Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2407–2411, April 1981
Cell Biology

Identification of mitochondrial proteins and some of their precursors in two-dimensional electrophoretic maps of human cells

(two-dimensional gels/mitochondria/uncouplers/signal peptides)

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Communicated by James V. Neel, January 15, 1981

ABSTRACT **A set of at least 30 proteins disappears from the two-dimensional electrophoretic pattern of human lymphoid cells treated with various antimitochondrial agents. This set is similar to the set of proteins found in isolated mitochondria (except for the presence of actin in the latter group), indicating that the inhibitor effect stops production of a majority of mature mitochondrial proteins. Several proteins having the characteristics of precursors to the major cytoplasmically synthesized mitochondrial proteins can be observed in cells during fast-pulse experiments and in a reticulocyte lysate system fed with total lymphoid cell RNA. In the three major instances of mitochondrial precursor–product processing, the removed peptide is quite basic in each case, suggesting that a lysine- or arginine-rich terminal sequence may be necessary for initial recognition by the mitochondrial protein uptake apparatus. The inhibitor effect allows easy identification of a large set of mitochondrial proteins in two-dimensional maps of various cells, thereby specifying a particularly tractable and functionally distinctive subset of the cellular proteins. The nature and wide scope of the effect support the concept of energy-dependent "vectorial processing" [Schatz, G. (1979) *FEBS Lett.* 103, 203–211] and indicate that such a mechanism is generally applicable to the major class of cytoplasmically synthesized mitochondrial proteins in mammalian cells.**

By using high-resolution two-dimensional electrophoresis (1), several thousand distinct proteins can be resolved among the constituents of a mammalian cell such as the lymphocyte. A majority of these molecules probably have never been described, nor is it likely that their functions have yet been imagined in any detail. Because the shape and position of a protein spot in such a two-dimensional map indicates almost nothing about that protein's function or relative importance in the cell, it is sometimes concluded that the technique presents an overabundance of unorganizable data. In fact, order readily can be brought to such data and, ultimately, a detailed blueprint of the cell's activities can be extracted if those subsets of proteins that comprise specific functional or structural units can be defined (2). Information of this kind provides a foundation upon which to base interpretations of changes in the pattern of protein synthesis resulting from gene regulation, differentiation, or, over a longer time span, evolution.

The proteins of the mitochondrion form such a well-defined subset of the cell's constituents, being both physically sequestered and functionally specialized. The fact that mitochondria can be isolated from various sources has made it possible to study in detail the biochemistry of their energy generation and other functions, but it has left the relationship of the organelle to the cell it serves largely unexplored. It is the latter relationship that is of particular interest in the study of cells by two-dimensional mapping techniques. What has been lacking is a convenient and reliable way of identifying mitochondrial protein spots in the total cell pattern.

This paper describes such a method, based on the observation that nonactin (a K^+ ionophore) causes the disappearance of a specific and reproducible group of proteins from the map of labeled lymphocyte proteins (unpublished data). This group correlates well with the pattern of proteins found in isolated mitochondria.

MATERIALS AND METHODS

Labeling of Cells. Two sublines of the human lymphoblastoid cell line GM607 (Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) were used in this study. The original line was derived from peripheral lymphocytes of a normal individual; the two sublines differ only in the level of expression of a presumed μ-type immunoglobulin chain. Cells at a density of approximately 10^6/ml were labeled (with or without inhibitor as indicated) in RPMI-1640 medium containing 5% fetal bovine serum and antibiotics and lacking the labeling amino acid (GIBCO Selectamine kit). [^{35}S]Methionine was used at a concentration of 60 μCi/ml (1 Ci = 3.7×10^{10} becquerels). Incubation was carried out at 37°C in 5% CO_2/95% air at 95% relative humidity, with continuous gentle shaking.

