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ELECTROPHORETIC ANALYSIS OF THE PLASMA MEMBRANE PROTEINS OF A MUTANT OF *NEUROSPORA CRASSA* WHICH LACKS A CELL WALL

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Summary

The plasma membrane proteins of a mutant of *Neurospora crassa* (FGSC No. 326) which lacks a cell wall were analyzed by two-dimensional polyacrylamide gel electrophoresis. Approximately 180 different proteins were detected in purified plasma membrane preparations. Nonpermeant labeling experiments indicated that approximately 40% of these proteins were exposed on the extracytoplasmic surface of the plasma membranes of these cells. The studies demonstrate the complexity of the protein composition of *N. crassa* 326 plasma membranes to be greater than has been suggested by previous investigations.

Introduction

The mutants of *Neurospora crassa* which lack a cell wall (slime) are particularly useful for studies of eukaryotic plasma membrane structure and function. Their nutritional requirements are well defined, and relatively large numbers of cells (e.g., 10^{10}) are easily harvested from liquid cultures. Biological activities of plasma membrane proteins such as adenyl cyclase [1—3], adenosine triphosphatase [4—6], and glucose transport systems [7] have been characterized in these mutants. Scarborough [8] has described a rapid method for the preparation of highly purified plasma membranes from *N. crassa* FGSC No. 326. The method

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Abbreviation: SDS, sodium dodecyl sulfate.

employs concanavalin A stabilization of the plasma membranes during purification. The purity of the preparation was evaluated by enzymatic analysis, surface labeling, chemical composition, and electron microscopy.

Previous reports describing one-dimensional SDS-polyacrylamide gel electrophoresis of proteins solubilized from purified plasma membranes of *N. crassa* slime mutants [6,9] have indicated approximately 15—30 protein species. This number is similar to that reported for the proteins of human erythrocyte plasma membranes [10] using this technique. The introduction by O'Farrell [11] of a two-dimensional electrophoretic technique has, however, extended the analytical capacity of acrylamide gel methods to resolve complex mixtures of proteins. Metzenberg and Nelson [12] have reported the separation of 480 nuclear proteins from *N. crassa* 326 by this method. These authors point out that the genome of *N. crassa* is approximately 7 times the size of that of *Escherichia coli*, and estimate that as many as 10^4 different proteins may be present in *N. crassa* at any one time. Furthermore, Krumlauf and Marzluf [13] have reported the presence of 2000 mRNAs of three abundance classes in the cytoplasm of rapidly growing *Neurospora* vegetative cells. These observations suggest that *Neurospora* has the potential for more diverse gene expression at the level of the plasma membrane than indicated by one-dimensional electrophoretic analysis of plasma membrane proteins.

Because of the utility of these mutants in studying plasma membrane structure and function, it is of interest to investigate the component proteins of their plasma membranes by a high resolution method such as two-dimensional electrophoresis. This study addresses the complexity and orientation of plasma membrane proteins of *N. crassa* 326.

Materials and Methods

Growth of an N. crassa mutant which lacks a cell wall

Stock cultures of *N. crassa* FGSC No. 326 (*fz; sg; arg*-1 (B369); *cr*-1 (B123); *aur* 34508; *os*-1 (B135)A), an arginine-requiring, adenyl-cyclase-deficient strain, were maintained on 1.5% (w/v) agar slants of Vogel's N medium [14] supplemented with 2 mM arginine, 0.25 M sorbitol, and 1.5% (w/v) sucrose. Cultures were incubated at 30°C and transferred at 7 day intervals. Liquid cultures were obtained by inoculating 50 ml of Vogel's N medium containing 0.25 M sorbitol, 1.5% (w/v) sucrose, and 2 mM arginine with cells obtained by resuspension of cells from one 7-day slant culture. The 50-ml cultures were incubated in 250-ml Erlenmeyer flasks at 30°C in a New Brunswick rotary shaker at 150 rev./min for 16 h. Cell counts were performed with a hemocytometer. 500-ml cultures in a 2 l Erlenmeyer flask were inoculated with $5 \cdot 10^7$ cells from the 50 ml starter cultures. After 16 h growth the 500-ml cultures yielded approximately 10^{10} cells.

