The molecular composition of the volutin granule of yeast

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The volutin granule was isolated from yeast by disruption of freeze-dried cells in an organic solvent and density-gradient centrifugation. The granule is composed of two types of macromolecule, a linear-chain polyphosphate and four basic proteins, of molecular weights ranging from 10 000 to 20 000. In the dissolved granule these macromolecules are in a complex that is uniform by hydrodynamic criteria ($s_{20,w}^0 = 22.3$ S). The polyphosphate separated from this complex gives a single ^{31}P n.m.r. resonance and in the analytical ultracentrifuge behaves as a monodisperse solute of molecular weight $245\,000 \pm 1000$. In the ^{31}P n.m.r. spectrum of yeast used for its isolation, this polyphosphate accounts for 14% of total cell polyphosphate.

A correlation between the polyphosphate content of yeast cells and the number and size of volutin granules was observed by Wiame (1947). Attempts to isolate volutin granules from yeast and other organisms were only partially successful (for a review see Harold, 1966). Linear-chain polyphosphate of relatively high molecular weight (>5000 ; Glonek *et al.*, 1971) was identified by ^{31}P n.m.r. spectroscopy in volutin granules obtained from *Micrococcus lysodeikticus*. Of the methods used to study the granule in the whole cell, X-ray-energy-dispersive analysis of the volutin granule in a blue-green alga showed the presence of phosphorus and calcium as the major elements of atomic number greater than 11 (Sicko-Goad *et al.*, 1975). Thus only fragmentary information on the molecular composition of the volutin granule is available at present.

We have approached the study of the volutin granule in yeast by adapting the non-aqueous-solvent procedure that Hase *et al.* (1963) have used for isolation of the volutin granules of *Chlorella*. The volutin granule, described in the present work, was isolated by density-gradient centrifugation. Polyphosphate of the granule was isolated by gel filtration of the solubilized granule. We find that the polyphosphate of the isolated granule is monodisperse and that in the granule it is associated with low-molecular-weight basic proteins.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Growth of yeast and preparation of a cell-free homogenate

Polyphosphate-rich yeasts were prepared as described by Liss & Langen (1962). A 150 g batch of fresh baker's yeast (obtained locally) was incubated in 50 litres of phosphate-free medium for 9 h. The yeast was then harvested in a Sharples centrifuge and washed with an equal volume of distilled water. The yield was 350 g. Yeasts thus obtained were designated phosphorus-starved. Phosphorus-starved yeasts (300 g) were resuspended in 10 litres of phosphate-rich medium. After 2 h yeasts were harvested in a Sharples centrifuge and washed with cold distilled water. Yeasts obtained in this step were designated polyphosphate-rich. They were resuspended in cold distilled water and freeze-dried.

A 20 g portion of freeze-dried yeast and 25 g of chromium-steel balls (diam. 0.8 mm) were mixed by shaking, and carbon tetrachloride was added to a final volume of 50 ml. The suspension was transferred to a metal container of a Braun model MSK homogenizer and treated for ten periods of 1 min each at a speed of 1000 rev./min with 1 min interruptions for cooling. Microscopic examination of the homogenate showed only few intact cells.

Chemical analyses

All analyses of the isolated volutin were performed on samples dried over P_2O_5 in a desiccator.

Tightly bound water content was determined by weight loss on drying the volutin sample at 110°C over P_2O_5 in a vacuum gun.

Total phosphorus was determined after digestion in 1 ml of constant-boiling $HClO_4$ for 6 h. Orthophosphate was determined by the method of King (1932).

Extraction of polyphosphate from yeast for chemical determination was performed as described by Harold (1966).

The possible presence of lipids was determined by the method of Folch *et al.* (1957) and of poly-4-hydroxybutyrate by the method of Schlegel *et al.* (1961).

Metals were determined in a dry sample of the volutin granules after it had been subjected to the wet-ashing procedure of Ballentine & Burford (1957). Sodium, potassium, magnesium and calcium were determined with a Perkin-Elmer model 306 atomic-absorption spectrophotometer.

Protein was determined with the phenol reagent of Lowry *et al.* (1951), with bovine serum albumin as standard; amino acid analysis was performed with a Beckman model 121 automatic amino acid analyser on 6 M-HCl digests; neutral carbohydrates were determined with the phenol/ H_2SO_4 reagent of Dubois *et al.* (1956).