Preparation of Mitochondria. Cells prelabeled with [^{35}S]methionine were homogenized in 0.25 M sucrose/0.3 mM EDTA, pH 7.4, and spun at 600 × *g* for 10 min. The supernatant was centrifuged at 10,000 × *g* for 10 min, and the resulting pellet was washed twice. The last pellet was resuspended in 50% (wt/wt) sucrose/0.3 mM EDTA and layered under a step gradient of 44.5% and 41% sucrose. This was centrifuged for 1 hr at 60,000 rpm in a Beckman type 65 rotor. The material banding between 41% and 44.5% sucrose (density, 1.187 to 1.205 g/cm^3) was diluted, pelleted, and dissolved in 9 M urea/2% Nonidet P-40/2% Ampholines (LKB, 3.5–10)/1% mercaptoethanol.

Reticulocyte Translations. Total GM607 RNA was prepared by phenol extraction and lithium chloride precipitation and was translated for 30 min at 30°C in a commercial rabbit reticulocyte system containing 1.2 mM $MgCl_2$ and 135 mM KCl but no added spermidine (Bethesda Research Laboratories, Rockville, MD). Mitochondrial uptake was demonstrated by adding crude mouse liver mitochondria after this translation period and incubating for a further 30 min before removing the mitochondria by sedimentation.

Two-Dimensional Electrophoresis. The method of O'Farrell (1) was used with modifications as described (3, 4). The acid end of the focusing dimension was to the left, and high apparent molecular weight ($NaDodSO_4$) was at the top. Isoelectric point standards derived from rabbit muscle creatine phosphokinase

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were used on all gels (5). Figures show either autoradiographs or fluorographs (6).

Nomenclature of Protein Sets. In this work I follow this laboratory's standard practice with regard to the naming of functionally or characteristically related protein sets; each set has a six-letter acronym, and each protein of the set has a serial number following the acronym. Thus, the set of proteins that disappear when mitochondrial function is impaired is designated Mitcon:1-n; the set of putative precursors to these is designated Mitpro:1-n. This nomenclature is useful in the two-dimensional mapping work itself, in which it is necessary to have short, meaningful names for particular sets of spots, but it is intended only to supplement, not replace, exact functional names of enzymes, etc., when these have been convincingly associated with specific spots. The numbering of all the Mitcon proteins is not shown here (for reasons of clarity) but is indicated on a standard lymphocyte map available from this laboratory on request.

RESULTS

Effect of Mitochondrial Inhibitors on Cellular Protein Synthesis. Treatment of human lymphoblastoid cells with the K^+ ionophore nonactin (7) results in the loss or radical diminution of a well-defined subset of spots from the two-dimensional pattern of labeled polypeptides (Fig. 1). The same effect can be produced by various other anti-mitochondrial agents: (*i*) valinomycin [another K^+ ionophore (9)], (*ii*) dinitrophenol (the classical uncoupler), (*iii*) oligomycin [a mitochondrial ATPase inhibitor (10)], (*iv*) antimycin [a respiratory inhibitor(11)], or (*v*) rhodamine 123 [a vital stain specific for mitochondria (12)]. Because the mitochondrion is the common site of action of these compounds, the set of protein spots affected has been called the "Mitcon" group. Altogether, 33 proteins visible on these gels, plus 6 more visible only on basic protein resolving gels [BASO gels (13), not shown], belong to the Mitcon group in these cells. Human peripheral lymphocytes lack detectable levels of 7 of

