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A Cytoplasmic Protein from *Neurospora crassa* Resembling Membrane Proteins*

Charles F. Shannon and John M. Hill†

ABSTRACT: A protein component, in association with nonprotein components, has been recovered from the cytoplasm of *Neurospora crassa* and has been partially characterized. The protein component was separated from the nonprotein components by gel filtration. This protein component represented over 50% of the total soluble protein during early logarithmic phase growth of the organism. The isolated protein component appeared to be a single protein species. It electrofocused as a single band with an isoelectric point of pH 5.3. The isolated protein migrated as a single band during analytical electrophoresis at pH 7.0 in polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Multiple bands were observed in the same gel system following analytical electrophoresis of the protein when it was associated with the nonprotein components. However, preparative electrophoretic separation of these bands and subsequent amino acid analyses indicated that these bands were very similar in amino acid composition, thus providing strong evidence that only a single protein was present. Gel filtration also indicated a single protein species. Molecular weight analyses by gel electrophoresis, sedimentation equilibrium centrifugation, gel filtration, and amino acid composition were consistent with a monomer molecular weight of 15,000 with a stable aggregated species of molecular weight 45,000. The amino acid composition of the protein indicated that the protein was extremely hydrophobic. This would explain the aggregation phenomena encountered in molecular weight analyses. The amino acid composition, solubility properties, and electrofocusing pattern shows this protein is very similar to proteins isolated from cellular and organelle membranes.

A protein component was obtained from the cytoplasm of *Neurospora crassa* by Kuehn *et al.* (1969) using a mild isolation procedure. This protein component appeared to be similar to protein components isolated from cellular and organelle membranes. However, the protein components of membranes have proved to be so numerous and complex as to preclude a unified concept of membrane structure. Schnaitman (1969) has described inner mitochondrial membranes containing 23 proteins and endoplasmic reticulum with 15 proteins. Neville (1967), as well as Kiehn and Holland (1968, 1970a,b), found a complex array of proteins in mammalian cell membranes. The plasma membranes of several strains of Mycoplasma contain over 20 proteins (Rottem and Razin, 1967). The protein complexity of erythrocyte membranes has been described by several laboratories (Schneiderman, 1965; Lenard and Singer, 1966; Carraway and Kobylka, 1970). It is possible that many of these components observed in cell and organelle membranes were not different proteins, but were oligomers of the same protein.

Only relatively specialized membranes have shown an apparent simplicity in protein structure, and these appear to be the exception rather than the rule. The gas vacuole membranes from blue-green algae are assembled with a single protein of molecular weight of 14,000 and without the lipids usually found in biological membranes (Jones and Jost, 1970). Yu and Masoro (1970) claim that sarcotubular membranes from rat muscle cells are composed principally of a single protein of molecular weight of 6000–10,000.

The apparent complexity of membrane structure has been magnified by the controversy concerning homogeneity and molecular weights of protein fractions isolated from membranes of various cells and organelles. Criddle *et al.* (1962) and Richardson *et al.* (1964) solubilized organelle membranes from bovine cells using detergents and surface activating agents, and fractionated the protein components. A major protein fraction was obtained which was labeled "structural

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protein." This material was claimed to be homogeneous, nonenzymatic, and to have a molecular weight of about 25,000. A protein fraction isolated from *Neurospora crassa* mitochondrial membranes by Woodward and Munkres (1966), using essentially the same methods, also appeared to be homogeneous and was labeled structural protein. However studies by Lenaz *et al.* (1968a,b) have shown the bovine material to be a heterogeneous family of proteins with molecular weights in the range of 55,000–70,000. Recently Senior and MacLennan (1970) demonstrated heterogeneity in bovine structural protein preparations isolated by the procedures of Criddle and Richardson. In addition, they claimed a similarity of a major component of this material with ATPase which had been denatured by the isolation procedure.

Kuehn *et al.* (1969) used a much milder method to obtain the protein component in quasicrystalline form from the cytoplasm of *N. crassa*. This gentle procedure reduced the possibility of protein denaturation. The present paper is a report on further studies of this protein component as a step toward answering the following important questions: (1) the number and molecular weights of proteins contained in this component; (2) the other characteristics of this material which would be relevant to its possible role as a membrane component.

Materials and Methods

Growth and Extraction. N. crassa, St. Lawrence strain 74 A, was used. It was grown with vigorous aeration in Vogel's medium (Vogel, 1964) at 39° and harvested during early logarithmic phase growth by filtration through cheesecloth. The mycelial pad was washed with distilled water, pressed dry, weighed, and ground in liquid nitrogen. Powdered, frozen *N. crassa* (1 g) was extracted with 2 ml of 0.1 M Tris-sulfate (pH 7.4, 25°) containing 0.5 M $NaHCO_3$ and 2 mM β-mercaptoethanol. The extract was centrifuged at 105,000*g* for 1 hr at 4° and the supernatant was decanted. Quasicrystalline material was obtained during dialysis of the supernatant against 1 mM Tris-sulfate (pH 7.4, 25°) containing 5 mM $NaHCO_3$, 2 mM β-mercaptoethanol, and 1 mM $MgCl_2$.

The quasicrystals were recovered from the dialyzed supernatant by centrifugation and were washed five times with 100 volumes of glass-distilled water.

Standard Assays and Treatments. PROTEIN concentrations were determined by the method of Itzhaki and Gill (1964). Quasicrystals were dissolved in 2% SDS[1] before assaying.