Steady-state labeling of cultures with L-[4,5-^{3}H(n)]leucine (60 Ci/mmol, New England Nuclear) was performed by addition of 50 μCi of the tritiated amino acid to 50 ml of freshly inoculated culture. The label was present for the 16 h growth period.

Preparation of plasma membranes

Plasma membranes of *N. crassa* FGSC No. 326 were prepared by the method of Scarborough [8]. Cells from 16-h cultures were harvested by centrifugation at 160 × *g* for 10 min, and washed twice in Scarborough's buffer A (0.05 M Tris-HCl, pH 7.5, containing 0.01 M $MgSO_4$ and 0.25 M mannitol). In experiments involving surface labeling the washed cells were treated as described below prior to membrane purification. All subsequent steps were as described by Scarborough [8], with 0.25 mM phenylmethylsulfonyl fluoride added to all solutions. Concanavalin A was eluted from purified membranes by suspension in 1.0 M α-methyl-D-mannopyranoside for 10 min at 4°C, followed by centrifugation at 250 × *g*. This step was repeated twice. The pellet was then washed five times by repeated centrifugation and resuspension in 0.01 M Tris-HCl, pH 7.5, containing 0.25 M phenylmethylsulfonyl fluoride. The washed membranes were either stored frozen in this buffer at −70°C or lyophylized and stored at −70°C.

Surface labeling

Washed cells of *N. crassa* FGSC No. 326 were suspended to $5 \cdot 10^6$/ml in Scarborough's buffer A [8]. The cell suspension was transferred to serum bottles fitted with rubber septa, and between 20 and 200 μCi (in different experiments) of $Na^{125}I$ (New England Nuclear, Boston, MA) was added per ml of cell suspension. Lactoperoxidase (Calbiochem-Behring, La Jolla, CA) was added to 0.3 I.U./ml, and the reaction initiated by addition of 5 μl 1.56 mM H_2O_2 per ml of suspension (to give an H_2O_2 concentration of 7.8 μM in the reaction vessel). Twenty sequential additions of H_2O_2 were made with a 15 s interval between additions. This procedure is essentially that employed by Poduslo et al. [15]. 2 min after the last addition of H_2O_2, 2 vol. 5 mM NaI in buffer A were added. The cells were collected by centrifugation at 160 × *g* for 10 min and washed five times in buffer A. Separate vessels were used for controls, omitting either lactoperoxidase or H_2O_2. The washed surface-labeled cells were then treated as described above for membrane preparation or, in some instances, were sonicated, and total soluble and total particulate fractions prepared.

Preparation of cell fractions

In experiments concerned with the distribution of proteins between total particulate and soluble fractions, the washed cells were suspended in 0.05 M Tris-HCl (pH 7.5) containing 0.25 mM phenylmethylsulfonyl fluoride, and ruptured by sonication with a Branson 184 sonifier (Heat Systems Ultrasonics, Plainview, NY) at 40 W for 30 s at 0°C. Microscopic examination of each sample indicated 100% cell disruption. The sonicate was then centrifuged at 100 000 × *g* for 1 h in a Beckman L-350 ultracentrifuge. Aliquots of the supernatants and resuspended pellets were taken for subsequent analysis.

Determination of radioactivity

Tritium and iodide radioactivity was determined using a Beckman LS-3133T liquid scintillation counter. Aquasol II (New England Nuclear Corp., Boston, MA) was used as the scintillation cocktail.

Protein determination

The method of Lowry et al. [16] was used for protein determination. Bovine serum albumin (Fraction V, Sigma) was used to construct the standard curve.

Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis was performed as decribed by O'Farrell and co-workers [11,17]. Samples to be analyzed were lyophilized prior to solubilization in a solution consisting of 0.5% (v/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.2% Ampholines (pH range 3.5—10, LKB Instruments Inc.). After brief (30 s) sonication in this solution, an equal volume of a solution consisting of 9.5 M urea (Ultrapure, Schwarz/Mann), 2% (w/v) Nonidet P-40 (Particle Data Labs., Elmhurst, IL), 2% Ampholines (LKB Instruments, pH range 3.5—10), and 5% β-mercaptoethanol was added.