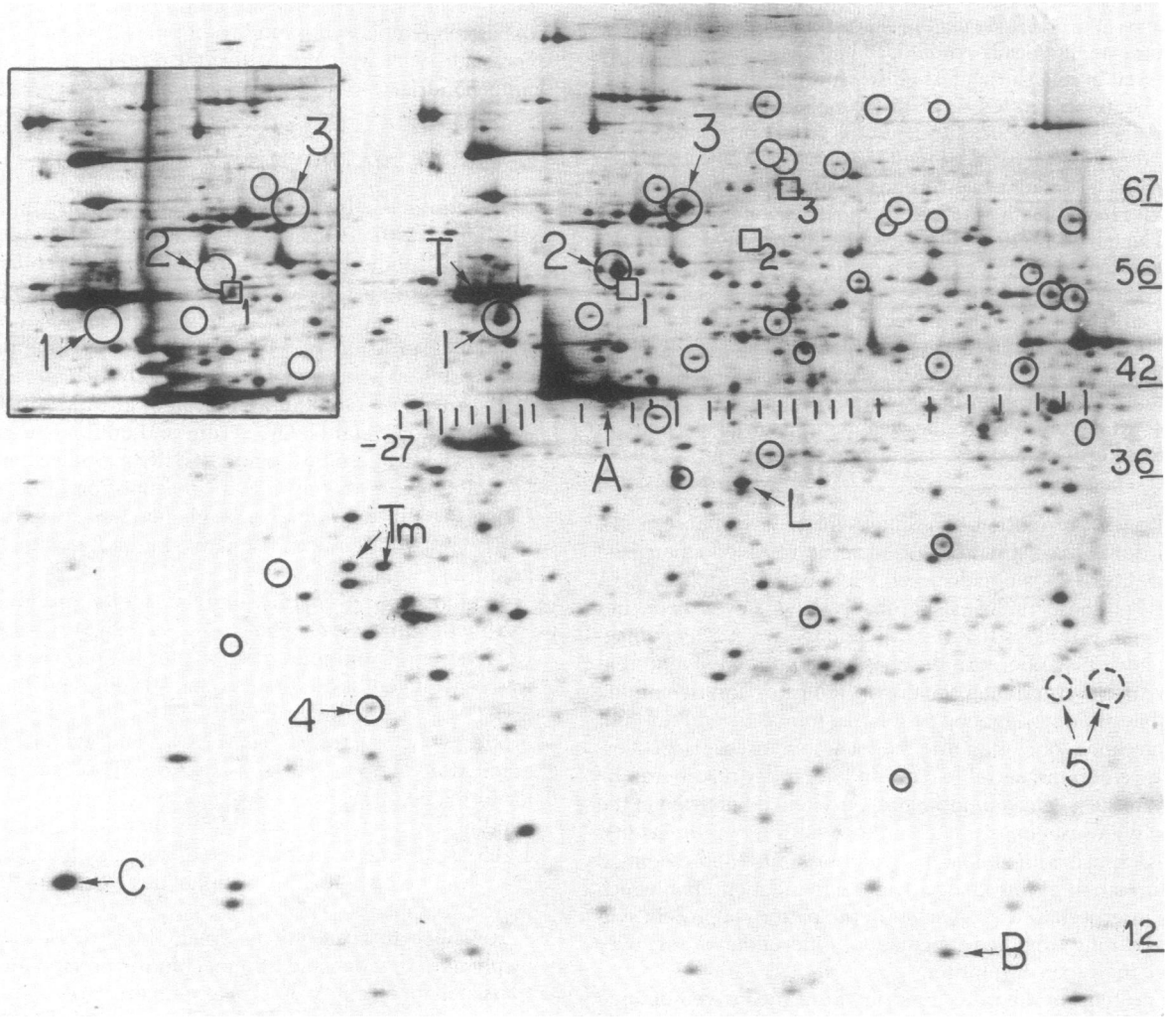

FIG. 1. Two-dimensional electrophoretic pattern of [^{35}S]methionine-labeled human lymphoblastoid cells (GM607). *Inset* shows a portion of the pattern obtained when these cells are treated with 10 μM nonactin throughout an 18-hr labeling period. Spots that disappear in nonactin-treated cells (the Mitcon group) are indicated by circles; the positions of spots that appear due to treatment (Mitpro) are indicated by squares. Large and small numerals indicate specific members of the Mitcon and Mitpro groups, respectively. Comparison of the original autoradiographs indicates that all the Mitcon proteins are rendered undetectable by nonactin treatment except Mitcon:3; here the small remaining labeled material may be due to continued synthesis of a protein with nearly identical two-dimensional position or to a low, persistent production of authentic Mitcon:3. Dashed circles indicate the position of a major member of the Mitcon group in fresh human peripheral lymphocytes (Mitcon:5) which is absent from all lymphoblastoid lines so far examined. The scale running horizontally across the figure from 0 to −27 marks the positions of rabbit muscle creatine phosphokinase isoelectric point standards (5); the numbers along the right margin indicate approximate molecular weights ($\times 10^{-3}$) in $NaDodSO_4$. Several identified proteins are indicated for reference: L, LDH B chain (8); T, tubulin; A, actin; Tm, nonmuscle tropomyosins; C, calmodulin; B, β_2-microglobulin.

the 33 proteins indicated in Fig. 1 but express 3 additional Mitcon proteins, of which one (Mitcon:5) is quite abundant (dashed circles in Fig. 1). Mitcon:5 disappears from lymphocytes upon treatment with phorbol ester tumor promoters (unpublished data). This property, together with its absence from all permanent lymphoblastoid lines thus far examined, indicates that the expression of Mitcon:5 is sensitive to cell transformation.