LIPID was qualitatively analyzed by thin-layer chromatography on silica gel G plates using two solvent systems: hexane–diethyl ether–acetic acid (90:10:1, v/v) for neutral lipids; and chloroform–methanol–acetic acid–water (50:25:8:4, v/v) for polar lipids. Quasicrystals were treated with 2-chloroethanol adjusted to pH 8.0 with ammonium hydroxide, and the lipid fraction was separated by gel filtration on Sephadex LH-20. The standards used in the lipid analyses were: ergosterol (Nutritional Biochemical Corp.); 1,2-diolein, cholesterol, oleic acid, 1,3-diolein, triolein, cholesterol oleate, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine (Applied Science Laboratories, State College, Pa.). The lipids were visualized by oxidation at 200° with 50% sulfuric acid containing 0.5% sodium dichromate, and identified by their relative mobilities. Ultraviolet spectral analysis of the lipid fraction in 2-chloroethanol at pH 8.0 was performed with a Cary 14 spectrophotometer. Lipid was quantitated gravimetrically following extraction of the quasicrystals with absolute methanol, 95% ethanol, and acetone.

TOTAL PHOSPHORUS was assayed by the method of Ames and Dubin (1960).

CARBOHYDRATE analysis was performed by the orcinol procedure (Horecker, 1957). Deoxyribose was assayed using diphenylamine (Burton, 1956).

CARBOXYMETHYLATION of the isolated protein component was accomplished by the method of Crestfield *et al.* (1963) using 0.10 M β-mercaptoethanol and 0.14 M iodoacetic acid.

Protein Isolation. The protein component was isolated from the quasicrystalline material after alkaline treatment overnight at room temperature using 1.0 M NaOH. It was separated from the nonprotein components of the quasicrystals by gel filtration on a 20 × 1.5 cm column of Sephadex LH-20 (Pharmacia) equilibrated with freshly distilled 2-chloroethanol adjusted to pH 8.0 with ammonium hydroxide. Elution of samples was monitored at 254 nm using an Isco 270 ultraviolet flow monitor. The isolated protein component precipitated when dialyzed against glass-distilled water. This product was lyophilized and stored in a desiccator over $CaCl_2$.

Amino Acid Analyses. Samples of quasicrystals and isolated protein component were hydrolyzed under vacuum in sealed vials for 14, 24, 48, and 72 hr at 110° in constant-boiling HCl (5.7 N). Following hydrolysis, the HCl was evaporated to dryness and the dried amino acids dissolved in 0.2 M citrate buffer (pH 2.2). Amino acid analyses were carried out on a Beckman Spinco 120C Automatic amino acid analyzer by the method of Moore and Stein (1963). Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956), and tryptophan by spectroscopic analysis in 2-chloroethanol. Extinction coefficients of tyrosine and tryptophan in 2-chloroethanol were determined in our laboratory. The amounts of all other amino acids were determined from timed hydrolyses. The values for valine and isoleucine were taken at 72 hr. Other values were obtained by graphic extrapolation to zero time.

Electrofocusing. Samples of the protein component were prepared for analytical electrofocusing by solubilization in 5 M H_3PO_4. Electrofocusing was conducted in 7% polyacrylamide gels containing 8 M urea and 1% LKB ampholine (LKB-Produkter AB, Stockholm, Sweden), pH 4–6. Electrofocusing continued for 1 hr at 150 V between an upper bath of 5% H_3PO_4 (positive electrode) and a lower bath of 5% ethylenediamine (negative electrode). Following the electrofocusing the gels were removed from the tubes and fixed in 7% trichloroacetic acid. They were then scanned at 280 nm on a Gilford 2400 recording spectrophotometer with a 2410 linear transport. A sample gel from each electrofocusing experiment was sliced into 5-mm sections and the ampholyte from each section was eluted into 1 ml of distilled water. The pH of the resulting solution was determined to estimate the pH gradient within the gel.

Preparative electrofocusing was performed on a LKB electrofocusing apparatus with a 110-ml column using 1% LKB ampholine, pH 4–6, in 8 M urea. The anode solution was 1% phosphoric acid and the cathode solution was 2% ethanolamine. Electrofocusing continued for 30 hr at 600 V and the column was drained through an ISCO ultraviolet flow monitor.

Electrophoresis. Analytical polyacrylamide electrophoresis of the protein component was conducted at pH 3 (Takayama *et al.*, 1964) and at pH 9.5 (Davis, 1964) using 7 × 0.7 cm gels of 7.5% acrylamide. Electrophoresis was also conducted

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; V_e, elution volume; V_0, void volume; *D*, deviation function.

at pH 7.0 (Weber and Osborn, 1969) using 10% acrylamide gels containing 0.1% SDS. For molecular weight determinations using the SDS system the following pure protein standards were used: catalase and lysozyme (Mann); aldolase, ribonuclease A and insulin (Sigma); pepsin and chrmotrypsinogen A (Worthington). Following electrophoresis, the gels were either fixed with 7% trichloroacetic acid and scanned at 280 nm on a Gilford 2400 recording spectrophotometer, or they were stained with aniline black or with coomassie blue. The stained gels were destained electrophoretically in an aqueous destaining solution of 7.5% acetic acid and 5% methanol, or by diffusion in 7% acetic acid before scanning at 550 nm.

