The mitochondrion is a unique cellular organelle containing a distinct and separate genome. However, the DNA contained within mitochondria is limited in size and does not possess sufficient information to encode all mitochondrial proteins. Thus, a great majority of these proteins must be encoded by the nuclear genome, translated in the cytosol, and imported to their final destinations within the mitochondrion (1). This import process of nuclear derived precursor proteins into mitochondria has been well studied in lower eukaryotes such as Neurospora crassa and Saccharomyces cerevisiae (cf. Refs. 2–5 for reviews). Extensive work being conducted on these organisms continues to provide revealing insight into the mechanisms of protein import, as well as the identification of some of the key components involved in precursor-protein translocation. In contrast, limited work has been devoted to the examination of protein import in mammalian mitochondria (6–13), and no studies exist that have investigated the mitochondrial protein import process in mammalian skeletal muscle. This is important for two reasons. First, skeletal muscle has a great capacity for mitochondrial biogenesis in response to physiological stimuli such as thyroid hormone treatment (14) or chronic contractile activity (cf. Refs. 15 and 16 for reviews), and the composition of mitochondria is altered as a result of these treatments (17). These compositional changes include increases in the expression of mitochondrial chaperonins, which are critical components of the mitochondrial import and assembly process (18). Thus, increases in mitochondrial biogenesis and altered composition may be due, in part, to an acceleration of protein import, thereby implicating protein import as a physiologically regulated step in organelle synthesis. Second, skeletal muscle mitochondria consist of two distinct, heterogeneous subfractions, the subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, located in distinct cellular compartments. Each subfraction possesses different protein and lipid compositions as well as differing capacities for mitochondrial respiration and endogenous protein synthesis (19, 20). We have previously reported that the SS mitochondrial subfraction contains a greater cardiolipin content than the IMF subfraction (19). Interestingly, it is now established that mitochondrial phospholipids, particularly cardiolipin, play an early role in the binding of precursor proteins destined for the mitochondrion (21–24). If cardiolipin is uniquely important for import into mammalian mitochondria, the SS subfraction should demonstrate a greater rate of protein import than the IMF mitochondria.

The rate of precursor protein import is also dependent on both external and internal ATP supply (25–27). ATP is involved in the chaperone-mediated release of import-competent precursors to facilitate import- (3, 9, 25) as well as chaperonin (mthsp70, hsp60, and Cpn10) induced translocation and refolding of the mature, processed form of the internalized protein into mitochondria. The mitochondrial import process requires ATP to be delivered to the organelle in a timely manner (26). Inhibitors of mitochondrial respiration (e.g. antimycin A) or ATP synthesis (e.g. oligomycin) significantly decrease import rates of protein precursors into the isolated mitochondrial subfractions (25, 27). This observation relates to the physiological dependence of precursor protein import on ATP availability and highlights the importance of the mitochondrial ATP supply in determining the final mitochondrial phenotype.

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(28, 29). In skeletal muscle, an approximate 3-fold greater rate of ATP synthesis occurs in IMF, compared with SS mitochondria (19). This may be an important determinant of import and could account for some of the observed differences in protein composition between these two subfractions. Thus, the primary purpose of the present study was to establish the measurement of protein import into skeletal muscle IMF and SS mitochondrial subfractions and to examine mechanisms of import related to the abundance of intramitochondrial chaperones, the ATP dependence of import, and the potential role of cardiolipin in the translocation process.

MATERIALS AND METHODS

Mitochondrial Isolation—IMF and SS mitochondrial subfractions were isolated by differential centrifugation following a brief polymer homogenization from whole rat gastrocnemius plantaris muscle as described previously in detail (19, 20), with the exception that the nagarse concentration was reduced from 2.5 to 0.25 mg/ml, wet muscle weight, without sacrificing the yield of IMF mitochondria. IMF and SS mitochondrial subfractions were induced by homogenating of 10 ml HEPES, 0.25 mM sucrose, 2.5 mM potassium phosphate dibasic, 10 mM succinate, 0.21 mM ADP, and 1 mM dithiothreitol (pH 7.4). For import assays, IMF and SS mitochondrial protein concentrations were determined photometrically (A260, Ref. 30), and the concentrations were adjusted to 1 mg/ml.