Only one of the proteins of the Mitcon group is synthesized on mitochondrial ribosomes (Mitcon:4); this spot alone disappears when cells are treated with chloramphenicol. The remaining mitochondrially encoded proteins are probably too hydrophobic to be resolved in this two-dimensional system.

Similarity of the Mitcon Group to the Protein Pattern of Isolated Mitochondria. [^{35}S]Methionine-labeled human lymphoblastoid mitochondria isolated by isopycnic banding yielded the two-dimensional pattern shown in Fig. 2. All of the major Mitcon proteins are visible in the pattern with relative abundances similar to those seen in whole cells. Conversely, all major proteins in the mitochondrial pattern (except actin) are members of the Mitcon group. The presence of actin could be due to contamination of the mitochondria with actin filaments or possibly to a specific physical association with the organelle. On the basis of molecular weight and relative abundance (14), it seems likely that Mitcon:1 and :2 are the β and α subunits of the F_1 ATPase, respectively.

Precursors of the Major Mitcon Proteins. In cells concurrently labeled and treated with nonactin for 24 hr, a few new spots appeared (squares in Fig. 1 and in Fig. 3). These proteins could be seen at much greater relative abundance in untreated cells pulse-labeled briefly (2–3 min) and immediately solubilized (Fig. 3*A*). After short chase periods, these proteins disappeared from untreated cells [coincident with increases in abundance of the major Mitcon proteins (Fig. 3*B*)] but not from inhibitor-treated cells [where no Mitcon proteins are made and the normally short-lived species persist (Fig. 3 *C* and *D*)]. Such behavior is what is expected of precursors that are converted to mature Mitcon proteins through the action of functioning mitochondria. This set is therefore called "Mitpro."

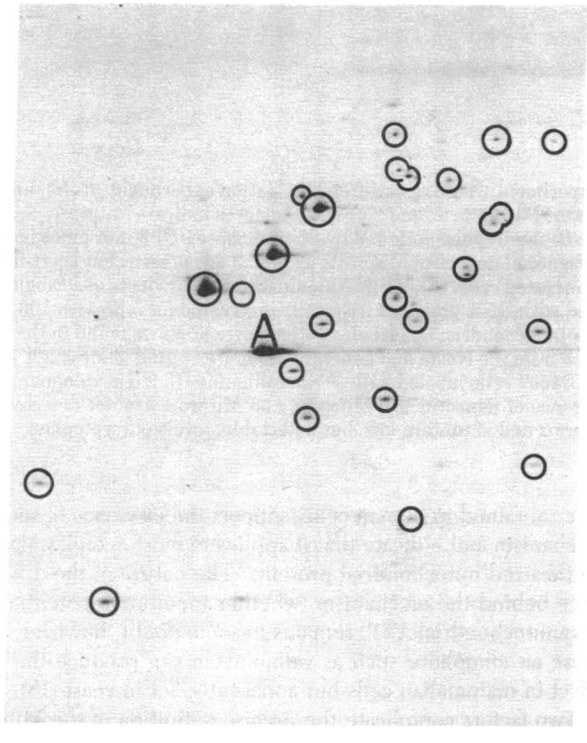

FIG. 2. Two-dimensional pattern of isolated GM607 mitochondria. Circles indicate the expected positions of Mitcon proteins (Fig. 1) on a gel of this pH range, as established by coelectrophoresis and the use of internal standards. Virtually all of the spots observed are in the Mitcon group (with the exception of actin, labeled A), and all of the expected Mitcon proteins are present.