Preparative electrophoretic separations were obtained using the 0.1% SDS analytical electrophoretic system of Weber and Osborn (1969). Samples of quasicrystalline material were solubilized in 0.01 M sodium phosphate buffer (pH 7.0), 5% SDS, and 0.1% β-mercaptoethanol and then dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.0), 0.1% SDS, and 0.1% β-mercaptoethanol. The samples were loaded onto a 10% polyacrylamide column 4.0 cm long containing 0.1% SDS, in a cylindrical chamber 2.5 cm in diameter with a central cold finger 0.7 cm in diameter. Electrophoresis continued for 24–36 hr at 120 V and 45 mA. Fractions of 1.5 ml were collected and monitored using an Isco ultraviolet flow monitor. The absorbance at 280 nm was then determined. The contents of peak fractions were lyophilized and redissolved in a minimum amount of distilled water. A ninefold excess of cold acetone was added to precipitate the protein leaving the detergent in solution. The proteins were recovered by centrifugation and hydrolyzed in constant-boiling HCl for 24 hr before analysis for amino acids.

Subunit Molecular Weight. A minimum subunit molecular weight was calculated from the amino acid composition of the protein component by plotting the total per cent deviation from integers of the amino acid residues *vs.* the molecular weight. The per cent deviation from integers for a particular quantity of an amino acid residue is the decimal portion of that quantity divided by the nearest whole number. This value is calculated for each amino acid residue at a given molecular weight, and summed over all residues to give a total per cent deviation from integers for that molecular weight. A minimum total per cent deviation which is consistent with the amino acid composition (no residue present less than one) indicates the minimum subunit molecular weight.

Gel Filtration. A modification of the method of Fish *et al.* (1969) was used to estimate molecular weight by gel filtration. A 90 × 1.5 cm Sephadex column (Pharmacia) was packed to a height of 85 cm with Sephadex G-75 equilibrated with 8 M urea in 25% formic acid. Samples of the protein component were carboxymethylated as described previously and solubilized in the eluting solvent. Ten milligrams of each of the following pure proteins [pepsin, chymotrypsinogen A, and trypsin (Worthington); ribonuclease A and insulin (Sigma); lysozyme (Mann)] was dissolved in 0.5 ml of 8 M urea in 25% formic acid and used to standardize the column. The column void volumn (V_0) was measured with Blue Dextran 2000 (Pharmacia). Fractions of 2 ml were collected and assayed for protein by absorbance at 280 nm. Elution volumes (V_e) were measured, and a plot was made of V_e/V_0 (to normalize data from different runs) *vs.* log molecular weight.

Ultracentrifugation. High-speed sedimentation equilibrium studies (Yphantis, 1964) were performed on a Spinco Model E ultracentrifuge equipped with an electronic speed control and RTIC temperature control unit. Samples of the protein component were solubilized in and dialyzed against 6 M guanidine hydrochloride containing 1 mM β-mercaptoethanol. Ultracentrifugation was performed using a six-channel Yphantis cell run at 40,000 rpm (20°) using Raleigh interference optics. Fringe displacements on all photographic plates were measured with a Gaertner microcomparator. A value of 0.736 for $\bar{v}$, the partial specific volume, was calculated from the amino acid composition (Cohn and Edsall, 1943). A density of 1.45 for 6 M guanidine hydrochloride was independently determined.

Hydrophobicity. The average hydrophobicity per amino acid residue ($H\Phi_{av}$) of the isolated protein was calculated on the basis of amino acid composition by the method of Bigelow (1967), and the polarity ratio (p) calculated according to Fisher (1964).

Extinction Coefficient. The extinction coefficient of the protein isolated from the quasicrystals was determined on a Cary 14 spectrophotometer from the absorption spectrum of 2-chlorethanol solutions of weighed samples of the lyophilized protein. Subsequent protein concentrations were determined spectrophotometrically.

Mitochondrial Membrane Protein. Protein was isolated from the mitochondrial membranes of *N. crassa* by the method of Woodward and Munkres (1966). This membrane protein was provided by D. O. Woodward.

Results

Components of the Quasicrystals. The quasicrystalline material, which was obtained from the cytosol of *N. crassa* by Kuehn *et al.* (1969), was analyzed extensively by the present authors. This material, which was recovered during early logarithmic phase growth, contained an average of 0.2 mg of protein/mg dry weight of quasicrystals. Organic phosphates were present at a minimum of 0.08 mg of phosphate per mg dry weight of quasicrystals. Carbohydrate analysis by the orcinol procedure indicated 0.11 mg of ribose/mg dry weight of quasicrystals. The diphenylamine assay for deoxyribose was negative. Lipid represented 0.47 mg/mg dry weight of quasicrystals. Thin-layer chromatographic analysis for lipids indicated the major lipid in the quasicrystals was ergosterol. Traces of phospholipids were also present. Ultraviolet spectral analysis of this lipid fraction confirmed the presence of ergosterol by comparison with the published spectrum (Fox *et al.*, 1952). The balance of the quasicrystalline material was a component with an absorption maximum at 260 nm.

Protein Isolation. The quasicrystals were treated overnight with 1 M NaOH at room temperature prior to gel filtration on Sephadex LH-20 equilibrated with 2-chloroethanol. Two components of ultraviolet-absorbing material were separated by the gel filtration, only one of which contained protein. Without treatment of the quasicrystals with 1 M NaOH prior to gel filtration, this protein component could not be separated from the nonprotein material.

