1972). Upon appropriate mechanical or chemical stimulation, the trichocyst contents (matrices) are discharged into the external medium through a Ca²⁺-mediated series of events (Hausmann, 1978). The function of these organelles has not been defined.

Trichocysts differ in at least three ways from many other secretory granules. First, the proteins of the trichocyst matrix are organized in a very stable, highly ordered array (Hausmann, 1978). Such structures have been observed in a limited number of other secretory granules of metazoan cells (Caulfield et al. 1980; Zuccarello, 1980; Michael et al. 1987). Second, discharge of the trichocyst matrix into the external medium is accompanied by an eightfold increase in the body of the trichocyst matrix length and a twofold decrease in body diameter (Bannister, 1972). Electron microscopy of condensed and extruded trichocyst matrices reveals a striking, highly regular lattice in both (Hausmann, 1978; Peterson et al. 1987a; Sperling et al. 1987). The third difference between trichocysts and the secretory granules of higher organisms is that Paramecium mutant cell lines containing altered trichocysts are available for detailed, comparative study. Numerous mutants have been isolated that exhibit varying degrees of defective trichocyst assembly and discharge (Pollack, 1974; Ruiz et al. 1976; Aufderheide, 1978; Plattner et al. 1980), but their trichocysts have not been thoroughly characterized biochemically.

Little is known about the proteins comprising the trichocyst matrix. Jakus (1945) performed the initial chemical stability studies of the trichocyst matrix. The first biochemical characterization described matrices as being composed of a single, acidic 17,000 Mₘ protein (Steers et al. 1969). Subsequent studies have indicated a greater complexity of the subunits (Adoutte et al. 1980; Tindall, 1986). Calmodulin has been reported to be a major component of the trichocyst (Rauh & Nelson,
1981). Recently, certain trichocyst matrix proteins have been shown (Peterson et al. 1987b) to be immunologically related to chromogranin A, the major protein component of chromaffin granules from bovine adrenal medulla cells. To determine whether the similar size and isoelectric points of the many trichocyst matrix proteins reflect similar (or identical) sequences, and whether the trichocyst matrix proteins show sequence homology with chromogranin A, we have isolated and partially sequenced two trichocyst matrix proteins. We have made detailed comparisons of the proteins of condensed and extended trichocyst matrices to determine whether the remarkable structural rearrangement of trichocyst matrix body during secretion involves changes in the protein subunits themselves. We have also re-investigated the association of calmodulin with trichocysts.

Materials and methods

Trichocyst matrix isolation

Cells were cultured in axenic medium and harvested as described by Rauh & Nelson (1981). Trichocyst discharge was induced by suspending cells in cold (4°C) Dryl's solution (Dryl, 1959) and, after low-speed centrifugation, a fluffy layer of trichocysts and debris was present above the cell pellet. The material in the fluffy layer was washed in 10 mM-Tris-HCl, 3 mM-EDTA (pH 7), then loaded onto a sucrose step gradient (Adoutte et al. 1980) where it banded at the 55%/58% (w/w) sucrose interface. Condensed trichocyst matrices were isolated according to Peterson et al. (1987a), using the same buffer as described above. Viewed in the electron microscope these preparations consisted of trichocyst matrices that had lost both the body sheath and the enclosing membrane. No other particles or debris were visible in the light (phase-contrast) or electron microscope.

Electrophoretic methods

The 10 cm long IEF (isoelectric focusing) gels used in one-dimensional analyses contained 8 M-urea, 6% T acrylamide, 3% C bisacrylamide, 3% Bio-Lyte 4/6, and 1% Bio-Lyte 3/10, and were run for 18 h at 200 V and for an additional 1-5 h at 400 V (Tindall, 1986). The IEF tube gels used for two-dimensional polyacrylamide gel electrophoresis (PAGE) contained 8 M-urea, 6% T acrylamide, 12% C dihydroxyethyl-cellulose, 1-2% Bio-Lyte 4/6, 0-8% Bio-Lyte 3/10, and 75 mM-Mops. The details of the two-dimensional methods are reported elsewhere (Tindall, 1986). SDS-PAGE for immunoblots was performed using the method of Laemmli (1970).

Immunological methods

Male Balb/c mice were immunized with whole-cell calmodulin that was isolated from P. tetraurelia cells as described by Rauh & Nelson (1981). This antisera reacted with Paramecium calmodulin but not with bovine calmodulin, troponin C, parvalbumin or other Paramecium proteins (DeVito, 1985).