At least three of the principal Mitpro spots are readily synthesized in a rabbit reticulocyte cell-free translation system fed with total RNA from lymphoblastoid cells (Fig. 3*E*). These products also were seen when RNA from nonactin-treated cells was used (Fig. 3*F*), demonstrating that the abundance of Mitpro message is not substantially affected by antimitochondrial treatment over a period of many hours. No mature Mitcon proteins were observed among the direct products of the reticulocyte system except for Mitcon:4 (not shown), the sole mitochondrially encoded protein so far identified in these patterns. However, when mouse liver mitochondria were incubated with the reticulocyte mixture after a period of protein synthesis and then removed by centrifugation, the putative precursors were removed specifically (though not completely) from the lysate supernatant (Fig. 3*G*); labeled mature Mitcon molecules appeared in the mitochondrial pellet (Fig. 3*H*). Thus, mouse mitochondria are capable of recognizing and correctly processing some of the major human mitochondrial proteins.

Neither Mitpro:1 nor Mitcon:1 contains any detectable tryptophan (Fig. 3 *I–L*). This unusual property, not shared by the other Mitcon or Mitpro molecules, suggests that the two are the precursor and mature forms of the same protein. The similarity of proteolytic digestion patterns (Fig. 4) supports such an assignment and suggests that Mitpro:2 and :3 are similarly related to Mitcon:2 and :3, respectively. This pairing of precursors and products indicates that a weight loss of about 3000–4000 daltons ($NaDodSO_4$) is associated with maturation in each case. Because the mature proteins are considerably more acidic than their respective precursors, the removed peptides must be quite basic. Each is estimated to carry between 4 and 8 net positive charges, based on comparison of the sizes of single-charge shifts in the mature forms with the total precursor–product pI differences.

DISCUSSION

The basic observation presented here is that the inhibition of mitochondrial function (by various agents) specifically halts production of the major mature mitochondrial proteins visible in the two-dimensional system used. This phenomenon has both practical and theoretical significance. From the practical viewpoint, it means that a set of mitochondrial proteins can be identified in high-resolution two-dimensional maps of whole cells without the necessity of isolating the organelle. This makes it particularly easy (*i*) to investigate relationships between the control of the expression of these genes and others active in the cell (as in the case of Mitcon:5 and cell transformation), (*ii*) to search for genetic variants of nuclear-encoded mitochondrial proteins (and to determine whether their variability is characteristically different from that of the cytosolic, cytoskeletal, or other protein sets), and (*iii*) to compare mitochondrial proteins of various species with a view toward establishing a measure of relatedness based on two-dimensional spot shifts [charge and molecular weight ($NaDodSO_4$) changes] alone. Questions of this kind can be addressed whenever a meaningfully large subset of cellular proteins can be rigorously defined and easily identified. Because classical isolation techniques must often be modified to produce mitochondria from different types of cells (perhaps leading to variations in contamination with other subcellular fractions and in loss of intermembrane molecules), it may be that