Gel electrophoresis

A low-pH discontinuous electrophoresis system was used as described by Reisfeld *et al.* (1962). The separation gel was 15% polyacrylamide (buffer 0.75 M-acetic acid/KOH, pH 4.3) and spacer gel was 3% polyacrylamide (buffer 60 mM-acetic acid/KOH, pH 6.8). Protein samples contained 15% (w/v) sucrose and 0.001% benzyl-2-naphthylamine as a tracing dye.

SDS/15%-polyacrylamide-gel electrophoresis was performed as described by Weber *et al.* (1972).

Analytical ultracentrifugation

Sedimentation experiments were performed with a Spinco model E analytical ultracentrifuge. The rotor temperature was controlled at 20°C. Photographing and comparator reading techniques were standard. Sedimentation-velocity experiments were conducted either at 44 000 rev./min or at 56 000 rev./min in a double-sector centrepiece of aluminium-filled Epon. Diffusion coefficients were measured at 5000 rev./min in a capillary-type synthetic-boundary cell. Photographs of the boundary were taken with the schlieren optics at time intervals, and from these photographs diffusion coefficients were calculated by the height-area method. Sedimentation and diffusion coefficients were recalculated to standard conditions, water at 20°C and extrapolated to a zero solute concentration.

Sedimentation equilibrium was performed at

3000 rev./min in an aluminium-filled Epon double-sector centrepiece. With the solution column height of 7 mm and initial overspeeding at 4500 rev./min, equilibrium was attained within 7 days. Photographs of the equilibrating system were taken with the interference optics. Vertical fringe displacements were measured relative to the hinge point (Richards & Schachman, 1959).

^{31}P n.m.r.

All spectra were recorded on a Bruker HX-270 n.m.r. spectrometer operating at 109.29 MHz in the pulse Fourier-transform mode and interfaced with a Nicolet BNC-12 computer. Probe temperature was maintained at a desired value with cooled N_2 flow. Chemical-shift values are quoted positive upfield from external 85% orthophosphoric acid.

Spectra of whole yeast were recorded in the following manner. Immediately after the polyphosphate-rich yeasts were harvested, they were washed twice with ice-cold 10 mM-sodium succinate buffer, pH 5.8, and resuspended in the same buffer to give an approx. 50% (w/v) suspension. Then 2–2.5 ml of this suspension was transferred to the 10 mm n.m.r. tube and left for 15 min. After the cells had sedimented to the bottom, the supernatant was taken off. A 4 mm coaxial capillary filled with 2H_2O (field lock) was inserted into the tube (the sample height was 1 cm) and the latter was put into the magnet probe. Sample spinning during the accumulation of free induction decay was not used. Preliminary experiments established that there was no observable change in spectra of yeast cells during the first 2 h if they were kept at 3–5°C. Chemical shifts are referenced by substitution of a sample of 85% orthophosphoric acid in place of the yeast suspension.

Intracellular concentrations of phosphorus-containing metabolites were determined by comparing their respective resonance areas with known orthophosphate concentrations measured under similar conditions. Peak areas were determined by the computer integration subroutine. The relaxation times for all phosphorus atoms were estimated to be shorter than the interval between pulses by at least 5-fold, assuring proportionality between the integrated areas and concentrations.

Results

The volutin granule was isolated from freeze-dried yeast, rich in polyphosphate, by first disrupting the cells as described in the Experimental section. The homogenate was filtered through Miracloth (Calbiochem AG, Lucerne, Switzerland) and the cell debris on the filter was repeatedly washed with carbon tetrachloride. The filtered homogenate was con-

centrated approximately 10-fold by evaporating it on a rotary evaporator at room temperature. Portions (1 ml) of the homogenate were layered on top of a linear gradient of a mixture of carbon tetrachloride and sym-tetrabromoethane (density range 1.59–2.5 g/ml) in cellulose nitrate tubes (2.5 cm × 8.9 cm) and run for 6 h at 60 000 g ($r_{av.} = 11.7$ cm) in the SW 27 rotor of a Beckman ultracentrifuge at 20°C. At the end of the run tubes were removed and inspected visually. Most of the material accumulated at the top of the gradient. Only one narrow band of white material was located in the middle of the gradient, and this was isolated with a fractionating device. The buoyant density of the isolated fraction was 1.95 g/cm³. Under a phase-contrast microscope the isolated fraction was seen to consist of spherical granules, 0.5–1.5 µm in diameter. The isolated fractions from several tubes were combined and dried *in vacuo* at room temperature. The yield of granule was 28 mg from 1 g of freeze-dried yeast.