The first dimension non-equilibrium pH gradient gel electrophoresis was performed as described by O'Farrell et al. [17]. Gels were cast to a height of 100 mm in 130 × 3.5 mm glass tubes, and were not pre-run prior to sample application. Sample volume was usually less than 100 μl and contained at least 100 μg protein. The gels were run at a constant 400 V for 5 h at 4°C (2000 V/h). The gels were removed from the tubes and stored frozen at −70°C prior to equilibration and second dimension SDS-polyacrylamide gel electrophoresis. One gel to which no protein was applied was run with each set of samples to establish the pH gradient for each non-equilibrium pH gradient gel electrophoresis experiment as described by O'Farrell [11].

First dimension gels were equilibrated for 1 h in SDS sample buffer (10% glycerol/5% β-mercaptoethanol/2.3% SDS/0.0625 M Tris-HCl (pH 6.8)) prior to electrophoresis in the second dimension. SDS-polyacrylamide gel electrophoresis in the second dimension was performed as described by O'Farrell [11] using a 10% acrylamide separating gel 0.75 mm thick and 100 mm in height, with a 25 mm 4% acrylamide stacking gel.

The 10% acrylamide second dimension SDS-polyacrylamide gels were standardized using the following proteins: phosphorylase a (M_r 94 000), catalase (M_r 60 000), ovalbumin (M_r 43 000), aldolase (M_r 40 000), chymotrypsinogen (M_r 25 700), trypsin (M_r 23 300), and lysozyme (M_r 14 300). The molecular weight scale is shown on the left side of each second dimension gel. The pH gradient of the first dimension is indicated at the top of the second dimension gels.

Gel drying, fluorography, and autoradiography

The second dimension SDS-polyacrylamide gels were dried on a Pharmacia gel drier (Pharmacia, Piscataway, NJ) either after destaining or after processing for fluorography. Gels were processed for fluorography by the method of Bonner and Laskey [18]. The dried gels were loaded into X-ray film holders (8 × 10 inch, Eastman Kodak Co., Rochester, NY) with Kodak X-Omat R film. The films were exposed at −70°C for periods up to 30 days. Gels for autoradiography (^{125}I samples) were dried after destaining, and loaded into X-ray film holders with GAFMED GP/MS medical X-ray film (GAF Corp., Binghampton, NY) and exposed at −70° C for periods up to 30 days. Film holders used for autoradiography of ^{125}I samples were fitted with DuPont Cronex Lightning-Plus intensifying screens (E.I. DuPont, Wilmington, DE).

Electron microscopy

Electron microscopy was performed on the purified plasma membrane preparation. The membranes were washed, but concanavalin A was not eluted prior to fixation in 3% glutaraldehyde (buffered at pH 7.4 with 0.1 M sodium phosphate) at 4°C for 12 h. The samples were post-fixed in buffered 1% (w/v) OsO_4, and dehydrated in graded solutions. Samples were embedded in Epon and thin sections cut. The sections were viewed on a Phillips 300 EM electron microscope.

Results

An electron micrograph of plasma membranes prepared from *N. crassa* 326 is shown in Fig. 1. The preparation is free of mitochondria and nuclei. Based upon the evidence presented by Scarborough [8], the beaded appearance of one side of the membrane is due to the presence of concanavalin A aggregates rather than to ribosomes bound to the plasma membrane. The plasma membranes are observed to be primarily in the form of sheets and large vesicles.

The addition of L-[^{3}H]leucine to freshly inoculated *N. crassa* 326 cultures and the growth of the cells for 16 h in the presence of the labeled amino acid results in steady-state labeling of the cells [8]. Preparation of plasma membranes from such steady-state labeled cells results in approximately 8% of the total cellular protein being recovered as plasma membrane protein (Table I). Sonication of washed steady-state labeled cells followed by centrifugation at

