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Energy-dependent processing of cytoplasmically made precursors to mitochondrial proteins

(mitochondrial biogenesis/import of proteins/bioenergetics)

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ABSTRACT Earlier work has shown that mitochondrial proteins synthesized in the cytosol are initially made as larger precursors which are then transferred into the organelles and processed to their mature size in the absence of protein synthesis. It is now demonstrated that depletion of the mitochondrial matrix ATP in intact yeast spheroplasts by various combinations of inhibitors and mutations prevents the processing of precursors to the three largest subunits of the mitochondrial F_1-ATPase and two subunits of the cytochrome bc_1 complex. These polypeptides are all synthesized outside the mitochondria and transported to the mitochondrial matrix or inserted into the mitochondrial inner membrane. In contrast, depletion of the matrix ATP does not inhibit processing of the precursor to cytochrome *c* peroxidase; this enzyme is located in the mitochondrial intermembrane space which is freely accessible to ATP made in the cytosol. The processing of extramitochondrially made precursors or the transfer of these precursors across the mitochondrial inner membrane is thus dependent on ATP.

Most of the proteins of mitochondria and chloroplasts are synthesized on cytoplasmic ribosomes and transferred into these organelles (1). Several polypeptides imported in this manner have been shown to be initially made as larger precursors (2–7) which can then be transported across the organelle membranes and processed to their mature size in the absence of concomitant protein synthesis (3–5, 7).

This communication demonstrates that processing of these precursors *in vivo* requires ATP. Our experiments exploited the fact that the ATP level in the mitochondrial matrix of intact yeast cells can be specifically lowered by appropriate inhibitors and mutations. Because ATP can diffuse only through the mitochondrial outer membrane (8) but not through the inner one (9), the matrix space must obtain ATP through the following two major routes: (*i*) respiration-driven phosphorylation of ADP on F_1-ATPase directly in the matrix itself; (*ii*) import of glycolytically generated ATP from the cytoplasm via a specific "adenine nucleotide transporter" located in the mitochondrial inner membrane. Each of these two major routes can be inhibited independently of the other. Oxidative phosphorylation can be blocked either by inhibitors of the respiratory chain such as KCN or antimycin or by mutations such as the pleiotropic *rho*$^-$ mutation which causes the loss of several mitochondrial cytochromes as well as the proton channel of mitochondrial ATPase (1). Import of glycolytically generated ATP into the matrix can be blocked by inhibitors of the adenine nucleotide transporter such as bongkrekic acid (9) or by the nuclear op_1 mutation which specifically lowers the efficiency of this transporter (10–12). If both oxidative phosphorylation and ATP import are blocked, the matrix space can be depleted of ATP without affecting protein synthesis on cytoplasmic 80S ribosomes. This unique situation made it possible to show that several cytoplasmically made mitochondrial polypeptides need ATP not only for their synthesis but also for their conversion to the mature forms.

MATERIALS AND METHODS

Growth of Cells and Preparation of Spheroplasts. Wild-type *Saccharomyces cerevisiae* strain D273-10B (α; ATCC 25657), the corresponding *rho*$^-$ mutant D273-10B-1, and the op_1 nuclear mutant 777-3A (α ade 1 op$_1$; ref. 9) were grown in sulfate-free semisynthetic medium (13) supplemented with 0.3% galactose. The growing medium for strain op_1 was supplemented with 30 mg of adenine per liter. Wild-type cells were grown to 140 Klett units; *rho*$^-$ and op_1 cells were grown to 80 Klett units. Spheroplasts were prepared as described (5, 6) and suspended to 200 mg wet weight per ml in 0.061% $MgCl_2 \cdot 6H_2O$/0.1% KH_2PO_4/0.05% NaCl/0.04% $CaCl_2$/1% ethanol/0.3% galactose/1.3 M sorbitol at pH 6.0.