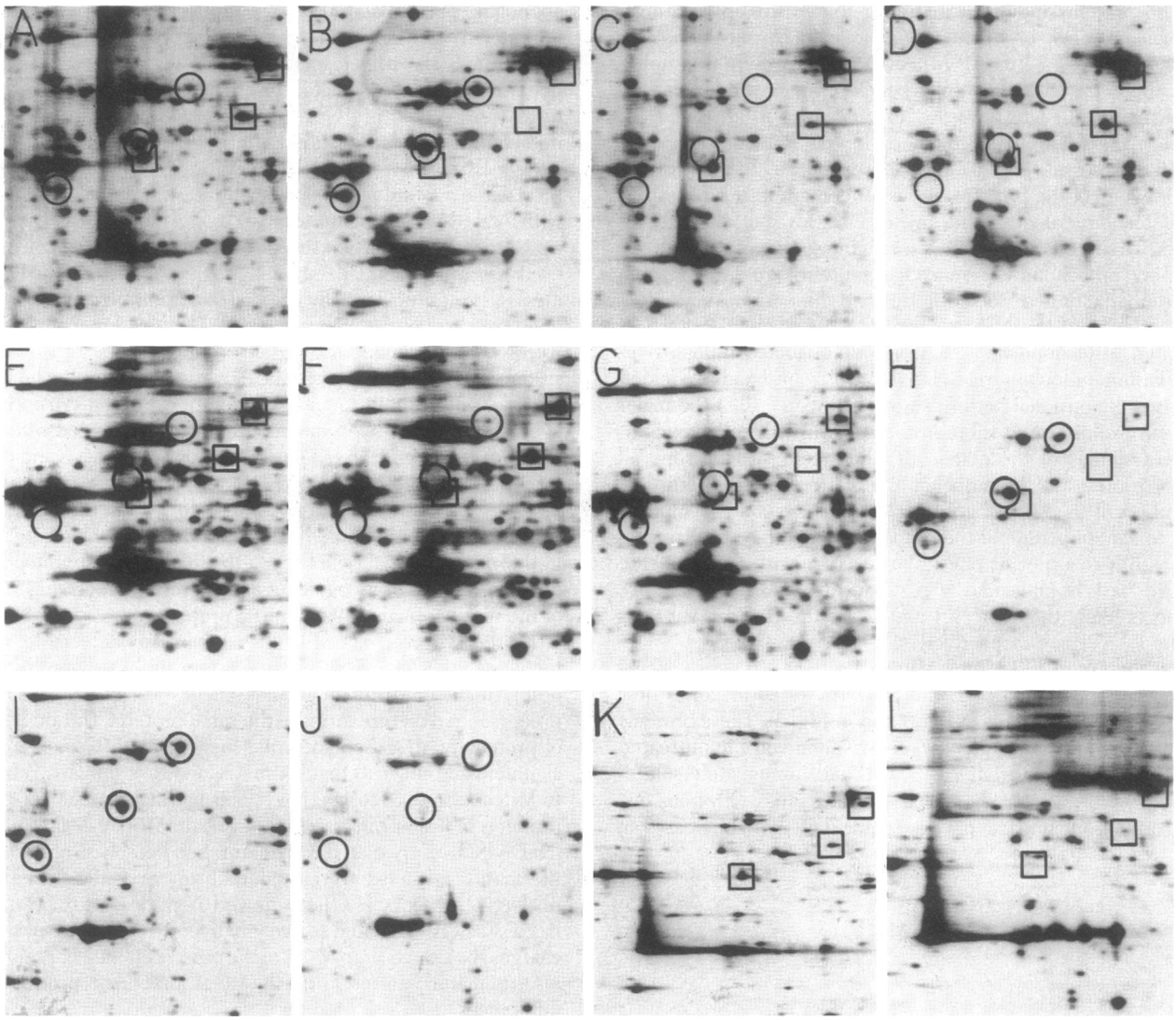

FIG. 3. Analogous regions of two-dimensional gels from a pulse-labeling experiment (*A–D*), a cell-free translation experiment (*E–H*), and a methionine vs. tryptophan labeling study (*I–L*). Circles indicate (in ascending order) Mitcon:1, :2, and :3 (Fig. 1); squares indicate Mitpro:1, :2, and :3. (*A*) GM607 cells pulse-labeled for 2 min with [^{35}S]methionine at 1 mCi/ml; (*B*) 2-min pulse followed by 30-min chase; (*C*) 2-min pulse in the presence of nonactin (100 μM); (*D*) 2-min pulse and 30-min chase, all in the presence of nonactin. Mitpro:1, :2, and :3 are present but short-lived in untreated cells; they persist (in the absence of Mitcon production) in nonactin-treated cells. (*E*) [^{35}S]Methionine-labeled products of a rabbit reticulocyte lysate system fed with total GM607 RNA; (*F*) products of a system fed with RNA prepared from cells pretreated for 5 hr with 100 μM nonactin; (*G*) products remaining in *E* after addition and removal of mouse liver mitochondria; (*H*) labeled reticulocyte products found in the mitochondria removed from *E*. Mouse liver mitochondria remove Mitpro:1, :2, and :3 from the lysate and generate Mitcon:1, :2, and :3 although with quite different efficiencies in the case of each protein. Bottom four panels are GM607 cells labeled with [^{35}S]methionine (I), [^{3}H]tryptophan (*J*), [^{35}S]methionine in the presence of nonactin (*K*), or [^{3}H]tryptophan in the presence of nonactin (*L*). Mitcon:1 and Mitpro:1 are not detected in [^{3}H]tryptophan-labeled cells (even on long exposures). Mitcon:2 and :3 and Mitpro:2 and :3 contain low, but detectable, levels of tryptophan.

the inhibitor effect is a more reliable way of identifying a specific set of mitochondrial proteins.