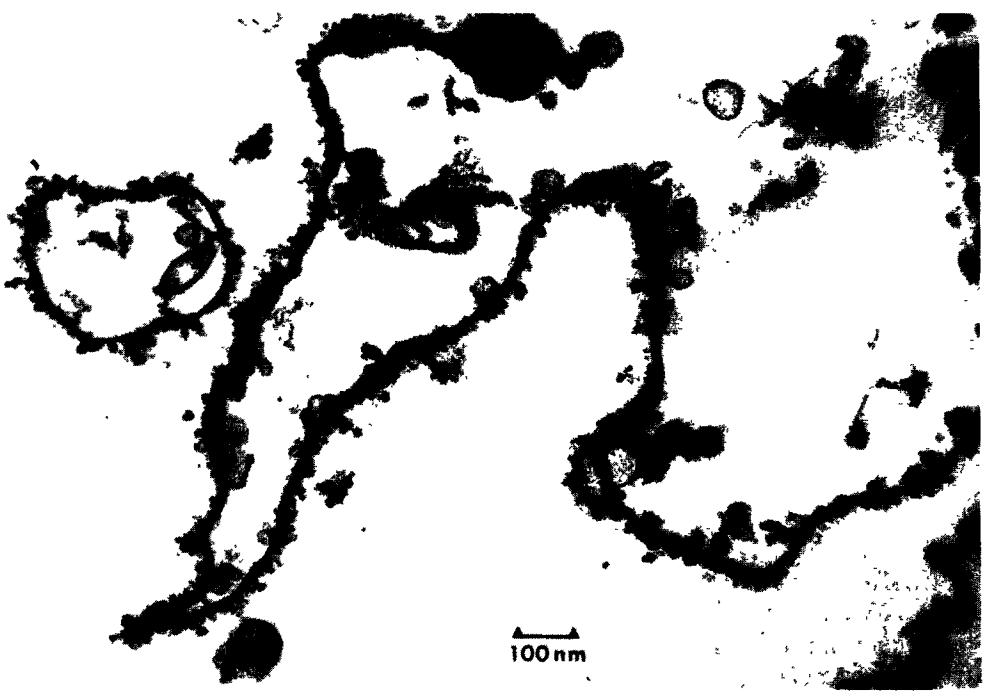

Fig. 1. Plasma membranes from *N crassa* 326. Membranes were prepared as described in Materials and Methods.

TABLE I

DISTRIBUTION OF STEADY-STATE AND SURFACE LABEL RADIOACTIVITY IN CELL FRACTIONS

Fraction	Total L-[^{3}H]-leucine cpm in each fraction	Percentage of total	Total ^{125}I cpm after non-permeant labeling procedure	Percentage of total
A. Lysate	$1.23 \cdot 10^7$	100	$1.05 \cdot 10^7$	100
Plasma membranes	$9.21 \cdot 10^5$	8	$5.33 \cdot 10^6$	51
B. Sonicate	$2.53 \cdot 10^6$	100	$1.22 \cdot 10^6$	100
100 000 × *g* supernatant	$1.01 \cdot 10^6$	40	$1.65 \cdot 10^5$	14
100 000 × *g* pellet	$1.52 \cdot 10^6$	60	$1.01 \cdot 10^6$	84

100 000 × *g* for 1 h shows 40% of the total cellular protein to be soluble (Table I).

Also shown in Table I is the distribution of ^{125}I after surface iodination of *N. crassa* 326 cells. Cells used for the iodination procedure were grown at the same time as those used to assess the distribution of the L-[^{3}H]leucine steady-state label. The cells used for iodination, however, were grown in the absence of a radioactive label for 16 h, and were then harvested and iodinated as described under Materials and Methods. After iodination the cells were washed and an aliquot of the suspension removed for sonication and centrifugation at 100 000 × *g* for 1 h. The remainder of the cells were used for preparation of plasma membranes. As can be seen from Table I, 51% of the total incorporated ^{125}I was recovered in the purified plasma membrane fraction. This probably reflects some loss of plasma membrane, since the pellet of the first density centrifugation step during membrane purification contained 90% of the total incorporated iodide (not shown). In the aliquot of the iodinated cells which was sonicated and centrifuged, 84% of the ^{125}I radioactivity was found to be associated with the total particulate material. The 14% of the total ^{125}I radioactivity present in the 100 000 × *g* supernatant fraction was not precipitable by 10% trichloroacetic acid, suggesting no covalent association with protein. Juliano and Behar-Bannelier [19] reported a similar observation with Chinese hamster ovary (CHO) cells iodinated by the lactoperoxidase method. Thus, essentially all of the incorporated ^{125}I appeared in the 100 000 × *g* pellet fraction. This fraction contains all the cellular particulate material, including, of course, the plasma membranes.

Comparison between the recovery of cell protein in steady-state L-[^{3}H]leucine-labeled cells with recovery of iodinated protein in surface-labeled cells, shown in Table I, indicates an enrichment of approximately 7-fold with respect to surface label in the purified plasma membranes.