Labeling of the Spheroplasts. Aliquots (0.25 ml) of the spheroplast suspension were preincubated for 60 min in 15-ml glass centrifuge tubes at 29°C with gentle shaking. Then, 0.25 ml of the suspending medium was added, followed by 10–20 μl of [^{35}S]methionine [10 mCi/ml (1 Ci = 3.7×10^{10} becquerels)]. After 15-min incubation at 29°C, 2-μl samples were removed for measuring incorporation of [^{35}S]methionine into proteins (14). Then, 10 μl of a solution of protease inhibitors in dimethylsulfoxide (*p*-aminobenzamidine, phenylmethylsulfonyl fluoride, *N*-tosyl-L-phenylalanylchloromethane, and *N*-tosyl-L-lysylchloromethane at 0.1 M each) was added and the spheroplast suspension was mixed with 1 ml of 4% (wt/wt) sodium dodecyl sulfate ($NaDodSO_4$) at 100°C in 10-ml steel centrifuge tubes. The dissociated mixture was cooled to room temperature, diluted with 5 ml of 20 mM Tris·HCl, pH 7.4/140 mM NaCl/5 mM EDTA/1% Triton X-100 (Triton buffer), and clarified by centrifugation at $50,000 \times g$ for 15 min.

Immunoprecipitation. The clarified supernatant was diluted to 40 ml with Triton buffer, mixed with 0.1 ml of the desired antiserum, and incubated overnight at room temperature. Then, 0.2 ml of a 10% (wt/vol) suspension of fixed *Staphylococcus aureus* cells (5) was added and the mixture was shaken at room temperature for 1 hr. The *S. aureus* cells were isolated by centrifugation (10 min at $5000 \times g$) and washed four times with 10 ml of Triton buffer by centrifugation. The antigen was extracted for 3 min at 95°C with 0.25 ml of 2% $NaDodSO_4$/0.1 M Tris, pH 6.8/2 mM EDTA/0.03 mg of dithiothreitol per ml/10% (wt/vol) glycerol. The cells were removed by centrifugation and the supernatant was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in 12.5% polyacrylamide

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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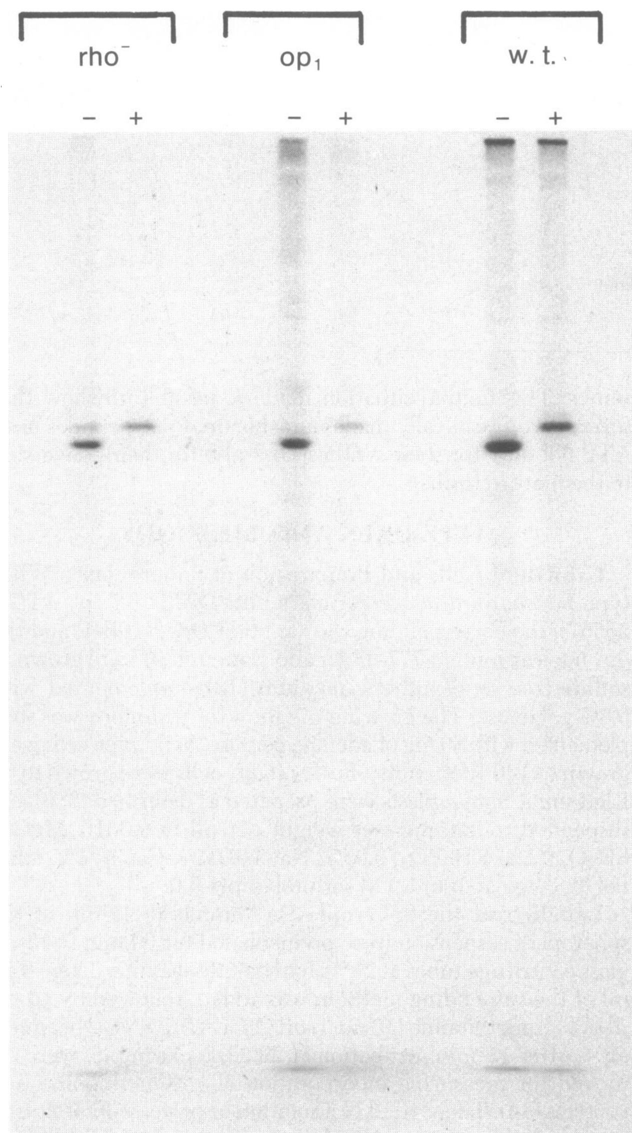