In Vitro Synthesis of Precursor Proteins—Full-length cDNAs encoding 33-kDa yeast mitochondrial OCT and MDH (pGMDH) were generous gifts from Dr. A. Strauss (Washington University School of Medicine) and Dr. G. Shore (McGill University), respectively. pSP019 DNA was isolated using mini-prep plasmid DNA isolations, followed by RNase treatment (1.0 μg of RNase, 2.5 μg of DNA) for 1 h at room temperature. pSP019 was linearized by SacI, treated with proteinase K (0.1 mg/ml final concentration) for 1 h at 37 °C, and recovered by phenol extraction and ethanol precipitation. pGMDH DNA was isolated using CaCl2 gradient centrifugation and linearized using BamHI. Both linearized plasmids were resuspended in Tris-EDTA (pH 7.8) to a final concentration of 0.8 mg/ml. Transcription reactions were carried out with SP6 RNA polymerase as described by Sambrook et al. (31) for 90 min at 40 °C. Both MDH and OCT mRNAs were isolated by phenol extraction followed by ethanol precipitation, and final concentrations were adjusted to 2 mg/ml. In vitro translation was performed at 30 °C using a cell-free rabbit reticulocyte lysate (Ambion, Texas) in the presence of [35S]methionine (400 Ci/mmol) for 15 min. The intensity of photon emission from the reaction was recorded using a scintillation counter (Packard model 2200) essentially as described previously (31). Counts were recorded 1 min after the addition of extract to the reaction buffer.

Western Blot Analysis of Mitochondrial Chaperones hsp60 and Grp75—Isolated IMF and SS mitochondrial samples were lysed by freezing and thawing twice in liquid N2. Protein concentrations were determined (34), and equal amounts of SS and IMF proteins were added to each lane of a 10% SDS-polyacrylamide gel. Preliminary experiments indicated that 20 μg of SS and IMF mitochondrial proteins were required per lane for the detection of hsp60, whereas 80 μg of each were required for Grp75 detection. Following overnight electrophoresis, gels were then stained with Coomassie blue tetrazolium. The color products were quantified by laser densitometry.

Determination of Mitochondrial Respiratory Rates—Samples of isolated IMF and SS mitochondrial subfractions (0.9–1.7 and 2.3–3.7 mg of IMF and SS, respectively) were incubated with 2 ml of a respiration buffer (250 mM sucrose, 50 mM potassium chloride, 25 mM Tris-HCl, 10 mM potassium phosphate dibasic, pH 7.4) at 30 °C in a respiratory chamber. State 4 and state 3 respiration rates (nanoatoms of oxygen/min/mg of protein) were measured using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). Unless otherwise stated, the final concentrations of respiratory substrates were 35 mM glutamate and 0.12 mM ADP, where indicated, or at concentrations ranging from 0 to 100 μM. The degree of mitochondrial intactness, determined using the addition of 2.5 mM NADH, indicated that IMF and SS mitochondrial subfractions were 82 ± 4% and 92 ± 3% intact, with respiratory control ratios (state 3/state 4 respiration rates) ranging from 5 to 7, similar to our previous findings (19).

Determination of Mitochondrial MDH Activity—Rates of mitochondrial MDH activity in isolated, freeze-thawed mitochondria were kinetically determined based upon the rate of NADH oxidation at 340 nm (35).

Statistics—Paired Student’s t tests (α = 0.05) were used for analysis of enzyme and protein data. A two-factor analysis of variance was used for both the time course and concentration data. A nonlinear least squares first-order kinetics computer analysis was used to calculate the transition half-time values (36) from the import data, as a function of time and mitochondrial protein concentration. All data are reported as means ± S.E.

RESULTS

Criteria for Protein Import Are Fulfilled in Skeletal Muscle IMF and SS Mitochondria—We used well-established import criteria to confirm the measurement of protein import into both IMF and SS skeletal muscle mitochondria. These included membrane intactness, the requirement of a membrane potential, accessibility of the bound precursor protein to proteolytic digestion, and the processing of the precursor protein to a lower molecular weight mature protein (37). A typical import reaction with IMF and SS mitochondrial subfractions is shown in Fig. 6 and 12. An upper band representing the 33-kDa precursor protein and a lower band consisting of imported 33-kDa MDH are evident. With the addition of proteinase K, the higher
molecular weight precursor MDH was digested (lanes 7 and 13), indicating that it represents bound, external precursor protein. The lower band remained intact, indicating that it represents internalized, mature MDH protein protected from the action of the protease. The requirement for an inner membrane potential was evaluated with the use of the respiratory chain uncoupler valinomycin. MDH import was eliminated in mitochondria exposed to valinomycin as evidenced by the lack of a lower 33-kDa band (lanes 2 and 8). Upon the addition of proteinase K following the import incubation, the upper 35-kDa band was also eliminated (lanes 3 and 9). Thus, in the presence of a respiratory chain uncoupler, precursor MDH is only capable of binding to the exterior of the mitochondrion. The requirement for intact mitochondria to allow for import and processing of the precursor is illustrated by the use of the membrane-solubilizing detergent Triton X-100. Preincubation of mitochondria with this agent to disrupt both inner and outer membranes led to limited precursor binding (lanes 4 and 10), which upon exposure to proteinase K resulted in the elimination of the upper band (lanes 5 and 11). This indicates that measurable import and precursor processing requires the normal, undiluted matrix environment as well as an intact membrane system. Both IMF and SS demonstrated very similar qualitative responses to each treatment, indicating that the protein import machinery is fully functional in both mitochondrial subfractions.