The isolated granule is not soluble at pH values lower than 6.5 or in the presence of bivalent metal cations, but it is soluble in low-ionic-strength buffers at neutral or alkaline pH. The u.v.-absorption spectrum of the dissolved granule is characteristic of protein ($\lambda_{max.} = 277$ nm, $\lambda_{min.} = 251$ nm, $A_{277}/A_{251} = 2.02$), and its ³¹P n.m.r. spectrum, which is characterized by a single resonance at 23.4 p.p.m. with a half-width of 47 Hz, is of polyphosphate. The isolated granule was analysed for phosphate and metals by digesting it in constant-boiling HClO₄, and for amino acids by digesting it in 6 M-HCl as described in the Experimental section. Polyphosphate (as PO₃) accounted for 32%, protein for 31%, metals for 21%, and tightly bound water for 19% of the material. Table 1 gives the metal analysis of the isolated granule. The amino acid composition of the protein in the granule is given in Table 2. Lipids, neutral carbohydrates and poly-4-hydroxybutyrate were not detected in the granule.

The polyphosphate was separated from protein by dissolving the granule in 6 M-urea and gel filtration. In a typical separation, 60.3 mg of dry volutin granules (19.4 mg of PO₃) was dissolved in 43 ml of 6 M-urea in buffer A (2 mM-Tris/HCl buffer, pH 7.5).

The resulting solution ($A_{280}^{1cm} = 11.6$) was applied to a 2.5 cm × 30 cm column of Sephadex G-100 pre-equilibrated with the same solution. The column was eluted with 100 ml of buffer A containing 6 M-urea and subsequently by a linear gradient of 0.1–1 M-NaCl in the same solution. As shown in Fig. 1, polyphosphate is eluted with the buffer run and protein with 0.62 M-NaCl. Recoveries from the column were 95% in polyphosphate (18.3 mg of PO₃) and 92% in protein ($A_{280}^{1cm} = 10.7$). Pooled polyphosphate and protein fractions were dialysed separately against several changes of buffer A at 4°C and were stored frozen. All the polyphosphate and protein are eluted in the front when a solution of the granule is chromatographed in buffer A.

The protein of the granule was separated by electrophoresis on a polyacrylamide gel as described in the Experimental section. Under these conditions the protein is resolved into four components, all of which move towards the cathode. In Fig. 2 a photograph of a gel stained with Amido Black after an electrophoretic separation of the protein is reproduced. Four components were also obtained when electrophoresis was performed in an SDS-containing gel. The molecular weights of the four components were then all between 10 000 and 20 000, as determined with protein markers.

Hydrodynamic analyses

For the hydrodynamic analyses the granule was first dissolved in buffer A and then NaCl was added to the concentration of 0.4 M. Sedimentation velocity was determined in an ultracentrifuge running at

Table 1. *Metal content of volutin granule*
Experimental details are given in the text.

Metal	Content (mg/g)
Na	2.1
K	150.1
Mg	52.9
Ca	3.1

Table 2. *Amino acid composition of the protein of volutin granule*

Experimental details are given in the text. Tryptophan was not determined.

Amino acid	Composition (mol of residue/mol of methionine)
Lysine	13.5
Histidine	1.6
Arginine	7.6
Aspartic acid	4.8
Threonine	5.7
Serine	6.2
Glutamic acid	7.9
Proline	4.3
Glycine	8.3
Alanine	13.4
Cysteine	0
Valine	5.9
Methionine	1.0
Isoleucine	3.7
Leucine	7.6
Tyrosine	2.1
Phenylalanine	1.7

Fig. 1. Gel-filtration chromatography of solubilized volutin granule

The volutin granule was dissolved and chromatographed in 6 M-urea in buffer A on a Sephadex G-100 column. Protein was retained on the column and a gradient of NaCl was used to elute it. Full experimental details are given in the text. Symbols: ●, phosphate; ▲, protein; ----, concn. of NaCl.

Fig. 2. Electrophoretic separation of volutin-granule protein

The gel was 15% in polyacrylamide and the buffer was of pH 4.3. A 30 μ g portion of protein was applied in buffer A. The conditions were: 3 mA/gel, 4 h, 4°C. Staining was with Amido Black. Full experimental details are given in the text.

29.6 mg/ml solution of the granule sedimentation coefficients, $s_{20,w}$, were 21.3 ± 0.6 S and 21.8 ± 1.1 S from the schlieren pattern and the u.v. scan respectively. The extrapolated value of the sedimentation coefficient, $s_{20,w}^0$, is 22.3 S.