Two-dimensional electrophoresis

Two-dimensional electrophoretic analysis was performed on plasma membrane as well as 100 000 × *g* supernatant and pellet fractions. It was reasoned that membrane-associated proteins should constitute a subset of the total particulate fraction, whereas the presence of a substantial number of soluble proteins in plasma membrane preparations would be indicative of cytoplasmic contamination.

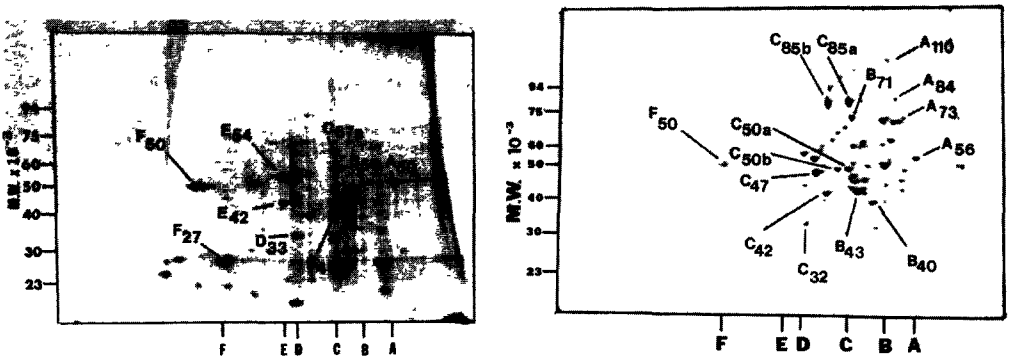

Fig. 2. Fluorogram prepared from non-equilibrium pH gradient SDS-polyacrylamide gel electrophoretogram of purified plasma membrane proteins of *N. crassa* 326. The cells were steady-state-labeled with L-[^{3}H]leucine prior to plasma membrane purification. 100 000 cpm were applied to the first dimension gel. The fluorogram was exposed for 21 days at −70° C.

Fig. 3. Fluorogram prepared from non-equilibrium pH gradient SDS-polyacrylaamide gel electroporetogram of 100 000 × *g* supernatant of sonicated *N crassa* 326 whole cells. Cells were steady-state-labeled with L-[^{3}H]leucine prior to sonication and centrifugation. 100 000 cpm were applied to the first dimension gel. The fluorogram was exposed for 20 days at −70° C.

To facilitate comparison of the two-dimensional electrophoretograms, zones were established by drawing vertical lines through prominent proteins in electrophoretograms of purified plasma membrane samples. The proteins were detected by both Coomassie-blue-staining and by fluorography of ^{3}H-labeled samples. The specific proteins selected to establish the zones were chosen to facilitate orientation with respect to pH regions and molecular weight range. In non-membrane samples the lines were drawn at corresponding pH values to establish similar zones. Regions A—F were established for the non-equilibrium pH gradient SDS-polyacrylamide gel electrophoretograms. A protein was considered to be in the A zone if it appeared on the A line or in the region to the left of the A line limited by the B line. The B zone begins with the B line and extends left to the C line, etc. Using this nomenclature system a protein was designated by the letter of the zone in which it appeared with a subscript denoting its approximate molecular weight. If more than one protein appeared in a zone with the same molecular weight (different isoelectric point), lower case letters were appended to the subscript (e.g. A_{47a}, A_{47b}). The convention proposed by O'Farrell [11] was observed, i.e., all two-dimensional electrophoretograms are shown with the basic end of the (first dimension) pH gradient on the left.

A two-dimensional non-equilibrium pH gradient SDS-polyacrylamide gel electrophoretogram of *N. crassa* 326 purified plasma membrane proteins is shown in Fig. 2. Careful inspection determined 180 separate proteins, of which approximately 70% appear in zones A—D (pH 4.0—7.0). The subunit molecular weights range from 19 000 to 90 000, with 73% between 25 000 and 60 000. Prominent proteins present in the plasma membrane sample are A_{50}, B_{50}, C_{40a}, C_{67a}, D_{33}. E_{42}, F_{27}, and F_{50}. All the plasma membrane proteins were present in the 100 000 × *g* pellet samples from sonicated *N. crassa* 326 whole cells (not

shown). The 100 000 × *g* pellet material contained a larger number of proteins than the plasma membrane sample, and there were many instances of differences in relative intensities of corresponding protein spots between the two samples.