**IMF Mitochondria Import Precursor Proteins at a Faster Rate than SS Mitochondria**—The kinetics of protein import were measured as a function of time using equal (40-μg) mitochondrial concentrations for both subfractions. The times to half-maximal import of MDH into IMF and SS mitochondria were similar, ranging between 6.9 and 8.8 min. However, the IMF mitochondria were capable of importing an average of 3.8 ± 0.9-fold more precursor MDH between 4 and 30 min of incubation (Fig. 2A).

To further understand mechanisms of protein import into skeletal muscle mitochondria, studies were conducted using OCT (Fig. 2B), an enzyme that is localized in a tissue-specific fashion within the matrix of liver mitochondria. The existence of similar import kinetics between homologous (MDH) and heterologous (OCT) import systems (38) would provide evidence for a generalizable mechanism of matrix precursor protein import into skeletal muscle mitochondria. Kinetic analyses revealed that IMF and SS mitochondria imported OCT with half-times of 6.7 and 5.3 min, respectively. The capacity of IMF mitochondria to import OCT was approximately 3.3 ± 0.5-fold greater than SS mitochondria between 4 and 30 min (Fig. 2B). The similarity of import kinetics in IMF and SS mitochondria for the two precursor proteins suggests that shared mechanisms within the two mitochondrial subfractions exist for the import of these two proteins. However, the capacity of the mitochondrial subfractions to import OCT was 2–3-fold less than for MDH.

**Precursor Protein Binding to Mitochondrial Subfractions Is Equal When Import Is Inhibited by Valinomycin**—To account for the possibility that the binding of precursor proteins may contribute to the differential rates of import observed between the two subfractions and to obtain a preliminary index of the number of binding sites available in SS and IMF subfractions, mitochondria were pretreated with valinomycin and then incubated with varying amounts of synthesized MDH precursor. Under these conditions of import inhibition, the mitochondria demonstrated binding of 9.1 ± 1.8% and 7.7 ± 2.6% of the total protein available (n = 3 experiments) to the SS and IMF mitochondria, respectively. These data suggest that differences in import between the two subfractions are not a simple consequence of precursor binding to the outer membrane but rather may be related to the efficiency of import brought about...
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Fig. 3. Protein import into IMF and SS mitochondria is markedly reduced by small decrements in external ATP. To evaluate the effect of graded reductions in external ATP on protein import into the two mitochondrial subfractions, the reticulocyte lysate was preincubated with 0–3 units of apyrase (APY). The import data are plotted as a function of the ATP concentration in the lysate resulting from the apyrase treatment (n = 3 experiments). Autoradiogram, import into mitochondrial subfractions under normal (0) or apyrase- (APY) treated conditions. Inset, expression of MDH import (percentage of 0 apyrase) into IMF (●) and SS (○) mitochondria and ATP concentration (×) as a function of apyrase concentration.

SS Mitochondria Possess Greater Quantities of hsp60 and Grp75—Two constituents of the mitochondrial protein import machinery that are vital for the import process are the molecular chaperones hsp60 and Grp75 (mthsp70). To evaluate whether endogenous levels of these were related to the differences in protein import observed, immunoblot analyses of the intramitochondrial content of these chaperones were performed (not shown). Laser densitometric quantification of blots that possessed equal IMF and SS mitochondrial protein per lane indicated that the SS mitochondria contained 1.6 ± 0.3-fold (p < 0.05, n = 8) and 1.7 ± 0.3-fold (p < 0.05, n = 8) higher Grp75 and hsp60 concentrations, respectively, compared with the IMF mitochondria.