The isolated protein component precipitated when dialyzed against glass-distilled water. This product was lyophilized and stored in a desiccator over $CaCl_2$. Carbohydrate analysis of the protein component by the orcinol procedure was negative. Total phosphorus determinations detected less than 0.002 μg of phosphorus/μg of the protein component. Lipid analysis by thin-layer chromatography indicated no lipids present in this material. The isolated protein component showed a strong absorption at 277 nm (Figure 1). The protein component was insoluble in aqueous buffers except at extremes of pH.

Amino Acid Analyses of the Isolated Protein Component. To

FIGURE 1: Absorption spectrum of the protein component isolated from the quasicrystals following treatment with 1 M NaOH. The solvent is 2-chloroethanol adjusted to pH 8.0 with ammonium hydroxide.

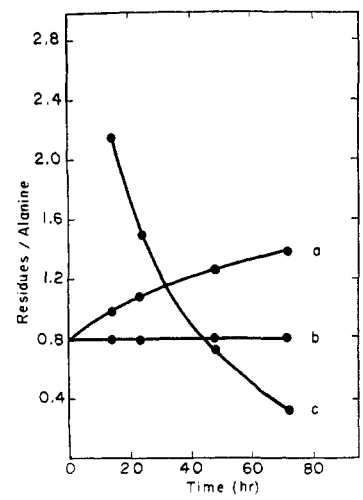

FIGURE 2: Kinetics of acid hydrolysis of (a) glycine residues of the quasicrystals, and (b) glycine residues of the isolated protein component. Curve c represents a product of acid hydrolysis of the quasicrystals which is absent in hydrolysates of the isolated protein component.

determine if the isolated protein component was the same as the protein contained in the quasicrystals, amino acid analyses of the two proteins were compared (Table I). An apparently high value for lysine was observed in the quasicrystalline material. The expected kinetics of hydrolysis of the amino acid residues were observed for both protein samples with the exception of an apparent increase in glycine content with increased time of acid hydrolysis of the quasicrystals (Figure 2a). The amount of glycine in the isolated protein component remained constant with increased time of acid hydrolysis (Figure 2b). The glycine content of both the quasicrystals and the isolated protein component extrapolated to a common point of origin. A product of hydrolysis of the quasicrystals, which eluted from the amino acid analyzer column before the lysine residues, exhibited a sharp decrease in content with increased time of acid hydrolysis (Figure 2c). This material was absent in the hydrolysates of the isolated protein component.

The similarity of the isolated protein component and the protein contained in the paracrystals was determined, based on their amino acid compositions, by using the deviation function (D) defined by Harris *et al.* (1969): $D = [\Sigma(X_{1,i} - X_{2,i})^2]^{1/2}$, where $X_{1,i}$ represents the mole fraction of amino acid i in protein 1, and $X_{2,i}$ represents the mole fraction of the same amino acid in protein 2 (by typographical error the square term was omitted in the original Harris publication). A ±3% difference in amino acid composition data of two identical proteins would result in a D value of 0.001. The deviation function would increase with increasing dissimilarity in the amino acid

TABLE I: Amino Acid Composition.[a]

Amino Acid	Isolated Protein	Quasicrystals
Aspartic acid	83.79	83.79
Threonine	51.95	51.95
Serine	54.46	55.30
Glutamic acid	90.49	91.33
Proline	43.40	43.57
Glycine	66.36	66.36
Alanine	83.79	83.79
Valine	70.13	69.55
Methionine	17.18	17.60
Isoleucine	54.38	52.79
Leucine	77.25	76.25
Tyrosine	26.98	26.81
Phenylalanine	33.52	31.67
Lysine	58.23	74.99
Histidine	19.44	20.11
Arginine	55.72	56.31
Tryptophan	8.34	8.34
Cysteic acid	18.69	18.69

[a] Expressed in μmoles/100 mg of protein. The values represent a typical analysis which was confirmed by seven separate experiments.

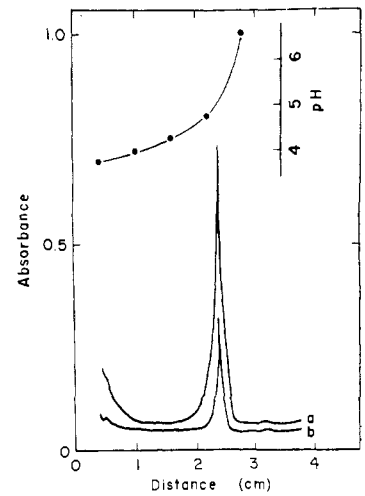

FIGURE 3: Polyacrylamide gel electrofocusing of (a) protein from *N. crassa* mitochondrial membranes and (b) protein component isolated from the quasicrystals. Samples were applied to 7% acrylamide gels in 5 M H_3PO_4. Electrofocusing continued for 1 hr at 150 V. The gels were fixed in 7% trichloroacetic acid and scanned at 280 nm.