The electrophoretogram of the 100 000 × *g* supernatant fraction from sonicated whole cells of *N. crassa* 326 is shown in Fig. 3. Approximately 300 proteins are present, with the majority distributed in the A—C region (pH 4.0—6.8) of the gel. Because of the complexity in this region, careful comparisons between plasma membrane and total soluble protein (100 000 × *g* supernatant) samples were made by overlaying the respective X-ray films. Comparisons were also made between Coomassie-blue-stained electrophoretograms of plasma membrane proteins and total soluble protein to verify the respective protein identities. Proteins A_{56}, A_{73}, A_{84}, A_{110}, B_{40}, B_{43}, B_{71}, C_{32}, C_{42}, C_{47}, C_{50a}, C_{50b}, C_{85a}, C_{85b} are prominent in the 100 000 × *g* supernatant sample and are not present in the plasma membrane sample (Fig. 2). Protein F_{50} was observed in electrophoretograms of purified plasma membranes (Fig. 2), 100 000 × *g* pellets from sonicated whole cells (not shown), and in the 100 000 × *g* supernatants (Fig. 3) from the whole cell sonicates. With this exception, however, there appears to be no significant homology between the 100 000 × *g* supernatant proteins and the purified plasma membrane proteins.

Intact cells of *N. crassa* 326 were iodinated as described in Materials and Methods prior to purification of the plasma membranes. An autoradiogram prepared from a two-dimensional (non-equilibrium pH gradient SDS-polyacrylamide gel electrophoresis) separation of *N. crassa* 326 iodinated plasma membrane proteins is shown in Fig. 4. The slightly 'fuzzy' appearance of the autoradiogram was characteristic of iodinated samples and is probably due to the high energy emission and the use of an intensifying screen. In the examination of autoradiograms of this type care was taken to avoid counting of streaked proteins as more than one protein. Protein identification in ^{125}I autoradiograms was made by overlaying duplicate Coomassie-blue-stained gels with the devel-

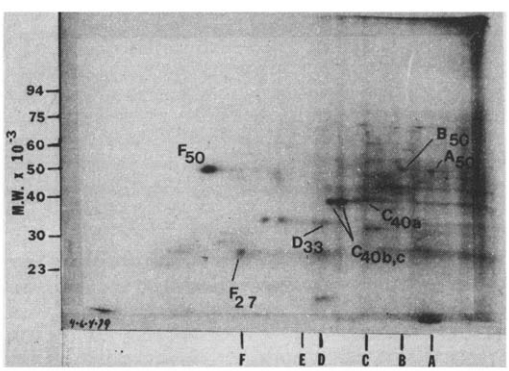

Fig. 4. Autoradiogram prepared from a non-equilibrium pH gradient SDS-polyacrylamide gel electrophoretogram of purified plasma membrane proteins of *N. crassa* 326. Intact cells were iodinated by the lactoperoxidase-catalyzed procedure described under Materials and Methods. After iodination, plasma membranes were prepared as described. 90 000 cpm of ^{125}I was applied to the first dimension gel. The autoradiogram was exposed for 30 days at —70°c with an intensifying screen.