Immunoblots

SDS-polyacrylamide gels were first soaked in 25 mM-Tris-HCl, 192 mM-glycine, pH 8-6, for 20 min and were then layered between two sheets of 7% acetic acid-soaked 0-45 mm nitrocellulose paper. Approximately 5-10% of the protein in the gel was then vacuum-transferred to the membrane. The nitrocellulose sheets were blocked with 3% BSA (bovine serum albumin) in TBS (Tris-buffered saline: 10 mM-Tris-HCl, 150 mM-NaCl, pH 8-6) and were subsequently incubated with serum raised against Paramecium calmodulin. After a wash with 0-05% Tween-20 in TBS, the nitrocellulose sheets were incubated with alkaline phosphatase-linked goat anti-mouse IgG antibodies and were subsequently visualized using the chromogenic alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate.

Protein purification

Approximately 100 mg of sucrose density gradient-purified discharged trichocyst matrices were incubated for 20 min at 35°C in 8 M-urea, 25 mM-Mes, 1% (v/v) 2-mercaptoethanol, pH 6, and were then dialysed overnight at 4°C against column buffer (6 M-urea, 25 mM-Mes, 10 mM-2-mercaptoethanol, pH 6). The dialysed sample was applied to a 50 ml DE-52 (Whatman) column at 4°C, which was subsequently washed with one column volume of column buffer. The bound protein was eluted with a 400 ml linear gradient that contained 0-0-2 M-NaCl in column buffer. The column effluent was monitored at 280 nm and the conductance of the column effluent was used to calculate the salt profile. Fractions were pooled according to their IEF profiles and stored frozen. The weight of protein in these fractions was quantified by the method of Peterson et al. (1987a) where it banded at the 55%/58% (w/w) sucrose interface. No other particles or debris were visible in the light (phase-contrast) or electron microscope.

Preparative IEF was carried out at 13°C on milligram quantities of DEAE-cellulose chromatography fractions. Preparative IEF samples were dialysed overnight at 4°C against 6 M-urea, 10 mM-Mops, 10 mM-2-mercaptoethanol at pH 7 and were then mixed with other gel components until the final slurry also contained washed Sephadex G-200, 6 M-urea (Schwarz/Mein, Ultrapure), 1-2% Bio-Lyte 4/6 and 0-8% Bio-Lyte 3/10 (Bio-Rad), 100 mM-Mops, and 1% 2-mercaptoethanol in a total volume of 20 ml. The consistency of the bed was adjusted with dry Sephadex G-200. The electrode buffers were the same as for IEF in polyacrylamide gels (Tindall, 1986). Bands were located using paper contact prints that were stained with Coomassie Brilliant Blue G-250 in a perchloric acid buffer (Reinser et al. 1975). Regions of gel containing bands of interest were electroeluted in 10 mM-Tris-HCl at pH 8-2. Urea was removed from these final fractions by dialysis at 4°C against 10 mM-NH4HCO3, pH 7-9. The purified trichocyst matrix proteins remained soluble under this final condition.

Protein sequencing

Gas-phase protein sequencing of trichocyst matrix proteins was carried out by the method of Hewick et al. (1981) using an Applied Biosystems model 470A gas-phase sequencer at the University of Wisconsin Biotechnology Center.

Results

Changes in protein composition after exocytosis

Trichocyst secretion does not cause a dramatic alteration in the matrix's protein composition. Two-dimensional analyses of condensed and extruded trichocyst matrix proteins revealed very few differences in the protein patterns before and after exocytosis (Fig. 1). The most noticeable difference associated with exocytosis was the reduction in spot 30 (Fig. 1); a few other spots were also somewhat diminished in intensity. The presence of basic
proteins in the trichocyst matrix was investigated using non-equilibrium pH-gradient electrophoresis, NEPHGE (O'Farrell et al. 1977), as the first dimension of the two-dimensional analyses (data not shown). Approximately 20 additional minor spots and no additional major spots were observed. The gels of condensed trichocyst matrices contained five minor spots not prominent in gels of extruded trichocyst matrices (data not shown).

Isolation of subunits

Isolation of individual subunits was accomplished by combining DEAE-cellulose ion-exchange chromatography in urea with subsequent preparative IEF in urea. Neither technique alone provided pure subunits, but DEAE-cellulose chromatography yielded less complex fractions from which pure subunits could be obtained by preparative IEF. Fig. 2 represents a typical pH 6 DEAE-cellulose chromatography profile of all urea-soluble proteins from sucrose gradient-purified extruded trichocyst matrices. Fractionation of trichocyst matrix proteins using DEAE-cellulose columns run at pH 8 resulted in the retention of all of the proteins that did not bind at pH 6 (fractions a, b, c, Figs 3, 4), but no major changes in relative elution positions of individual proteins were noted. Since urea decomposition is favoured by basic pH, and since there are otherwise no outstanding advantages of pH 8 over pH 6, preparative chromatography was carried out at pH 6. Fig. 3 shows the isoelectric profile of proteins present in each of the pooled regions. Approximately 60% of the material loaded onto the column was recovered in the pooled fractions.