FIG. 1. The uncoupler CCCP blocks the processing of the precursor to the β-subunit of F_1-ATPase in all yeast strains tested. Cells of the wild-type (w. t.) and of mutants *rho*⁻ and op_1 were converted to spheroplasts and pulse-labeled in the absence (−) or presence (+) of 40 μM CCCP. F_1 β-subunit was isolated from the lysed spheroplasts by immunoprecipitation and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by radioautography.

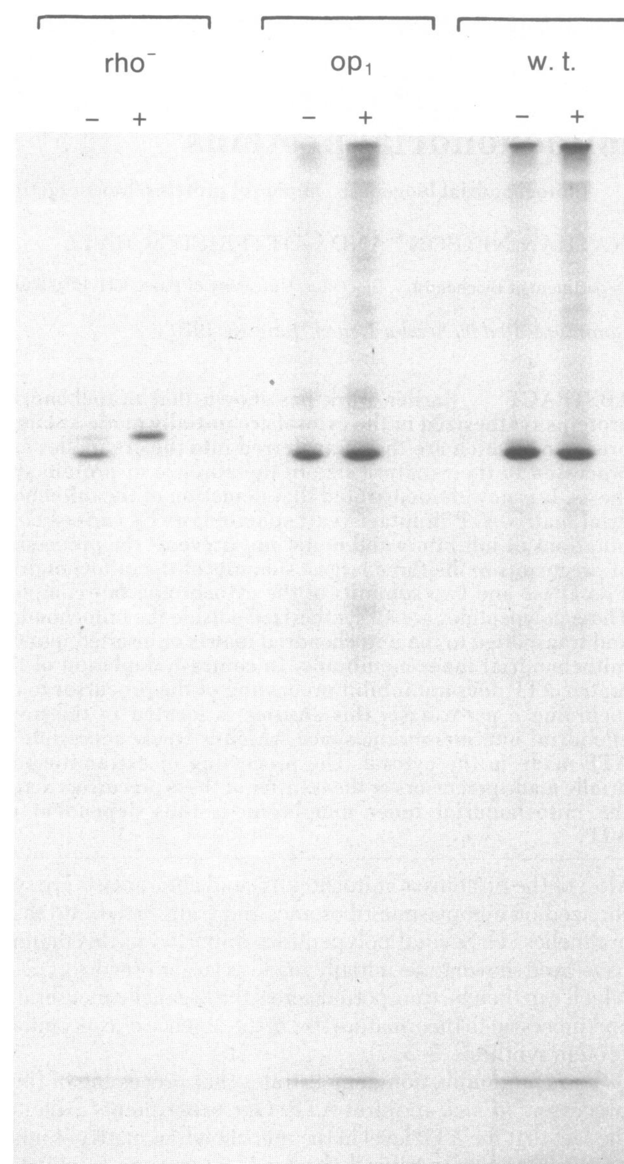

FIG. 2. Bongkrekic acid blocks the processing of the precursor to the β-subunit of F_1-ATPase in the respiration-deficient *rko*⁻ mutant but not in wild-type (w. t.) cells or the op_1 mutant. The conditions were the same as described in Fig. 1 except that CCCP was omitted and in the samples labeled "+," 14.6 μM bongkrekic acid was added to the spheroplast suspension 30 min before pulse-labeling.

gel slabs. Gel electrophoresis was performed as described (15) and the dry slabs were analyzed by autoradiography or fluorography (16). Published procedures were used to measure protein (17), to prepare antisera (18), and to synthesize L-[^{35}S]methionine (approximately 100 Ci/mmol) from $^{35}SO_4^{2-}$ (19). Bongkrekic acid was a gift from P. Vignais (Grenoble, France) and the antisera against subunits of the cytochrome bc_1 complex were gifts from M. Solioz and C. Côté.