TABLE II

Protein	^{125}I	Protein	^{125}I	Protein	^{125}I	Protein	^{125}I
A_{22}	—	B_{42}	—	C_{72}	—	E_{35b}	+
A_{24}	—	B_{46}	+	C_{84}	+	E_{36}	—
A_{26}	+	B_{49}	+	C_{90}	—	E_{37}	—
A_{27}	+	$B_{49.5}$	—	D_{20}	+	$E_{40.5}$	—
A_{29}	+	B_{50}	+	D_{24}	—	E_{42}	—
A_{33}	—	B_{53}	—	D_{25}	+	E_{50}	—
A_{34}	+	B_{57}	+	D_{26}	—	E_{51}	—
A_{39b}	+	B_{65}	+	D_{27}	—	E_{53}	—
A_{40}	+	B_{70}	+	D_{30}	—	E_{54}	—
A_{41}	—	C_{22}	—	D_{31}	—	E_{70}	—
A_{44}	+	C_{24}	+	D_{33}	+	E_{72}	—
A_{47b}	+	C_{25}	—	D_{37}	—	E_{76}	—
A_{50}	+	C_{27}	+	D_{39}	—	$F_{22.5}$	—
A_{52}	—	C_{28}	—	D_{42}	—	F_{23}	—
A_{69}	+	C_{32}	—	$D_{47.5}$	—	F_{24}	—
A_{70}	+	C_{33}	+	D_{52}	—	F_{27}	+
B_{24}	+	C_{35}	+	D_{54}	—	$F_{27.5}$	+
$B_{24.5}$	—	$C_{37.5}$	—	D_{56}	—	F_{28a}	+
B_{25}	+	C_{40a}	+	D_{60}	—	F_{28b}	—
B_{26}	—	C_{40bc}	+	D_{65}	—	F_{35}	—
B_{27}	+	C_{41}	+	D_{68}	+	F_{38}	—
B_{29}	+	C_{46}	+	D_{69}	—	F_{47}	—
B_{30}	+	C_{49}	+	D_{70}	—	F_{49}	+
B_{31}	—	C_{58}	—	E_{21}	—	F_{50}	+
B_{34}	—	C_{60}	+	E_{27}	—	F_{55}	+
B_{35}	+	C_{67a}	+	E_{29}	—	F_{56}	—
B_{37}	—	C_{67b}	—	E_{30}	—	F_{58}	—
B_{40}	+	C_{70}	+	E_{35a}	+	F_{61}	—
B_{41}	+						

oped X-ray films. A minimum of 75 protein spots were located in the autoradiogram of Fig. 4. Proteins A_{50}, B_{35}, B_{50}, C_{40a}, C_{40b}, D_{33}, F_{27}, and F_{50} are indicated on Fig. 4 for comparison with the steady state ^{3}H-labeled plasma membrane preparation shown in Fig. 2. Conspicuously absent from the iodinated sample were proteins E_{42} and E_{54}. A detailed comparison between surface-iodinated proteins (Fig. 4) and total plasma membrane proteins (Fig. 2) was performed and is shown in Table II. Only proteins which could be unambiguously determined as clearly present or absent in the iodinated preparation were considered in the comparison of Table II. Listed are 112 of the proteins present in the *N. crassa* 326 plasma membranes, those proteins labeled with ^{125}I (from Fig. 4) are indicated by a plus (+). 45% of these proteins are observed to contain ^{125}I from the surface-labeling procedure. Of particular note is the definite iodination of protein F_{50} in the *N. crassa* 326 plasma membranes. This protein was also intensely labeled in autoradiograms of 100 000 × *g* pellet material from sonicated whole *N. crassa* 326 cells which had been surface-labeled prior to sonication. The Coomassie-blue-stained electrophoretograms of 100 000 × *g* supernatants from the surface-labeled sonicated cells contained the F_{50} protein; however, prolonged (60 days) exposure of X-ray film on the electrophoretogram failed to show ^{125}I-labeling of any cytosolic proteins. Thus, the F_{50} protein was present in purified plasma membranes, 100 000 × *g* pellets, and

100 000 × *g* supernatants by Coomassie blue staining and ^{3}H fluorography, but was iodinated only in the plasma membrane and 100 000 × *g* pellet samples.

Discussion

The method of plasma membrane purification employed in this study results in preparations of substantial purity [8]. The use of concanavalin-A-agglutinated cells prevents plasma membrane vesiculation and entrapment of intracellular components, as well as membrane fragmentation upon lysis of the cells. The low centrifugal forces employed to sediment the concanavalin-A-stabilized membranes appears to preclude sedimentation of intracellular membranes (e.g. endoplasmic reticulum) and organelles during the plasma membrane purification. The possible presence of non-membrane carbohydrate, which Scarborough [8] reported to contaminate this type of preparation, was not investigated. However, for the purposes of these experiments such minor contamination by glycogen is irrelevant to the examination of the plasma-membrane-derived proteins.

The recovery of cellular protein in the plasma membranes prepared from *N. crassa* 326 ranged between 5 and 8% in five different experiments. These values are comparable to those reported by Scarborough [8] for the plasma membrane ghost fraction prepared from cells of *N. crassa* 326. Similarly, Benson [9] reported a 7% recovery of cellular protein in plasma membranes of *N. crassa* 1118 using a different purification method.