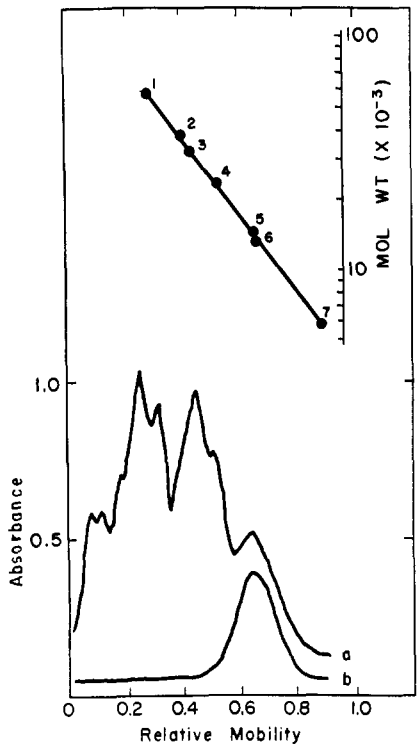

FIGURE 4: Analytical polyacrylamide gel electrophoresis at pH 7.0 in 0.1% sodium dodecyl sulfate. Samples applied to the 10% acrylamide gels were: (a) quasicrystalline material prior to separation of the protein component; (b) protein component isolated from the quasicrystals. Electrophoresis was conducted at a constant current of 5 mA/gel for 3–5 hr. Migration was from left to right. Gels were stained with coomassie blue and destained electrophoretically in an aqueous destaining solution of 7.5% acetic acid and 5% methanol before scanning at 550 nm. The mobility of standard proteins relative to the ion front is shown above the gel scans for molecular weight determination. Standard proteins used were: (1) catalase, (2) aldolase, (3) pepsin, (4) chymotrypsinogen A, (5) lysozyme, (6) ribonuclease A, and (7) insulin.

compositions of the two proteins (*i.e.*, the *D* value for unrelated proteins would be near 0.100). The *D* value for the isolated protein component and the protein in the quasicrystals, calculated from the amino acid composition data of Table I (including the lysine residues), was 0.011. The calculated *D* value was 0.006 if lysine was ignored.

The amino acid composition of the isolated protein component was consistent with its ultraviolet absorbance spectrum (Figure 1). This was demonstrated by a reconstruction of this spectrum using pure amino acids which were combined in amounts and ratios identical with those found in the protein component.

Electrofocusing. Analytical electrofocusing of the isolated protein component in the range of pH 4–6 resulted in a single sharp protein band in the gel at an approximate pH of 5.3 (Figure 3). Protein isolated from *N. crassa* mitochondrial membranes electrofocused in a corresponding manner under these conditions with a single band at a pH of approximately 5.3. To determine the isoelectric point more accurately, both proteins were electrofocused separately on a preparative column. A single band was found at pH 5.3 in both cases.

Electrophoresis. The isolated protein component was subjected to analytical electrophoresis using three different systems. When the isolated protein component was analyzed at pH 7.0 in 10% acrylamide gels containing 0.1% SDS (Weber and Osborn, 1969), a single protein band was observed

TABLE II: Deviation Functions of Peak Fractions From Preparative-Scale Electrophoresis of the Quasicrystals.[a]

	1	2	3	4	5	Isolated Protein
1	0	0.025	0.025	0.021	0.023	0.021
2	0.025	0	0.034	0.033	0.025	0.031
3	0.025	0.034	0	0.031	0.025	0.023
4	0.021	0.033	0.031	0	0.031	0.020
5	0.023	0.025	0.025	0.031	0	0.023
Isolated protein	0.021	0.031	0.023	0.020	0.023	0

[a] Calculated from amino acid compositions determined after 24-hr acid hydrolysis. For technical reasons, the only amino acids included in these calculations were: lysine, arginine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, isoleucine, and leucine.

(Figure 4). Electrophoreis of quasicrystalline material using the 0.1% SDS system resulted in multiple bands in the gels (Figure 4). These bands were separated by preparative electrophoresis in 0.1% SDS, recovered by precipitation, and hydrolyzed for amino acid analyses. The protein content of each band was compared to the protein content of all other separated bands, and to the protein component isolated from the quasicrystals, by calculating the deviation function (*D*) (Harris *et al.*, 1969) for all possible pairs (Table II). The low values calculated for the deviation functions indicated that all proteins were very similar and that the multiple bands observed were aggregated species of a single protein.

When the protein component was analyzed at pH 3.0 in 7.5% acrylamide gels (Takayama *et al.*, 1964), multiple bands of protein were observed (Figure 5a). Similar results were ob-

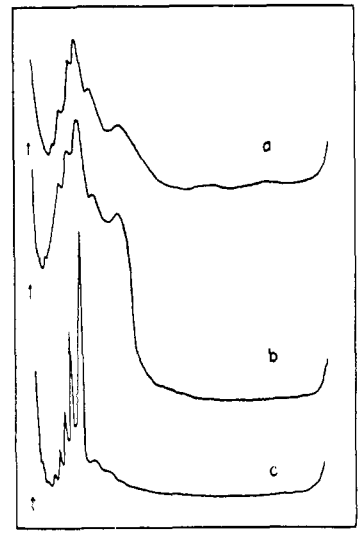

FIGURE 5: Analytical polyacrylamide gel electrophoresis at pH 3.0 in 5 M urea and 35% acetic acid. Samples applied to the 7.5% acrylamide gels were (a) protein component isolated from the quasicrystals; (b) protein isolated from *N. crassa* mitochondrial membranes; (c) recrystallized bovine serum albumin. The origins are marked with arrows and migration was from left to right. The gels were fixed with 5% trichloroacetic acid before scanning at 280 nm.