RESULTS

The experiments reported here were done with a wild-type yeast strain and two mutants with defective mitochondria: an extrachromosomally inherited *rho*⁻ mutant and the nuclear op_1 mutant. The *rho*⁻ mutant lacks oxidative phosphorylation (1) but can still exchange adenine nucleotides between the mitochondrial matrix and the cytoplasm (11, 12). In this mutant, most of the ATP in the matrix is derived from glycolysis in the cytosol. The op_1 mutant (10) has a normal oxidative phosphorylation system but can no longer efficiently exchange adenine nucleotides between the mitochondrial matrix and the cytoplasm (11, 12). As a result, most of the ATP in the matrix is derived from oxidative phosphorylation.

Fig. 1 depicts the effect of the uncoupler carbonyl cyanide *m*-chlorophenylhydrozone (CCCP) on the pulse-labeling of the β-subunit of F_1-ATPase in the three yeast strains. This subunit is synthesized outside the mitochondria as a larger precursor and imported into the mitochondrial matrix space with concomitant processing to the mature polypeptide (5). In each case, the uncoupler inhibited the processing of the larger precursor to the mature β-subunit. Although this was the first indication that processing of this precursor *in vivo* requires energy, the effects of uncouplers on whole cells are too complex to constitute clear-cut evidence.

Such evidence was provided by additional experiments that showed that processing of the β-subunit precursor was inhibited whenever both oxidative phosphorylation and ATP import into

FIG. 3. Processing of the precursor to the β-subunit of F_1-ATPase in wild-type yeast is inhibited by a combination of antimycin A and bongkrekic acid but not by either inhibitor alone. The experimental conditions were essentially as described in Fig. 1 except that pulse-labeling of wild-type spheroplasts was performed in the presence of different inhibitors. Lanes: 1, no inhibition; 2, bongkrekic acid (14.6 μM); 3, antimycin A (170 μM); 4, bongkrekic acid (14.6 μM) plus antimycin A (170 μM); 5, CCCP (40 μM); 6, 2 μM valinomycin; 7, 10 μM valinomycin. Control experiments showed that 10 μM valinomycin blocked oxidative phosphorylation in the wild-type cells.

the mitochondria were blocked. This was accomplished by the following conditions: (*i*) *rho*$^-$ mutation *plus* bongkrekic acid (Fig. 2), (*ii*) antimycin A *plus* bongkrekic acid (Fig. 3), and (*iii*) KCN plus the op_1 mutation (Fig. 4). It is also evident that blockage of *either* oxidative phosphorylation *or* ATP import did not prevent processing. Similarly, collapsing the mitochondrial membrane potential with valinomycin had no effect (Fig. 3, lanes 6 and 7). Control experiments confirmed that the inhibitors did not interfere with overall protein synthesis by the spheroplasts. The only exception was 5 mM KCN which inhibited protein synthesis by 30–40% in wild-type cells and by about 60% in the op_1 mutant.

Taken together, these data show that depletion of the mitochondrial matrix ATP interferes with the processing of the β-subunit precursor.