Reductions in External ATP Lead to Mitochondrial Subfraction-independent Decreases in Protein Import—To determine whether external ATP influenced protein import equally in both mitochondrial subfractions, ATP was reduced in the reticulocyte lysate by apyrase treatment (32). ATP concentration declined rapidly from 376 to 69 pmol of ATP/μg of lysate protein as the concentration of apyrase was increased from 0 to 0.5 units (Fig. 3). Increases in apyrase to 1.0 and 3.0 units (Fig. 3) did not lead to further significant decreases in ATP. Absolute rates of protein import were most dramatically reduced in IMF mitochondria, and these closely paralleled the decline in external ATP. It is evident that only very small decreases (approximately 7%) in lysate ATP levels were necessary to markedly reduce import in both mitochondrial subfractions. The residual import evident in both SS and IMF mitochondria either represents a fraction of import that is external ATP-independent or reflects the import capacity of these mitochondrial subfractions in the approximate ATP range of 70–350 pmol/μg of lysate protein (Fig. 3). It does not appear to be due to ATP provided by mitochondrial respiration, since a combination of apyrase and atractyloside (to block the export of synthesized ATP) did not lead to a further reduction in import (not shown). Importantly, these data do not appear to indicate a marked difference in the sensitivity of IMF and SS mitochondrial protein import to reductions of external ATP.

IMF and SS Mitochondrial Protein Respond Differently to Altered Rates of Respiration and Internal ATP Levels—We wished to determine if the difference in import rate between IMF and SS mitochondria could be attributed to the discrepant rates of endogenous mitochondrial respiration and ATP availability. In the absence of respiratory inhibitor, mitochondrial state 3 respiration and ATP content were approximately 3.1- and 1.8-fold greater in IMF compared with SS mitochondria, respectively (Fig. 4A, Table I). To examine import under equalized conditions of mitochondrial respiration, we evaluated the response of state 3 mitochondrial respiration to progressive doses of atractyloside (ATR), a competitive inhibitor of the adenine nucleotide translocase. ATR sharply decreased IMF mitochondrial state 3 respiration to a value that was not different from the rate of state 4 (non-ADP-stimulated) respiration at concentrations of ATR ranging between 10 and 100 μM (Fig. 4A). In the presence of 100 μM ATR, intramitochondrial ATP was reduced by 85% in the SS mitochondria and by 88% in the IMF mitochondria (Table I) to values that were not signifi-
significantly different from each other. These dramatic decreases of internal ATP were accompanied by compromised MDH import into the two mitochondrial subfractions, particularly in the SS mitochondria, where import was reduced by 72±3% of the untreated condition (Fig. 4B). MDH import in IMF mitochondria was less affected (p ≤ 0.05), since reductions of only 43±9% were apparent. Thus, IMF mitochondria are capable of maintaining a greater level of protein import under conditions of similar internal ATP levels. However, rates of state 3 respiration were not equalized by 100 μM ATR. Indeed, the approximate 3.5-fold difference in MDH import rate in the presence of 100 μM ATR was accompanied by a 2.4-fold difference in state 3 respiration. In contrast, when we measured MDH import into IMF and SS mitochondria during matching conditions of state 3 respiration, very similar rates of import were observed (compare untreated SS with IMF in the presence of 100 μM ATR; Fig. 4B).

Internal mitochondrial ATP content and respiratory rate were also manipulated using the specific F1-ATPase inhibitor oligomycin. In the presence of 20 μM oligomycin, IMF mitochondrial respiration was reduced to 132.7±11.1 nanoatoms/mg of protein/min, similar to that with 100 μM ATR. However, MDH protein import was more severely impaired compared with ATR treatment (Fig. 4B), despite slightly higher ATP levels (Table I). SS mitochondrial respiration was also reduced by oligomycin to values similar to that found with 100 μM ATR, yet in contrast to IMF mitochondria, import was not different from that found in the presence with ATR (Fig. 4B). Despite these variations in agent-specific effects, a close relationship between mitochondrial respiration and protein import is evident from a compilation of these data (Fig. 4C). These data strongly suggest the existence of a closer relationship between protein import and mitochondrial respiration than between import and absolute internal ATP concentrations.