Scarborough [8] labeled intact cells of *N. crassa* 326 with diazotized [^{35}S]-sulfanilic acid and recovered approximately 50% of this label in the plasma membrane ghost preparation. The ^{35}S : ^{3}H ratio in the ghost preparation compared to that of the whole cell lysate indicated almost a 12-fold enrichment for surface label in the ghost preparation. The lactoperoxidase method employed in this study resulted in a 50% recovery of ^{125}I in the plasma membrane preparations, with a 7-fold enrichment of surface label with respect to the L-[^{3}H]-leucine steady-state label for protein. The ^{125}I surface labeling was dependent upon added lactoperoxidase and H_2O_2, less than 2% iodination occurring when either reactant was omitted.

Microscopic examination of cells after lactoperoxidase-catalyzed surface labeling did not reveal cell lysis. Furthermore, surface labeling of intact cells followed by sonication and centrifugation at 100 000 × *g* showed essentially all the incorporated ^{125}I to be associated with the particulate fraction.

The plasma membrane preparations employed in this study were of substantial purity based on the following criteria: (1) The cellular protein recovery was quantitatively similar to previously published values for preparations characterized with respect to chemical composition and enzymatic content [8] from the same organism. (2) Electron microscopy did not reveal contamination by intracellular organelles. (3) Lactoperoxidase-catalyzed iodination of intact cells under non-permeant conditions resulted in a 7-fold enrichment of surface label in the purified plasma membrane fraction. (4) Two-dimensional polyacrylamide gel electrophoresis indicated the proteins from purified plasma membranes to be a subset of the total particulate proteins of the cell, without detectable contamination by cytoplasmic proteins.

The appearance of the F_{50} protein in cytoplasmic soluble as well as plasma membrane and total particulate samples does not appear to be artifactual. The possibility that F_{50} was loosely associated (peripheral) membrane protein was investigated. Treatment of purified plasma membranes with 0.1% solutions of octyl glucoside or Nonidet P-40 did not result in detectable solubilization of any proteins. Similarly, treatment of purified membranes with buffered EDTA solutions was ineffective in releasing membrane protein. These results indicated the absence of any loosely bound protein in the purified membranes. Indeed, appreciable solubilization of membrane protein was only achieved with anionic detergents such as SDS and lithium 3,5-diiodosalicylate, or the combination of 8.0 M urea/2% Nonidet P-40/5% β-mercaptoethanol described in Materials and Methods. The conclusion from these experiments was that the proteins resolved by two-dimensional electrophoresis of purified plasma membrane samples were all integral membrane proteins.

The results from the non-permeant iodination procedure also support the nonartifactual occurrence of F_{50} in cytoplasmic soluble and particulate fractions. The protein was intensely iodinated in purified plasma membranes and 100 000 × *g* pellet fractions from surface-labeled cells. However, no detectable ^{125}I radioactivity was observed in the 100 000 × *g* supernatant fraction from the same surface-labeled cells, even though the F_{50} protein was prominent in the Coomassie-blue-stained electrophoretogram.

Two other interesting features of the F_{50} protein are its abundance and its apparent high isoelectric point. Excising the F_{50} protein spot from electrophoretograms of steady-state labeled cells and counting in a scintillation counter suggests that F_{50} constitutes approximately 2% of the total cellular protein of *N. crassa* 326. Although the non-equilibrium pH gradient SDS-polyacrylamide gel electrophoresis system employed in this study does not give reliable p*I* values for basic proteins [17], the F_{50} protein is found at approximately pH 8.2 in the gradient, suggesting this as a minimum p*I* value.

The two-dimensional analytical scheme used in this study was selected to cover a broad pH range. The detection of at least 180 protein species in *N. crassa* 326 purified plasma membranes represents a 9-fold increase in resolution over previous one-dimensional analysis [9]. Seventy-five of the proteins were found to be exposed at the extracytoplasmic surface. It is probable, however, that the resolution can be further improved by analysis of the pH range in smaller increments, i.e. the 3—5 region, 5—7 region, 7—9 region, rather than in a single gradient as reported here. Therefore, the 180 proteins most likely represent an intermediate approximation rather than an absolute maximum number of plasma membrane proteins. The greater complexity of the protein composition of these plasma membranes appears to be consistent with the diverse biological functions contained within eukaryotic plasma membranes and the genetic potential of eukaryotic cells.

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