The sedimentation coefficient and the diffusion coefficient of the isolated polyphosphate in buffer B (0.4 M-NaCl/2 mM-Tris/HCl buffer, pH 7.5) were determined in an analytical ultracentrifuge as described in the Experimental section. In the concentration range studied (8.8–58.4 mM), the polyphosphate was homogeneous. Extrapolated values of the sedimentation coefficient and of the diffusion coefficient were: $s_{20,w}^0 = 14.6$ S and $D_{20,w}^0 = 1.83 \times 10^{-7}$ cm²/s.

The molecular weight of the polyphosphate in the same buffer solution was determined in a sedimentation-equilibrium experiment as described in the Experimental section. A plot of $\log J$ versus r^2 is given in Fig. 3, where J is the vertical fringe displacement, expressed as a number of fringes, from the zero-concentration fringe determined in a separate synthetic-boundary experiment and r is the distance (cm) from the centre of rotation. Symbols (●) represent experimental points and the continuous line represents the theoretical curve for a monodisperse solute of molecular weight 245 000. To calculate confidence limits of this molecular-weight estimate the data were fitted to the dependence (Svedberg & Pedersen, 1940):

$$\frac{d \ln J}{dr^2} = \frac{M(1 - \bar{v})^2}{2RT}$$

in its integrated form by using the Levenberg-Marquardt non-linear least-square algorithm (IMSL Library I, Edition 6, subroutine ZXSSQ, 1977). The

44 000 rev./min. The material, in the concentration range 7.4–74.2 mg/ml, sedimented with a single symmetrical boundary. The boundary movement was monitored either by schlieren optics or, at lower concentrations, by u.v. scanning. Both methods gave a comparable sedimentation coefficient. Thus for a

Fig. 3. Logarithmic plot of sedimentation-equilibrium data of polyphosphate

A 7.4 mg/ml solution of the polyphosphate (as PO_3) isolated from the volutin granule was analysed as described in the Experimental section. ●, Experimental results; —, theoretical curve for a monodisperse solute of molecular weight 245 000.

molecular weight calculated by this procedure was $245\,000 \pm 1000$, assuming a partial specific volume of $0.316\text{ cm}^3/\text{g}$ (Malmgren, 1952). This molecular weight corresponds to 3100 ± 12 phosphate units.

³¹P n.m.r. spectroscopic analysis

Fig. 4(a) is the ³¹P n.m.r. spectrum of the isolated polyphosphate. It is characterized by a single resonance at 22.9 p.p.m. with a half-width of 13 Hz. On the basis of the absence of resonances from end phosphates of polyphosphates in the vicinity of 7 p.p.m. and of the signal-to-noise ratio of 200:1, the minimal chain length of the polyphosphate is estimated to be not less than 400 phosphate units (cf. preceding section). Fig. 4(b) is the ³¹P n.m.r. spectrum of the dissolved granule. As in Fig. 4(a), it contains a single resonance from middle phosphate groups of a long-chain polyphosphate. This resonance is, however, shifted upfield to 23.4 p.p.m. and is also much broader, with half-width of 47 Hz. In Fig. 4(c) the ³¹P n.m.r. spectrum of the polyphosphate-rich yeast, from which the granule was isolated, is given. The assignment of the resonances in this spectrum was in accordance with

Fig. 4. ³¹P n.m.r. spectra of polyphosphate-rich yeast, volutin granule and polyphosphate

Spectra were recorded at 109.29 MHz. Samples, at 5°C, were irradiated with 45° radio frequency pulses (10 μs) with a repetition time of 5 s. A total of 1000–3000 free-induction-decay transients was accumulated by quadrature detection into 8 kbytes of a computer memory (acquisition time 0.9 s). (a) Polyphosphate (52.8 mM as PO_3) isolated from the volutin granule dissolved in buffer A; (b) volutin granules (5.2 mg/ml) dissolved in buffer A; (c) a suspension (70%, w/v) of polyphosphate-rich yeast in 10 mM-succinate buffer, pH 5.8. Resonance assignments are: 1, sugar phosphates; 2, orthophosphate; 3, unknown; 4, end phosphate groups of short-chain polyphosphates; 5, NAD^+ ; 6, penultimate phosphate groups of short-chain polyphosphates; 7, middle phosphate groups of short-chain polyphosphates; 8, middle phosphate groups of long-chain polyphosphates.