From a more theoretical viewpoint, it is of particular interest that the inhibitor effect applies to virtually all the proteins detected in a preparation of isolated mitochondria. This implies that the mechanism involved in the effect is intrinsic to the biogenesis of a large class of mitochondrial proteins. A number of recent papers by Schatz and coworkers (16–20) demonstrate that at least five nuclear-encoded mitochondrial components of yeast are synthesized in the cytoplasm as precursors larger than the mature protein. These precursors are subsequently taken up by the mitochondria and concurrently cleaved to mature form by means of a mechanism requiring mitochondrial energy production ["vectorial processing" (16)]. The results presented here, obtained in human cells, support the existence of such a mechanism and indicate that it applies to most cytoplasmically synthesized mitochondrial proteins. The nature of the driving force behind the mechanism (whether membrane potential or intramitochondrial ATP) remains in some doubt, however, because an ionophore such as valinomycin can produce the full effect in mammalian cells but apparently not in yeast (18).

Two factors complicate the precise definition of the Mitcon set in terms of submitochondrial compartments. (*i*) Cytochrome *c*, a small cytoplasmically made protein that functions between the inner and outer mitochondrial membranes, does not appear to be made as a larger precursor (21), and hence its transport into the organelle need not necessarily be subject to any control by mechanisms dependent on energy production. (*ii*) Cyto-

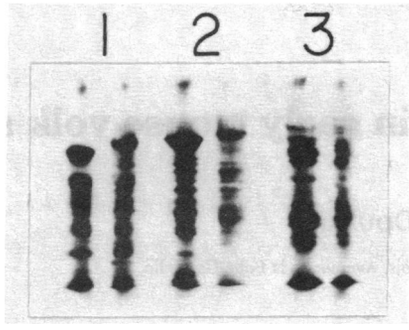

FIG. 4. Staphyloccocal V8 protease partial digests of spots cut from two-dimensional gels and analyzed according to Cleveland (15). Lanes: 1, Mitcon:1 and Mitpro:1; 2, Mitcon:2 and Mitpro:2; 3, Mitcon:3 and Mitpro:3.

chrome *c* peroxidase, a larger intermembrane enzyme, apparently is made as a larger precursor, but in yeast its processing is not interrupted by conditions that halt maturation of the mitochondrial matrix and inner membrane proteins so far examined (18). Thus, the production of mature or properly situated inter and outer membrane components in general may not be expected to cease if mitochondrial energy production stops. It therefore seems possible that the Mitcon group constitutes that set of proteins which pass through an energy-dependent processing channel in the inner mitochondrial membrane and not the total complement of mitochondrial proteins. The pattern of spots in isolated mitochondria would probably reflect a similar bias because the matrix and inner membrane proteins represent the vast majority of the mass of the mitochondrion and because leakage during isolation would selectively deplete the intermembrane molecules.

Each of the three mitochondrial protein precursors so far identified in the two-dimensional pattern is more basic than its mature form. The removed peptides fall in the range 3000–4000 daltons (approximately 20–25 amino acids) and carry between 4 and 8 net positive charges, indicating a high proportion of lysine or arginine. It therefore seems reasonable to suggest that either the mitochondrial protein transport site or some protein accessory to it has a high affinity for a lysine- or arginine-rich sequence and may use this sequence to identify proteins to be imported into the organelle. Such a highly charged, hydrophilic tail could prevent the precursor from folding prematurely into its mature conformation (particularly if it were attached to an end of the mature sequence that was hydrophobic and formed the core of the active structure). In any case, possession of a hydrophilic tail clearly distinguishes these mitochondrial precursors from the precursors of secreted proteins [which bear a hydrophobic tail (22)].

I thank Neal Sherberg for help in preparing RNA, Barbara Hickman, Sharron Nance, and Anne Gemmel for excellent technical help, and my colleagues in the Molecular Anatomy Program for useful discussions. This work was supported by the U.S. Department of Energy under Contract no. W31-109-ENG-38.

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