**Adriamycin Blocks Protein Import into Subsarcolemmal and Intermyofibrillar Mitochondria**—To further understand the role of phospholipids in the import process, isolated SS and IMF mitochondria were pretreated with adriamycin. Incubation of both the SS and IMF subfractions with adriamycin reduced protein import in both mitochondrial subfractions (Fig. 5). MDH import into the SS mitochondria was significantly reduced at 180 μM adriamycin (p ≤ 0.05, n = 10), with no further reduction evident at 360 μM (67±7% of untreated). The rate of MDH import into IMF mitochondria was less sensitive to adriamycin, since significant (p ≤ 0.05, n = 10) reductions in protein import into IMF mitochondria did not occur until 360 μM adriamycin was used, and the reduction (to 78±5% of untreated) was less than for the SS mitochondria.

**MDH Enzyme Activity Differences between Mitochondrial Subfractions**—The activity of MDH was determined in isolated mitochondrial subfractions. Activities were 6.6±0.6 and 13.8±1.3 units/mg of mitochondrial protein in the SS and IMF subfractions, respectively, representing a 2.2-fold difference between the two subfractions.

**DISCUSSION**

Morphological, biochemical, and functional investigations of mitochondria have clearly established the existence of organelar heterogeneity in a variety of tissues, including brain (39), liver (40–43), heart (44–51), and skeletal muscle (19, 52–54). Studies of skeletal muscle subject to conditions of chronic use or disuse have indicated that SS mitochondria are synthesized or degraded in more dramatic fashion than IMF mitochondria (20, 55–57). This heterogeneous response of the two mitochondrial subfractions implies that a different pattern or regulation of mitochondrial biogenesis exists in distinct regions of the muscle cell, perhaps influenced by surrounding nuclear domains (58) or the proximity to environmental factors (e.g., PO2; Ref. 59). Since most of the proteins within mitochondria are derived from the nuclear genome and imported into the organelle, we wanted to establish whether the import process could be responsible for a portion of the specific phenotypic differences between the IMF and SS mitochondria.

Our study is the first to report on the kinetics and characteristics of the protein import pathway in skeletal muscle mitochondria. As expected, the import processes in these mitochondria have similar characteristics (dependence on membrane potential, requirement for an intact inner membrane, proteolytic processing of precursors, and accessibility of bound, external precursor proteins to trypsin digestion) to those observed in other widely studied cellular systems (i.e., N. crassa and S. cerevisiae) as well as those found in commonly studied mammalian tissues such as liver and heart. Importantly, we have documented that mitochondria obtained from different cellular regions demonstrate remarkably divergent import kinetics, which can, in part, account for the phenotypic differences observed. For example, the 2-fold greater MDH enzyme activity found in IMF mitochondria is likely attributable in part to the 3–4-fold higher rate of MDH precursor protein import in this mitochondrial subfraction. Additional studies will address the role that endogenous proteolysis plays, relative to the rate of import, in determining the final concentrations of proteins within mitochondria.

We have also defined some of the factors that are important in determining the differential rate of import into the IMF and SS mitochondria. Since the extent of precursor binding is not different between the two subfractions, we examined other, more specific components of the import machinery. Previous work in other cellular systems has shown the essentiality of the molecular chaperone hsp60 for cell survival (60) and protein refolding (29) and the role of mitochondrial processing (20) (Grp75) acting as an ATP-dependent import “motor” in drawing the precursor into

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**TABLE I**

|                      | Un-treated | Atractyloside (100 μM) | Oligomycin (20 μM) |
|----------------------|------------|------------------------|--------------------|
| SS mitochondria      | 85.8±5.3   | 13.2±1.2 (15%)         | 26.2±5.7 (31%)     |
| IMF mitochondria     | 152.6±8.3  | 18.0±0.8 (12%)         | 29.5±4.0 (19%)     |

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**FIG. 5. Effect of adriamycin (Adr) on MDH import into SS and IMF mitochondria.** IMF and SS mitochondria were preincubated with 180 μM (lanes 3 and 7) or 360 μM (lanes 5 and 9) adriamycin or the appropriate volume of vehicle (mitochondrial resuspension medium; lanes 2, 4, 6, and 8), followed by the MDH import reaction. TL, 10 μM of reticulocyte lysate containing radiolabeled MDH precursor protein.
the matrix space during the initial import steps (61). We show that both of these chaperones are present in muscle mitochondrial subfractions but that the levels of these are not directly related to the magnitude of MDH or OCT import. Both hsps60 and Grp75 are found in greater abundance in SS mitochondria, despite lower rates of protein import compared with IMF mitochondria. These data suggest that mthsps70 may be differentially involved in the import process within the two mitochondrial subfractions. Further, although it appears that hsps60 is involved in the refolding of MDH into a mature, catalytically competent enzyme (62), our data indicate that the level of hsps60 is not a likely factor regulating the difference in MDH enzyme activity between SS and IMF mitochondria.