Salhany *et al.* (1975) and Navon *et al.* (1979). The multiple resonances that are not resolved and give a broad envelope at -3.4 p.p.m. originate from sugar phosphates. The resonance at -1.1 p.p.m. is of intracellular orthophosphate and corresponds to an intracellular concentration of 70 mM. The resonance at 1.1 p.p.m. is at the present time unaccounted for. The resonance at 10.9 p.p.m. is assigned to NAD^+ and its intracellular concentration is estimated as 30 mM. Resonances at 6.8 p.p.m., 20.6 p.p.m. and 22.0 p.p.m. were assigned to the end, penultimate and middle phosphate groups of short-chain polyphosphates, and the resonance at 22.6 p.p.m. was assigned to middle phosphate groups in long-chain polyphosphates (Glonek *et al.*, 1975). The amount of polyphosphate in polyphosphate-rich cells calculated on the basis of the resonances at 22.0 p.p.m. and 22.6 p.p.m. is 39.1 mg of P/g dry wt. of yeast, which accounts for 61% of the total cell phosphorus. Visual inspection of the resonance at 22.6 p.p.m. reveals that it does not conform to a single Lorentzian peak. Analysis of this resonance, assuming two Lorentzian components, was done by computer simulation. A computer generated sum of two Lorentzians was plotted with the relative intensities, positions and respective spin-spin relaxation rates as the variable parameters and was compared with the observed resonance. By this analysis two components suffice to account for the observed resonance: one, centred at 22.6 p.p.m. with a half-width of 50 Hz, corresponds to 85% of the area under the resonance, and the other, centred at 23.4 p.p.m. with a half-width of 180 Hz, represents the remaining 15% of the resonance.

Discussion

When the isolated volutin granule is dissolved at neutral pH in a non-dissociating buffer it gives a solution that is homogeneous by the criteria of ultracentrifuge analysis. The solute sediments with the same sedimentation coefficient (22.3 S) when monitored either by refractivity or by u.v. absorption. This shows that the polyphosphate and the protein are in a complex. This complex is not resolved when chromatographed on Sephadex G-100 in buffer A. The complex is resolved by 6 M-urea and is separated by gel filtration. The polyphosphate isolated by this procedure sediments in the ultracentrifuge as a homogeneous material ($s_{20,w}^0 = 14.6$ S), and the protein that has a high basic amino acid content (25%) can be separated into four low-molecular-weight ($10\,000$ – $20\,000$) components.

The isolated polyphosphate is characterized by a single ^{31}P n.m.r. resonance of a quite narrow linewidth (Fig. 4a), which in the granule is shifted upfield by 0.5 p.p.m. and is appreciably broadened

(Fig. 4b), as would be expected for the polyphosphate immobilized by interaction with protein. Additional factors can contribute to the observed broadening, e.g. broadening due to the inhomogeneity in the intimate environment of middle phosphate groups and/or the greater spread of P–O–P angles compared with free polyphosphate. The reason for the upfield shift of this resonance in the granule is apparently due to its relatively high magnesium content. An upfield shift of middle-phosphate-group resonance is observed when bivalent metal ions such as Mg^{2+} are added to high-molecular-weight polyphosphates (Glonek *et al.*, 1971).

The polyphosphate contained in the granule is only a small fraction of the total cell polyphosphate, which from the n.m.r. spectroscopical analysis was estimated to be 100 mg of PO_3 per 1 g dry wt. of yeast. However, the resonance from middle phosphate groups in long-chain polyphosphates in the ^{31}P n.m.r. spectrum of whole yeast (Fig. 4c) can be resolved into two components, with the chemical shift of the minor component (15%) corresponding to that of the isolated granule (see Fig. 4b). Thus it would appear that only a relatively small fraction of cell polyphosphate (14.3%) is in the volutin granule.

The isolated polyphosphate equilibrates in the ultracentrifuge as a homogeneous material of molecular weight $245\,000 \pm 1000$. For comparison, the molecular weight, calculated on the basis of the sedimentation and diffusion coefficients obtained from hydrodynamic measurements, is $286\,000$. Both these molecular-weight estimates, however, could be overestimates, since polyphosphates are known to form strong complexes with alkali-metal ions (Strauss *et al.*, 1957), and in an 0.4 M-NaCl solution, in which the experiments were done, the Na^+ ion could be part of the polyphosphate molecule.

The degree of dispersity of the polyphosphate isolated from the volutin granule, as determined by the sedimentation-equilibrium method, is at most several phosphate units out of several thousand units present in the molecule. The length of a coiled polyphosphate molecule of this size will be about $0.65\ \mu\text{m}$ (Thilo, 1962), which is similar to the minimal size of the isolated granule. In the granule this polyphosphate is complexed to low-molecular-weight basic proteins. Since, as discussed above, this complex sediments with a velocity not very different from that of the polyphosphate, a complex of a single molecule of polyphosphate with the attached proteins appears to be the structural unit of the granule.

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