The results of Figs. 5 and 6 demonstrate that the conclusions

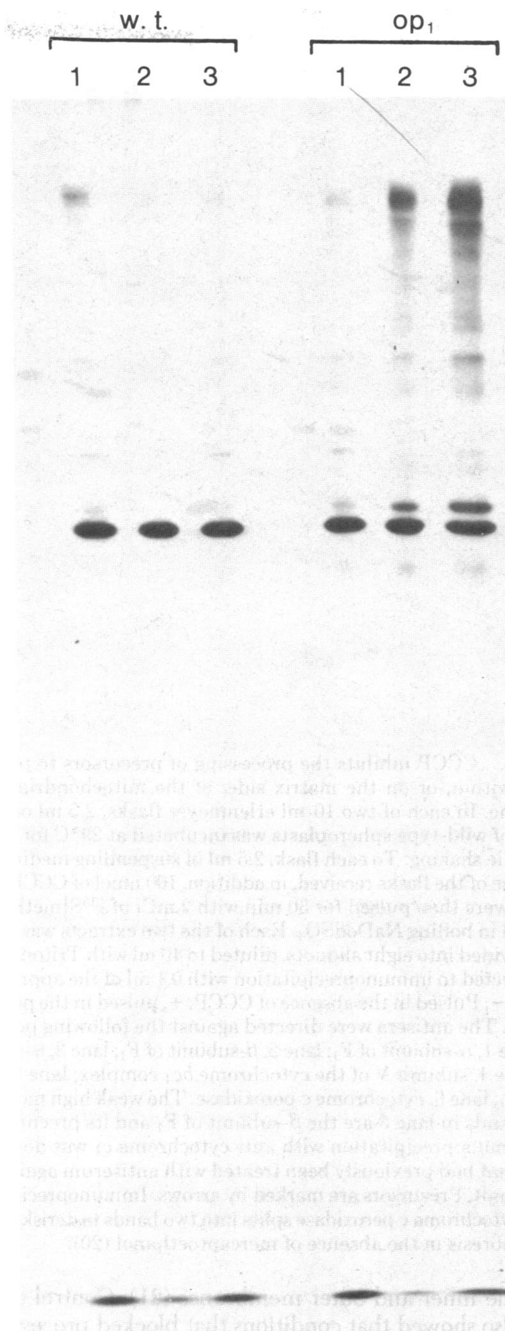

FIG. 4. Cyanide inhibits processing of the β-subunit of F_1-ATPase in the op_1 mutant but not in wild-type (w. t.) cells. Experimental conditions were as outlined in Fig. 1 with the following modifications: (*i*) after the 60-min preincubation, 0.25 ml of 1 M sorbitol/100 mM 2-(*N*-morpholino)-ethanesulfonate, pH 7.5/1% galactose was added instead of the suspending medium; (*ii*) pulse-labeling was performed in the absence of inhibitor (lanes 1) or in the presence of 1 mM KCN (lanes 2) or 5 mM KCN (lanes 3).

obtained for the β-subunit of F_1 are also valid for four other mitochondrial polypeptides made in the cytoplasm—the α- and γ-subunits of F_1 (5), cytochrome c_1 (unpublished data), and the 25,000-dalton subunit of the cytochrome bc_1 complex (6). The F_1 subunits are located on the matrix side of the mitochondrial inner membrane whereas the two subunits of the cytochrome bc_1 complex are located within the mitochondrial inner membrane. In contrast, CCCP or specific depletion of the matrix ATP does not interfere with processing of the precursor (20) to cytochrome c peroxidase. This enzyme is located be-