The DEAE-cellulose chromatography fractions depicted in Figs 2 and 3 were purified further by preparative IEF in the presence of 0·1 M-Mops, a pH-gradient modifier. A representative purification of DEAE-cellulose fraction (g) gave rise to subfractions shown in Fig. 4. This purification procedure yielded proteins that were devoid of ampholytes, that were >95% pure as assessed by density scanning of SDS–PAGE and IEF gels, and that were recovered in approximately 25% overall yields. From 2 to 5 μg of each of the pure proteins was obtained per mg of trichocyst matrix protein.

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Fig. 3. Composite IEF of the pH6 DEAE-cellulose chromatography fractions. The first lane contains 150 μg of total trichocyst protein and the remaining lanes contain 0-5 % of the material in each pooled fraction.

N-terminal sequencing of proteins
Gas-phase sequencing of proteins 3, 8, 12 and 27 (Fig. 1) was carried out on 500 pmol quantities of intact, unmodified samples. Proteins 3 and 8 were either N-terminally blocked or were not retained by the polybrene disk, since <10 % of the anticipated levels of PTH (phenylthiohydantoin) residues was recovered in the initial cycles. Proteins 12 and 27 gave >90 % of the expected level of PTH residues in the first cycle and had repetitive yields/lags of 94 %/4 % and 91 %/2 %, respectively. The sequences of more than 25 residues were obtained for both proteins (Table 1), and are presented in their optimal alignment without allowing gaps. The introduction of gaps does not significantly enhance the alignment. Since cysteine residues were not protectively modified, some unknown residues may be cysteines. The sequences were unrelated to any sequences within Paramecium calmodulin (Schaefer et al. 1987), and also differed completely from chromogranin A except for one similarity: the penultimate proline. No significant homology with any protein in the Protein Identification Resource data bank was detected.

Calmodulin content of gradient-purified trichocyst matrix
Rauh & Nelson (1981) reported that calmodulin rep-

Table 1. Amino acid sequences of proteins studied

<table>
<thead>
<tr>
<th>Trichocyst protein 3*</th>
<th>Not detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichocyst protein 8*</td>
<td>Not detected</td>
</tr>
<tr>
<td>Trichocyst protein 12*</td>
<td>SPLDTIKGVLDNFKSAVAEQXGXXDEVY</td>
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<tr>
<td>Trichocyst protein 27*</td>
<td>DPLDRLLSTLTDELDRYVAEQKEDDAKNQ</td>
</tr>
<tr>
<td>100K Chromogranin†</td>
<td>SPYHNHNNKGEVEVMMXVE</td>
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<td>MFPVIDHNEEQVTHKILEVXNAL</td>
</tr>
<tr>
<td>75K Chromogranin†</td>
<td>MPVNIHPMNKGEVEVMMK1VE</td>
</tr>
<tr>
<td>(chromogranin A)</td>
<td>LVVNSPMKGDTEVMMKCIREV15D</td>
</tr>
<tr>
<td>Mature chromogranin A‡</td>
<td>LPVN5SPMKNKDTEVMMKCIVEVISDTLSKP</td>
</tr>
</tbody>
</table>

The one-letter code for amino acid sequence is used.
* Determined here.
† Reported by Settleman et al. (1985): K = 10^3 M<sub>r</sub>.
‡ From DNA sequence. Reported by Iacangelo et al. (1986) and Benedum et al. (1986).
Fig. 4. Analytical IEF of fractions generated by preparative IEF of DEAE fraction g. The first lane contains 20 μg of the original sample (fraction g) and the remaining lanes contain 1% of the material in each pooled fraction.

Fig. 5. Immunoblot of condensed and extruded trichocyst matrices with and without exogenously added Paramecium calmodulin. Lanes 1 and 2 contain 100 μg of condensed trichocyst matrices. Lanes 4 and 5 contain 100 μg of extruded trichocyst proteins. Lanes 2, 3 and 5 also contain 1 μg of exogenously added calmodulin.

Discussion

Two decades ago the trichocyst matrix was thought to be composed of a single, acidic protein given the name 'trichynin' (Steers et al. 1969). Inspection of the two-dimensional gels of the trichocyst matrices discussed here reveals more than 150 protein spots, 34 of which can be classified as major constituents. The bulk of these proteins have molecular weights ranging from 14 000 to 21 000 and isoelectric points in 6 M-urea ranging from 4·8 to 5·2 (Tindall, 1986), uncorrected for the effect of urea. (The isoelectric points of proteins determined in urea cannot be directly compared with those determined under native conditions. See Tindall (1986) for a description of this phenomenon.)

Purification of monomeric trichocyst matrix subunits was complicated by the remarkable stability of the organelle, which cannot be dissociated into its subunits through 'mild' means such as heating, salt extraction, treatment with non-ionic detergents, or treatment with low levels of denaturants such as LiCl, urea or guanidine hydrochloride (J. B. Peterson, personal communication). The choice of urea-containing buffers permitted the application of ion-exchange chromatography and preparative IEF as purification techniques. When combined, these procedures yielded pure proteins that were soluble at neutral pH and room temperature.