Of greater importance in the regulation of protein import into muscle mitochondrial subfractions is the content of acidic phospholipids such as cardiolipin. Cardiolipin has been implicated in the preliminary binding of precursors and as a mediator of conformational changes in the presequences via electrostatic interactions (22, 23, 63–67). Studies in yeast mitochondria have indicated that blockage of acidic phospholipid-precursor interactions with the drug adriamycin has the potential to inhibit protein import (21). In the present study we have taken advantage of the naturally occurring 60% higher cardiolipin content in SS, compared with IMF, mitochondria (19) to evaluate the role of phospholipids in the import process. Our data indicate a greater reliance of SS mitochondria on acidic phospholipids such as cardiolipin for import; they establish the importance of precursor-phospholipid interactions for protein import in subtypes of mammalian mitochondria; and they represent another indication that the regulation of import in these two mitochondrial subfractions differs. These data also have implications for mitochondrial biogenesis, since we have previously shown that large and rapid increases in cardiolipin content occur in muscle responding to chronic contractile activity (36). Thus, an initial increase in cardiolipin content within the organelle could facilitate the subsequent import of proteins, particularly in SS mitochondria. This may represent an important mechanism responsible for the rapid increases in the content of SS mitochondria responding to contractile activity signals (20, 55, 56).

It is well established in other cellular systems that internally derived ATP is essential for the import of matrix and inner membrane proteins (25–27). However, it is unknown whether it is the absolute level of ATP or the rate of ATP production as determined by mitochondrial respiration that is most important for the import process. In addition, we wanted to determine whether the discrepant rates of protein import into IMF and SS mitochondrial subfractions could be equalized by modifying the respiration rate. The approximate 3-fold difference in import rate between IMF and SS mitochondria was reasonably well matched with the difference in endogenous, steady state ATP concentrations as well as differences in state 3 respiratory rates. However, import remained about 5-fold higher in IMF mitochondria when ATP levels were reduced to comparably low values in SS and IMF mitochondria by preincubation with ATR. IMF import in the presence of 100 μM ATR was very similar to that found in untreated SS mitochondria, in which respiration in the two subfractions was the same. In the presence of oligomycin a similar dissociation between ATP levels and import was found. These data support the contention that precursor import is more closely related to the rate of ATP production (Fig. 4C) rather than the steady state amount of ATP available (Table I). The ATP formed by respiration could be used internally to drive the mthsp70-mediated translocation of the precursor into the matrix (68). Alternatively, the ATP could be extruded by the adenine nucleotide translocase to the cytoplasm and immediately coupled to an ATP-dependent chaperone-mediated release reaction, thus permitting import of the precursor, as proposed for the function of mitochondrial import stimulation factor (9). This hypothesis remains to be tested, but it is a concept similar to that described for the metabolic coupling of ATP to hexokinase (69) or mitochondrial creatine kinase (70) in muscle and liver cells.

Previous studies have characterized the import of OCT and MDH into mitochondria (6, 10, 38, 71–73). Our data confirm and extend those obtained in other tissues, and indicate that the capacities of IMF and SS mitochondrial subfractions for MDH import are greater than those of OCT. Differences in the in vitro import of matrix-destined proteins have been noted previously (10, 38). In the case of MDH and OCT import, this is not likely, due to large variations in their import pathways, since competition assays with isolated presequence peptides indicate that they share some common steps in the import process (67, 74). The differences observed are more likely associated with 1) the amino acid compositions of the amino-terminal presequences and interactions with membrane anionic phospholipids, 2) the affinity of the presequences for protein factors (9) located in the reticulocyte lysate that promote import, or 3) differences in the targeting signals inherent to the mature portion of these proteins (75–77).

In summary, our results indicate that mitochondrial subfraction differences located in different cellular regions can derive at least a portion of their compositional and functional heterogeneity by differential regulation of the protein import pathway. Our current work is focusing on potential alterations in this pathway during conditions of mitochondrial biogenesis in skeletal muscle with the goal of identifying whether or not import could represent a potential rate-limiting step in the gene expression pathway of nuclear encoded proteins destined for mitochondrial compartments.

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