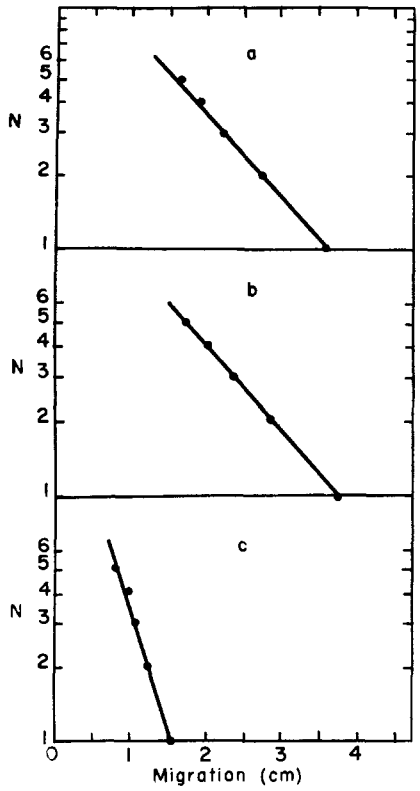

FIGURE 6: Smithies–Connell plots of the migration of protein bands during electrophoresis at pH 3.0. Plots a, b, and c refer to the corresponding samples in Figure 5. $N = 1$ is assigned to the fastest migrating band, and successive integral numbers (2, 3, etc.) are assigned to progressively slower moving components.

tained with *N. crassa* mitochondrial membrane protein (Figure 5b), and with recrystallized bovine serum albumin (Figure 5c). Linear Smithies–Connell plots (Smithies and Connell, 1959) of these protein bands (Figure 6) suggested that under acidic conditions these proteins electrophoresed as a series of aggregated species. The slopes of the lines for the isolated protein component and for the mitochondrial membrane protein were identical. Electrophoresis at pH 9.5 (Davis, 1964) caused the isolated protein to migrate at the ion front.

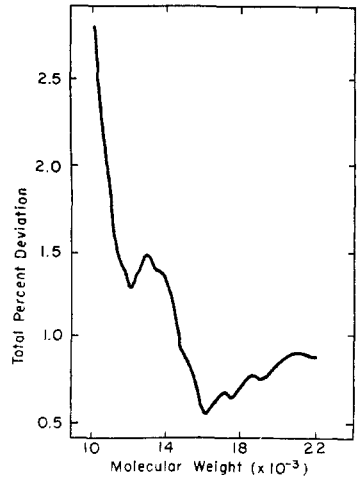

FIGURE 7: Minimum subunit molecular weight of the protein isolated from the quasicrystals. Total per cent deviation from integers of amino acid residues was calculated from the amino acid composition of the isolated protein (see Table I) as described in Materials and Methods.

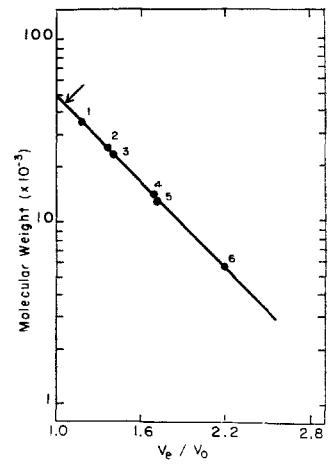

FIGURE 8: Molecular weight determination of the protein component of the quasicrystals by its elution behavior on Sephadex G-75 equilibrated with 8 M urea in 25% formic acid. The isolated protein component was carboxymethylated as described in Materials and Methods prior to gel filtration. The arrow marks the relative elution volume of the carboxymethylated protein in four separate experiments compared to the relative elution volume of standard proteins. Standard proteins used were: (1) pepsin, (2) chymotrypsinogen A, (3) trypsin, (4) lysozyme, (5) ribonuclease A, and (6) insulin.

Molecular Weight Determination. A molecular weight of 15,000 was determined for the isolated protein from its relative mobility on analytical electrophoretic gels containing 0.1% SDS (Weber and Osborn, 1969) compared to the relative mobilities of protein standards (Figure 4). A minimum subunit molecular weight of the protein of 15,000 was also indicated from the amino acid composition of the isolated protein (Table I) by the minimum total per cent deviation from integers of the amino acid residues (Figure 7). Gel filtration of the carboxymethylated protein on Sephadex G-75 equilibrated with 8 M urea in 25% formic acid gave a single protein species of molecular weight of 46,000 (Figure 8). While V_e of the isolated protein was close to V_0, it was significantly different than V_0 in four separate experiments. High-speed sedimentation equilibrium studies (Yphantis, 1964) of the protein in 6 M guanidine hydrochloride also indicated a protein species of mol wt 45,000. Evidence for homogeneity of this protein species was indicated by the linearity of a plot of ln y (y = fringe displacement in millimeters which is proportional to the concentration of the solute) *vs.* r^2 (r = distance in centimeters from the axis of rotation) (Figure 9).

Hydrophobicity. Two parameters have been defined which give an indication of the amount and availability of hydrophobic amino acid side chains in a protein. These parameters are the average hydrophobicity per residue ($H\Phi_{av}$) defined by Bigelow (1967), and the polarity ratio (p) defined by Fisher (1964). According to the interpretations of Bigelow and Fisher, proteins of low molecular weight and high average hydrophobicity (or low polarity ratio) would tend to aggregate forming higher molecular weight species. The values for these two parameters, calculated from the amino acid composition of the isolated protein, were 1116 cal/residue for the average hydrophobicity ($H\Phi_{av}$) and 1.00 for the polarity ratio (p). Assuming a monomer molecular weight of 15,000 for the isolated protein, the values calculated for these two parameters showed that this protein was similar to those proteins which

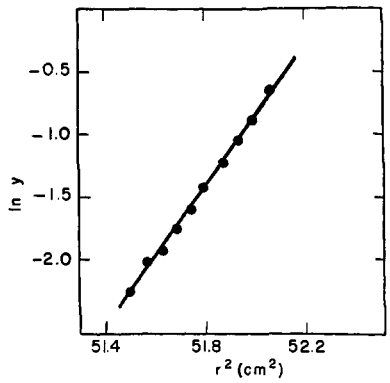

FIGURE 9: Plot of ln y (y = fringe displacement in millimeters *vs.* r^2 (r = distance in centimeters from the axis of rotation) for high-speed sedimentation equilibrium centrifugation of the isolated protein. Temperature 20°, speed 40,000 rpm. Protein dialyzed *vs.* 6 M guanidine hydrochloride containing 1 mM β-mercaptoethanol. Initial protein concentration was 0.3 mg/ml.

