Ribosomal Proteins of the Dimorphic Fungus, *Mucor racemosus*

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**Summary.** Ribosomal proteins of the dimorphic fungus *Mucor racemosus* were isolated and characterized by 2-dimensional gel electrophoresis. Proteins from ribosomes of the yeast and mycelial phase were compared, and were found to be qualitatively indistinguishable. The only consistent difference in the patterns of proteins was in a protein of the 40S subunit, S-6. This protein was phosphorylated in yeast and hyphae forms, but not in asexual sporangiospores. Studies on protein S-6 showed that it contained 3 phosphate residues per molecule of protein when maximally phosphorylated. In this form 3 different tryptic peptides were shown to contain a single phosphoserine. The S-6 protein also existed in forms containing 1 or 2 phosphates per molecule, depending on growth conditions.

**Introduction**

The dimorphic fungus *Mucor* offers many advantages for studying the relationship of macromolecular synthesis to the patterns of growth and differentiation in the eukaryotic protists. The organism grows in a spherical yeast form, demonstrating multipolar budding in 100% carbon dioxide. Growth in air, on the other hand, results in hyphal growth and ultimately the production of sporangiophores (Bartnicki-Garcia and Nickerson, 1962). Studies in this laboratory and others have laid a firm physiological and biochemical basis for many of the events which accompany morphogenesis when the organism is going through the transition from yeast to hyphae. At least two enzymes are synthesized specifically in response to a signal for hyphal development: NAD-dependent glutamate dehydrogenase (Peters and Sypherd, 1978) and ornithine decarboxylase (Inderlied and Sypherd, 1979).

During the transition of yeast to hyphae there is a dramatic increase in the rate of amino acid incorporation which occurs by an increase in the rate at which peptide bonds are formed and a decrease in the mean transit time of ribosomes on messenger RNA (Orlowski and Sypherd, 1977, 1978a). A similar phenomenon has been observed during the outgrowth of germ tubes from aerobically germinated sporangiophores (Orlowski and Sypherd, 1978b).

With the demonstrated change in the activity of the protein synthesizing system, we have begun a systematic analysis of the translational system in an attempt to identify the events which are responsible for the more rapid movement of ribosomes across the message during morphogenesis. In other systems undergoing development, it has been documented that certain changes in ribosomal proteins occur. These systems include the morphogenesis of *Drosophila* (Lambertsson, 1975), transformation and tumorigenesis (Rogers, 1973; Subramaniam et al., 1975), as well as certain differentiated tissues (Delaunay et al., 1972; Subramaniam et al., 1975). Phosphorylation of ribosomal proteins represents the most thoroughly characterized form of ribosome modification in vivo. While no definite functional role for the phosphorylation and dephosphorylation of ribosomal proteins has been identified, it is clear that the phosphorylation of the protein in the small subunit known as S6 responds to changes in cyclic AMP levels and hormone levels (Gressner and Wool, 1976), viral infection (Kaerlin and Horak, 1976), and cell proliferation (Lastick et al., 1977; Leader et al., 1976).

Incorporation of $^{32}$P into the protein S6 has been observed in a number of eukaryotic cells, including rat liver (Gressner and Wool, 1974), rabbit reticulocytes (Traugh and Porter, 1976), *Saccharomyces* (Zinker and Warner, 1976), cultured baby hamster kidney cells (Leader et al., 1976), and HeLa cells (Kaerlein and Horak, 1976; Lastick et al., 1977). Since morphogenesis in *M. racemosus* is at least partially regulated
by intracellular levels of cyclic AMP (Larsen and Sypherd, 1974), we have studied the relationship between the phosphorylation of ribosomal protein and the process of morphogenesis. In the course of these studies, we have found that the same set of major proteins is present in the ribosomes of ungerminated sporangiophores and in vegetative yeast-like and hyphal cells. The only consistent differences in these patterns result from changes in the extent of phosphorylation of a protein in the small subunit.

Materials and Methods

Organism and Culture Conditions

*Mucor racemosus* (M. isotianus) ATCC 1216B was used in all experiments. Aerobic hyphae used for ribosome preparations were grown in 400-600 ml of YPG media in a 2,800 ml Fernbach flask. After inoculation to 5 × 10⁵ spores per ml, the culture was flushed with 3 ml of air per ml of culture medium per minute while the culture was shaken on a rotary shaker. CO₂ yeast cells used for ribosome preparations were grown in 1,000-1,500 ml of YPG media in a 2,800 ml Fernbach flask. After inoculation to 1-2 × 10⁵ spores per ml, the culture was continuously flushed with 0.005-0.10 ml of CO₂ per ml of culture medium per minute. Shifts of CO₂ yeast cultures to atmospheres of air or nitrogen were carried out by replacing the CO₂ gas with the new gas at a flow rate of 4 ml of gas per ml of culture medium per minute. Labeling with ³²P was carried out in YPG medium by adding carrier-free ³²P₀₄ (ICN) to a final concentration of 50-100 gCi/ml. YPG medium was made up of 0.3% (W/V) yeast extract (Difco), 1% bactopeptone, 2% glucose, pH 4.5.