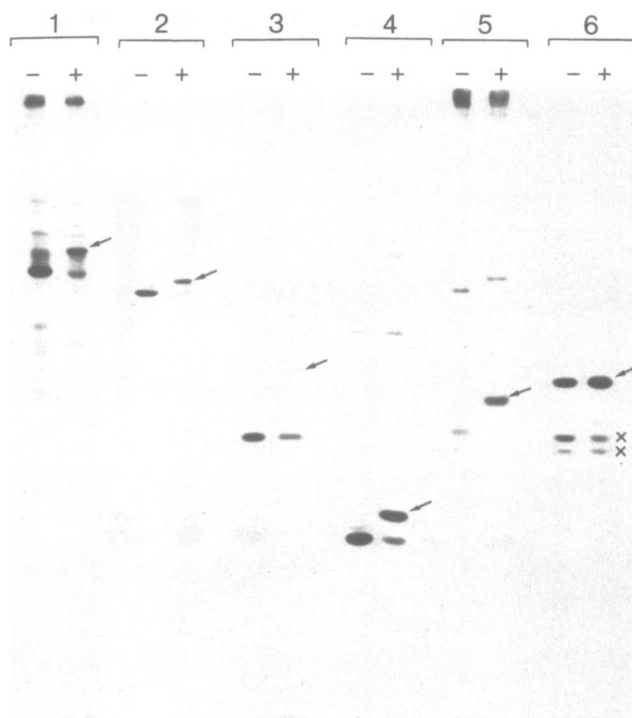

FIG. 5. CCCP inhibits the processing of precursors to proteins located within, or on the matrix side, of the mitochondrial inner membrane. In each of two 10-ml erlenmeyer flasks, 2.5 ml of a suspension of wild-type spheroplasts was incubated at 29°C for 60 min with gentle shaking. To each flask, 2.5 ml of suspending medium was added; one of the flasks received, in addition, 100 nmol of CCCP. Both samples were then pulsed for 30 min with 2 mCi of [^{35}S]methionine and lysed in boiling $NaDodSO_4$. Each of the two extracts was centrifuged, divided into eight aliquots, diluted to 40 ml with Triton buffer, and subjected to immunoprecipitation with 0.1 ml of the appropriate antisera. −, Pulsed in the absence of CCCP; +, pulsed in the presence of CCCP. The antisera were directed against the following polypeptides: lane 1, α-subunit of F_1; lane 2, β-subunit of F_1; lane 3, γ-subunit of F_1; lane 4, subunit V of the cytochrome bc_1 complex; lane 5, cytochrome c_1; lane 6, cytochrome c peroxidase. The weak high molecular weight bands in lane 5 are the β-subunit of F_1 and its precursor because immunoprecipitation with anti-cytochrome c_1 was done on a sample that had previously been treated with antiserum against the F_1 β-subunit. Precursors are marked by arrows. Immunoprecipitated mature cytochrome c peroxidase splits into two bands (asterisks) upon electrophoresis in the absence of mercaptoethanol (20).

tween the inner and outer membranes (21). Control experiments also showed that conditions that blocked processing of the precursors did not affect the pulse labeling of the cytosol enzyme glyceraldehyde-3-phosphate dehydrogenase (not shown). With both wild-type yeast and the *rho*$^-$ mutant, energy deficiency of the mitochondrial matrix appears to prevent maturation of those cytoplasmically made proteins that are transported across or into the mitochondrial inner membrane.

DISCUSSION

Transfer of polypeptides across biological membranes can occur by at least two basically different processes. The first one transports nascent (but not completed) polypeptides across the plasma membrane of prokaryotes and the endoplasmic reticulum of eukaryotes (22). Because transmembrane movement is obligately linked to translation, this process is termed "vectorial translation." The second process transfers completed precursor polypeptides from the cytosol into mitochondria and chloroplasts (3–5, 7). Because unidirectional transmembrane