TABLE III: Comparison of Isolated Protein Component to Other Cellular Proteins.[a]

Source	Deviation Function
Isolated protein component[b]	0
N. crassa mitochondrial membranes[b]	0.023
Yeast mitochondrial membranes[c]	0.040
Beef mitochondrial membranes[d]	0.030
Cytochrome *c*[e]	0.119
Ribosomal protein[f]	0.075

[a] All comparisons were made to the amino acid composition of the isolated protein component. [b] Composition determined after 24-hr acid hydrolysis. [c] Composition taken from Yang and Criddle (1970). [d] Composition taken from Woodward and Munkres (1966). [e] Composition taken from Ambler (1963). [f] Composition taken from Alberghina and Suskind (1967).

Bigelow and Fisher indicate would form higher molecular weight species by aggregation.

Extinction Coefficient. The extinction coefficient of the isolated protein in 2-chloroethanol at pH 8.0, calculated from the absorbance at 277 nm through a 1.0-cm light path, was $E_{277}^{1\%}$ 8.3.

Comparison to Other Proteins. The association of protein and lipid in the quasicrystals suggested that this material might be related to membrane components. To test this hypothesis the isolated protein component of the quasicrystals was compared to protein fractions isolated from membranes and to other cellular proteins by calculating the deviation function (D) in each case (Table III). The D values illustrated in Table III indicate that the protein component was more similar to protein fractions isolated from membranes than to other cellular proteins such as ribosomal proteins and cytochrome *c*.

Yield. The yield of the protein component was determined as a function of the growth cycle of *N. crassa*. The growth cycle was followed by determining the amount of total soluble protein per liter of culture suspension at various times after innoculation (Figure 10A). Quasicrystalline material containing the protein component was obtained throughout the growth cycle. Maximum yield of this protein component was obtained during logarithmic growth (Figure 10B). At the time of the first sample (0.15 of the growth cycle elapsed) the protein fraction of the quasicrystals represented 55.6% of the total protein contained in the cytosol (Figure 10C). The relative amount of the protein in the cytosol which was recovered as a component of the quasicrystalline material decreased through the rest of the growth cycle to a value of 2.4% at stationary phase. We emphasize that early log-phase material was used in all work reported in this paper.

Discussion

A quasicrystalline material of protein and nonprotein components has been recovered from the cytoplasm of *N. crassa* during early log-phase growth. The protein component can be separated from the nonprotein components by gel filtration following alkaline treatment of the quasicrystals.

The amino acid compositions of the isolated protein component and of the protein contained in the quasicrystals are very similar, with the exception of an apparently high lysine content observed for the quasicrystals. This could result from selective release of lysine from the protein during the isolation procedure. This could also be due to material from the nonprotein components of the quasicrystals which elutes with lysine. The apparent increase in glycine content of the quasicrystals with increased time of acid hydrolysis may be the result of the decomposition of a nonprotein component which elutes with glycine. A product of hydrolysis of the quasicrystals, which is absent in the hydrolysates of the isolated protein component, shows a decrease in content with increased hydrolysis time. This could cause the apparent increase in glycine content of the quasicrystals during hydrolysis. The similarity in amino acid composition and the low deviation function calculated (whether or not the lysine residues are included in this calculation) indicate that the isolated protein component is identical with the protein contained in the quasicrystals. During early log-phase growth, the protein com-

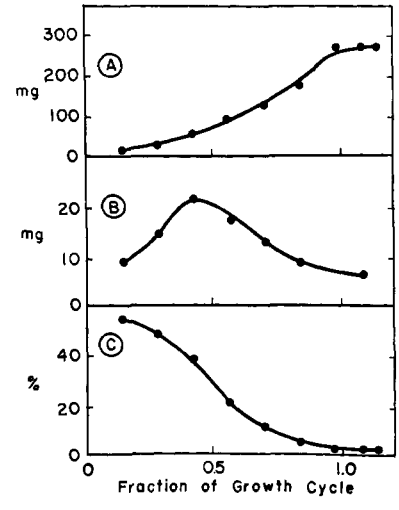

FIGURE 10: Yield of the protein component of the quasicrystals as a function of the growth cycle of *N. crassa*. (A) Milligrams of total soluble protein per liter of culture suspension. (B) Milligrams of quasicrystalline protein per liter of culture suspension. (C) Per cent of total soluble protein obtained as the protein component of the quasicrystals.

ponent appears to be a single protein since it electrofocuses as a single band on both analytical and preparative scales, and migrates as a single band during analytical electrophoresis at pH 7.0 in 0.1% SDS. Analytical electrophoresis of the quasicrystals in the same system without previous alkaline treatment and separation of the protein from the nonprotein components results in a multiplicity of bands. However, when these bands are separated by preparative electrophoresis in 0.1% SDS and analyzed for amino acids, they are found to be very similar in amino acid composition, providing strong evidence that only a single protein component is present.