It had been reported that calmodulin constitutes from 1 to 10% of the total trichocyst proteins (Rauh & Nelson, 1981). The immunological results presented here and the calmodulin-dependent phosphodiesterase assay data (L. D. DeVito, unpublished observations) argue that cal-
modulin is not a major component of the gradient-purified trichocyst matrix. Given the limits of detection in these experiments, calmodulin could account for no more than 0.05% of trichocyst matrix protein.

Maleki et al. (1987) suggest a role for calmodulin in the formation of exocytotic openings, and Satir et al. (1988) have postulated a role for calmodulin at the trichocyst membrane in controlling the access of Ca$^{2+}$ to the matrix. Garofalo (1983) localized calmodulin in trichocysts by immunocytochemistry at the light-microscopic level, and by electron-microscopic immunocytochemistry, Momayezi et al. (1986) localized calmodulin to the periphery of condensed trichocysts in situ. They suggested that calmodulin was associated with the trichocyst membrane, but their data would appear to be equally consistent with calmodulin localized in the sheath inside the membrane. Condensed trichocyst matrices in situ contain about their periphery a net-like layer of material (the sheath) and an enclosing membrane. The sheath is largely lost during trichocyst matrix extension, and appears absent from gradient-purified trichocysts (J. B. Peterson, personal communication). A re-examination of the original data from Rauh & Nelson (1981) reveals that the trichocyst matrix preparations most enriched in calmodulin were those least extensively washed, i.e. not purified by sucrose-gradient centrifugation. It seems probable that the difference between our present findings and the findings of Rauh & Nelson (1981) is due to the removal, by washing and centrifugation in sucrose gradients, of differing amounts of loosely associated material from the sheath or membrane, the structures that exhibit cross-reaction with antibodies against calmodulin (Garofalo, 1983; Momayezi et al. 1986). The whole condensed trichocyst, with intact sheath and membrane, has not been isolated; the conditions necessary to prevent extension (very low Ca$^{2+}$ concentration) apparently destroy the membrane and remove the sheath material. It is therefore difficult to test by isolation whether calmodulin is part of the intact secretary granule.

The absence of calmodulin from gradient-purified trichocyst matrices rules out that protein as a major structural element in the trichocyst shaft, and it is presumably not directly involved in the Ca$^{2+}$-triggered structural expansion that accompanies exocytosis, as that process occurs in vitro with gradient-purified condensed trichocyst matrices (Garofalo & Satir, 1984). One possible explanation for the dramatic structural rearrangement is some covalent modification(s) of trichocyst protein(s). We found, however, little evidence of covalent modification of trichocyst proteins; condensed and extended matrix materials show very little difference in two-dimensional gel electrophoresis, which would easily reveal charge-altering modifications such as phosphorylation. It seems likely that some non-covalent change, such as the binding of Ca$^{2+}$, forces the structural rearrangement.

The interrelationships among trichocyst matrix proteins were investigated by attempting to carry out N-terminal protein sequencing on four purified subunits. Two of these attempts were successful. These two trichocyst protein sequences have limited similarities with each other; they are identical at six of 25 positions. Another feature of these sequences is the occurrence of proline at position two, which is also a common feature of several chromogranins (Jacagelo et al. 1986; Benedum et al. 1986). The two sequences determined here do not otherwise share sequence homology with the known chromogranin sequences. Although the significance of proline at position two has not been reported, this proline may form part of a site recognized by processing proteases during maturation of the proteins.

Clearly, the trichocyst matrix is composed of a complex, possibly interrelated, group of proteins. The observed complexity could result from the actual presence of many different primary sequences. Alternatively, the complexity could result from proteolytic processing during secretory protein maturation (Adoutte et al. 1984) or from post-translational modifications of a limited number of primary sequences. It is not possible at this time to distinguish how many different primary sequences are present or whether any subunits have been post-translationally modified. As described previously (Tindall, 1986), numerous steps have been taken to ensure that artifacts due to carbamylation by urea contaminants did not take place. Should carbamylation occur, one would expect to see rows of spots at given molecular weights as described by Anderson & Hickman (1979). Furthermore, one would expect to see an alteration of the two-dimensional gel pattern as a function of sample preparation method and/or sample age. Such alterations have not been observed.

The trichocyst system has at least one unique feature to aid in the study of secretary granule protein structure and function: the existence of well-characterized Paramecium trichocyst mutants (Pollack, 1974; Ruiz et al. 1976; Auferheide, 1978; Plattner et al. 1980). An understanding of the genetically manipulable trichocyst system may provide insight into the functions of similar secretory granule proteins in higher organisms.

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References