Buffers

Buffer 50/10 consisted of 50 mM Tris-HCl of pH 7.2, 50 mM KCl, 10 mM magnesium acetate, 12 mM β-mercaptoethanol. Buffer 500/10 is the same as buffer 50/10 with the exception that KCl is 500 mM. Buffer 880/10 is the same as buffer 50/10 with the exception that potassium acetate is 500 mM. Buffer 500/0.2 is the same as buffer 500/10 with the exception that magnesium acetate is 0.2 mM.

Preparation of Ribosomes

Cells were disrupted by grinding in a mortar and pestle in liquid nitrogen for one minute. The frozen powder was transferred to centrifuge tubes held a 0 ° containing either buffer 500/10 or buffer 50/10. Spores and ³²P-labeled cells were disrupted in an appropriate buffer in the French pressure cell at 8,000 psi. A 30,000 × g supernatant was prepared by centrifugation for 30 min at 15,000 rpm in a Sorval SS-34 rotor. For the preparation of total unfraccionated ribosomes, a 100,000 × g pellet was obtained by layering 100-400 A₂⁶₀ units of the 30,000 × g supernatant in a volume of 5 ml over 3 ml of 10% sucrose in the appropriate buffer and centrifuging for 4 h at 45,000 rpm in the 50 Ti rotor at 4 °C. For the preparation of low salt “80S” ribosomes and “native 40S” subunits, 30,000 × g supernatants were prepared from cells disrupted in the French pressure cell in buffer 50/10. Approximately 140 A₂⁶₀ units of this extract were layered in a volume of 4 ml over a 33 ml 10-30% linear sucrose gradient in buffer 50/10 and centrifuged 7 hours at 26,000 rpm at 4 ° in SW 27 rotor. For preparation of derived subunits, 30,000 × g extracts were made 600 mM in KCl and 0.1 mM in puromycin-HCl, incubated for 20 min at room temperature and subjected to centrifugation. Polysomes and derived subunits were prepared from cells grown in liquid nitrogen and buffer of 500/10 containing 200 µg/ml cycloheximide. From 160-200 A₂⁶₀ units of 30,000 × g supernatant in a volume of 4 ml was layered onto a 32 ml 10–50% linear sucrose gradient formed over a 1.5 ml cushion of 60% sucrose, all in buffer 500/10. Centrifugation was for 4 h at 23,000 rpm in an SW 27 rotor at 10 °.

Isolation of Ribosomal Proteins

Ribosomal pellets were suspended in buffer 500/10 or 50/10 and then were made 100 mM in magnesium acetate. Glacial acetic acid was added to a final concentration of 66% and the mixture was stirred on ice for 1 h (Sherton and Wool, 1974). The RNA precipitate was removed and the supernatant was dialyzed for 24 h at 4 °C against 1,000 volumes of 5% acetic acid and 0.1% β-mercaptoethanol. The dialyzed solution was concentrated by lyophilization.

Two-Dimensional Polyacrylamide Gel Electrophoresis

A modification of the procedure of Kalschenidt and Wittmann (1970) was used throughout. The compositions of the first dimensional gel and tank buffer were modified as described by Lastick et al. (1977) except that the acrylamide concentration of the gel was increased to a final concentration of 6%, the pH of both solutions raised to 8.8 and 5 M urea was included in the upper reservoir buffer. Typically 200-400 µg of ribosomal pellet protein, or up to 3 mg of supernatant protein was dissolved in 150-400 µl of 10 M urea (Schwarz-Mann ultrapure), 3% β-mercaptoethanol and heated 10 min at 60 °C as described by Lastick et al. (1977). Constant-current electrophoresis was carried out for 17 h at 3 mA/gel towards the cathode (for basic proteins) or 6-8 h at 3 mA/gel towards the anode (for the acidic proteins). The second dimension was run for 26 h at 25 mA/gel. Gels were stained overnight on racks in a solution of 0.2% Coomassie Brilliant Blue R (Sigma) in 25% isopropanol, 10% acetic acid, and destained by soaking at least 48 h in several changes of 10% isopropanol, 10% acetic acid. Gel electrophoresis in sodium dodecyl sulfate (SDS) gels was carried out in the Tris glycine buffered system described by Laemmli (1970) in 12% or 8% polyacrylamide slabs (13 × 14 × 0.1 cm). Electrophoresis was for 6 h at 18 mA constant-current at room temperature.

 Autoradiography of two-dimensional and SDS gels was carried out with the wet gels wrapped in polyvinyl chloride plastic film (Saran Wrap) and exposed to Kodak NS-54T X-ray film for 4-21 days. Proteins were recovered from gel samples for determination of stain content, radioactivity, or further chemical analysis by crushing the gel in 0.6-1.2 ml of 50% acetic acid and incubating the suspension overnight at 30 ° in 10% acetic acid. Usually over 90% of the protein was recovered in the supernatant of the first extraction as determined by radioactivity or stain content.

Analysis of Proteins

Limited acid hydrolysis of protein for phosphoamino acid determination was carried out in vacuo for 4 h at 110 °C in 6 N HCl according to Bylund and Hung (1975). The hydrolyzate was concentrated by lyophilization. High voltage electrophoresis was carried out on Whatman 3 MM paper in 2.5% formic acid and