The relative mobility of the single band on electrophoresis of the isolated protein indicates a possible monomeric molecular weight of 15,000. This minimum subunit molecular weight is suggested also by the minimum total per cent deviation of the amino acid residues from integers. Gel filtration using 8 M urea in 25% formic acid as a solvent does not completely dissociate the protein into subunits and a single protein species of molecular weight of 46,000 results. High-speed sedimentation equilibrium centrifugation in 6 M guanidine hydrochloride also promotes aggregation of the protein and results in a single protein species of molecular weight of 45,000.

The amino acid composition of the protein indicates that the protein is very hydrophobic and would tend to aggregate forming higher molecular weight species. This hydrophobic nature of the protein makes it difficult to dissociate it into its smallest possible subunits. The discrepancy in molecular weight determinations of this protein can be explained by the very hydrophobic nature of the protein causing the formation of stable aggregated species under conditions which normally would result in complete dissociation of a hydrophilic protein. However, all evidence reported here for the molecular weight of the protein is consistent with a single aggregating protein.

The protein may be related to protein fractions which have been isolated from cell and organelle membranes. The protein appears to be more similar in amino acid composition to these membrane proteins than to other cellular proteins such as cytochrome *c* and ribosomal protein. Its solubility properties also resemble those of membrane proteins (Woodward and Munkres, 1966; Criddle *et al.*, 1962; Schneiderman and Junga, 1968). The electrofocusing pattern of this protein is identical with that of a protein fraction isolated from *N. crassa* mitochondrial membranes, and these two proteins have a common isoelectric point.

The present controversy concerning the heterogeneity and molecular weight of protein fractions isolated from membranes could be due to various isolation procedures or to varying degrees of removal of associated soluble proteins, depending on the amount of washing and the solvents used. Kiehn and Holland (1970a,b) do not attempt to isolate any single protein fraction from mammalian cell membranes and find a complex array of proteins following electrophoresis of these whole membranes, as one would expect. Senior and MacLennan (1970) report that the original procedures for isolation of structural proteins from bovine mitochondrial membranes causes protein denaturation. The mild method used to obtain the quasicrystals from the cytosol of *Neurospora*, as reported in the present paper, reduces the possibility of denaturing proteins.

Schneiderman and Junga (1968) have published an ultraviolet spectral analysis of a protein fraction isolated from human red blood cells which was inconsistent with their reported amino acid composition of this protein fraction, and which showed evidence of contamination by nonprotein components. The ultraviolet spectral analysis of the protein isolated from the quasicrystals reported by us is consistent with its amino acid composition. In addition, the isolated protein component is analyzed for carbohydrate, lipid, and phosphorus and is shown not to be contaminated by these materials.

An additional cause of the controversy could be data obtained by unsuitable methods of analysis of these protein fractions isolated from the membranes. We have shown that analytical electrophoresis under acidic conditions (Takayama *et al.*, 1964) of the protein isolated from the quasicrystals, and of protein isolated from *N. crassa* mitochondrial membranes results in multiple bands. Similar results are obtained with recrystallized bovine serum albumin. Smithies–Connell plots (Smithies and Connell, 1959) of the multiple bands observed for each protein suggest that in each case the bands represent aggregated species of a single protein. Identical slopes of the Smithies–Connell plots for the mitochondrial protein and the protein from the quasicrystals indicate that these two proteins aggregate in the same manner under these conditions.

The high degree of hydrophobicity, and possibly other properties unique to this cytoplasmic protein and to membrane proteins, could explain this diversity in purity and in other characteristics reported for these proteins from various laboratories. The molecular weight studies reported in this paper indicate that the resultant molecular weight of this protein, and perhaps of membrane proteins, depends on the isolation procedures and methods of analysis. Unique methods of isolation and analysis may be required to truly characterize these types of proteins.

The data presented here do not provide definitive evidence for the role of this protein in the cell. However, the presence of this protein in large amounts only during early logarithmic growth, the physical characteristics of the protein, and the composition of the quasicrystalline material suggest several possible roles. The quasicrystalline material could preexist in a soluble form as a lipid reservoir and in this sense closely resemble serum lipoproteins. The protein component could represent a heterogeneous collection of denatured enzymes. The mild method of preparation of this material as well as the demonstrated homogeneity of the protein by several analytical methods leads us to discount this possibility. Another possibility is that the dynamic state of biological membranes may allow a selective solubilization of a membrane component only during the early phases of the growth cycle.

An alternative hypothesis is that the protein represents a transient pool of soluble intermediates in membrane assembly and is subsequently incorporated into membranes. If the protein component of the quasicrystals is involved in membrane assembly, then it should be in relatively high concentration in the cytoplasm during early log-phase growth when membranes are being rapidly assembled. During stationary phase growth when membrane assembly is at a minimum, the concentration of this protein in the cytoplasm should be relatively low. Further studies using radioactive tracing methods and immunochemical techniques are presently under way to test this hypothesis. Further characterization of the nonprotein components of the quasicrystals is also in progress.

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Added in Proof

Dr. David Kaplan at the Department of Biological Sciences, Stanford University, has found that all peptides resulting from tryptic digestion of the protein from the quasicrystalline material correspond to peptides arising from tryptic digestion of mitochondrial membrane protein. He has also found that the protein from the quasicrystalline material has a single amino-terminal residue, aspartic acid.

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