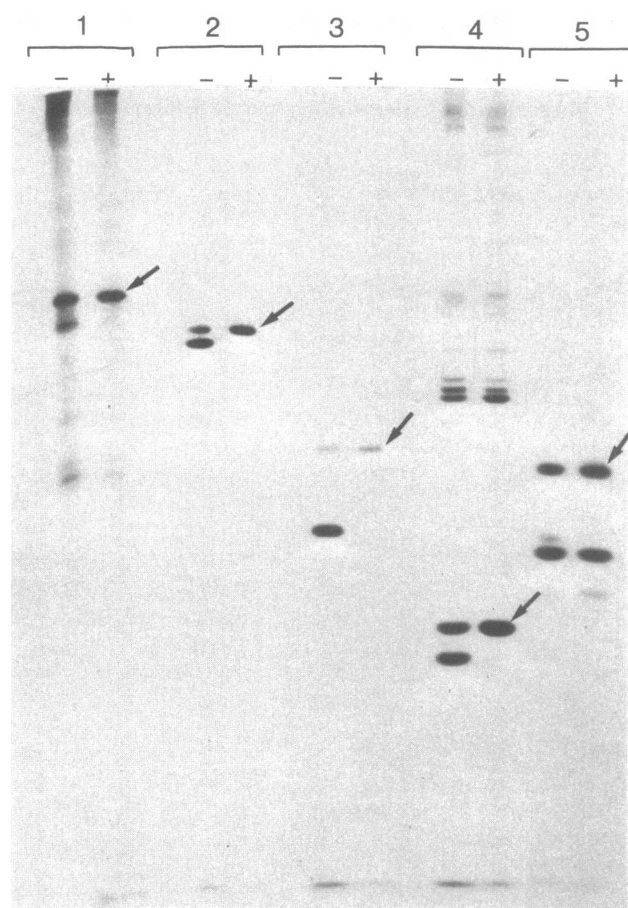

FIG. 6. Treatment of the *rho*$^-$ mutant with bongkrekic acid inhibits the processing of precursors to proteins located within, or on the matrix side of, the mitochondrial inner membrane. Experimental conditions were as in Fig. 5 except that 73 nmol of bongkrekic acid was added to one of the flasks (+) instead of CCCP. The labeling pulse was started 30 min after addition of bongkrekic acid and maintained for 20 min. The high molecular weight bands in lane 4 are impurities. Lanes 1–4 are numbered as in Fig. 5; lane 5, anticytochrome c peroxidase. Precursors are marked by arrows.

movement appears to be obligately linked to proteolytic processing of the precursors, we suggest that this mechanism be termed "vectorial processing." A similar vectorial processing mechanism appears to operate in the transfer of bacterial toxins across the plasma membrane of various cells (23).

The experiments reported here show that the correct *in vivo* processing of cytoplasmically made precursors to mitochondrial proteins requires energy. This demonstration was made possible by the following facts: (*i*) during import of polypeptides into mitochondria, transmembrane movement and proteolytic processing are not coupled to translation, (*ii*) the mitochondrial matrix, in contrast to all other cellular compartments, can be experimentally depleted of ATP independently of the remaining regions of the cell. Because depletion of the mitochondrial matrix ATP could be accomplished by several different combinations of inhibitors and mutations, we are confident that our data are not distorted by possible side-effects of individual inhibitors.

The fact that processing still occurs in *rho*$^-$ mutants in the absence of inhibitors makes it unlikely that a mitochondrial membrane potential, rather than ATP, provides the energy for processing: *rho*$^-$ mutants cannot form an appreciable membrane potential because they lack not only a respiratory chain but also a proton-translocating mitochondrial ATPase complex (1). The role of a membrane potential is further excluded by

the inability of valinomycin to block processing in the wild type.

The mechanism by which ATP drives vectorial processing is still unknown. Is it necessary for transmembrane movement of the precursor, for its processing, or for both?

The dependence of vectorial processing on ATP suggests some intriguing parallels to the ATP-dependence of protein degradation in bacterial as well as mammalian cells (24). Recently, ATP-dependent protein breakdown could be demonstrated in a cell-free system (25, 26). In all these instances, however, lack of suitable inhibitors has prevented identification of the intracellular locale involved in proteolysis. The present data suggest (but do not prove) that proteolytic maturation of at least some cytoplasmically made mitochondrial inner membrane proteins occurs in the matrix space.

Several years ago, Šubík *et al.* (27) showed that yeast cells stopped growing if the ATP level in the mitochondrial matrix was decreased by some of the procedures that were used in the present work. The present data provide the molecular correlate to this interesting phenomenon: ATP deficiency of the mitochondrial matrix blocks processing of mitochondrial precursor polypeptides and, as a result, mitochondrial assembly. This in turn suggests that eukaryotic cells require an intact mitochondrial compartment. This compartment is apparently not simply needed for generating ATP because yeast cells will grow on a fermentable carbon source even if they lack a respiratory chain or a proton-translocating F_1-ATPase complex or both (1). Rather, the mitochondrial compartment might be required for essential reactions that are catalyzed by enzymes imported from the cytoplasm.

Our data also raise the possibility that nuclear genes coding for mitochondrial proteins might be repressed if the cytoplasm contains increased levels of precursors to cytoplasmically made mitochondrial proteins. Although this hypothesis is at present purely speculative, it can be tested with presently available experimental techniques.

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