An Unusual Mitochondrial Import Pathway for the Precursor to Yeast Cytochrome c Oxidase Subunit Va

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Abstract. We have studied the import of the precursor to yeast cytochrome c oxidase subunit Va, a protein of the mitochondrial inner membrane. Like the majority of mitochondrial precursor proteins studied thus far, import of presubunit Va was dependent upon both a membrane potential ($\Delta\psi$) and the hydrolysis of ATP. However, the levels of ATP necessary for the import of presubunit Va were significantly lower than those required for the import of a different mitochondrial precursor protein, the β subunit of the F_1-ATPase. The rate of import of presubunit Va was found to be unaffected by temperature over the range 0 to 30°C, and was not facilitated by prior denaturation of the protein. These results, in conjunction with those of an earlier study demonstrating that presubunit Va could be efficiently targeted to mitochondria with minimal presequences, suggest that the subunit Va precursor normally exists in a loosely folded conformation. Presubunit Va could also be imported into mitochondria that had been pretreated with high concentrations of trypsin or proteinase K (1 mg/ml and 200 μg/ml, respectively). Furthermore, the rate of import into trypsin-treated mitochondria, at both 0 and 30°C, was identical to that observed with the untreated organelles. Thus, import of presubunit Va is not dependent upon the function of a protease-sensitive surface receptor. When taken together, the results of this study suggest that presubunit Va follows an unusual import pathway. While this pathway uses several well-established translocation steps, in its entirety it is distinct from either the receptor-independent pathway used by apocytochrome c, or the more general pathway used by a majority of mitochondrial precursor proteins.

THE vast majority of the proteins that constitute a functional mitochondrion are the products of nuclear genes that are targeted to the organelle after translation in the cytosol. The import of proteins into mitochondria represents, therefore, a fundamental problem in mitochondrial biogenesis as well as in cellular protein trafficking.

In recent years, a great deal of experimental effort has been focused on understanding the mechanism of protein import into mitochondria. From these studies a reasonably clear picture of several aspects of the import process has emerged (for recent reviews see Attardi and Schatz, 1988; Hartl et al., 1989; Hartl and Neupert, 1990). Most mitochondrial proteins are initially synthesized as precursors containing an NH₂-terminal extension called a presequence or leader peptide. It is now well-established that this peptide is necessary, and often sufficient, for correct mitochondrial delivery (Hurt et al., 1984, 1985; Horwich et al., 1985). The initial interaction between precursor proteins and mitochondria appears to be mediated first, by receptor-like components at the mitochondrial surface (Pfaller et al., 1988, Söllner et al., 1989, 1990), and second, by at least one protein of the outer membrane which has been referred to as the general insertion protein (GIP)[1] (Pfaller et al., 1988). It has been suggested that

GIP is a 38-kD protein in *Neurospora crassa* (MOM 38; Hartl and Neupert, 1990), and a 42-kD protein (ISP 42) in *Saccharomyces cerevisiae* (Vestweber et al., 1989; Hartl and Neupert, 1990). It should be noted, however, that the precise role of the presequence in the interaction with either the surface receptors or other outer membrane proteins (GIP) is not yet clear.

There is considerable evidence that the actual transfer of precursor proteins into intact mitochondria, excepting only proteins destined for the outer membrane and apocytochrome c, occurs at contact sites between the inner and outer mitochondrial membranes (Schwaiger et al., 1987; Pfanner et al., 1988a; Vestweber and Schatz, 1988; Pon et al., 1989; Skerjanc et al., 1990). Translocation also requires that precursor proteins adopt a specific conformation, which consists of a loose or unfolded state (Chen and Douglas, 1987b, Eilers et al., 1987, 1988; Pfanner et al., 1987; Pfanner et al., 1988b; Skerjanc et al., 1990). The ability of a given precursor to achieve and/or maintain this so-called "import-competent" conformation appears to be dependent upon ATP, and is a function of hsp70 and possibly other chaperone proteins located in the cytosol (Deshaies et al., 1988; Murakami et al., 1988). There is also evidence that acidic phospholipids in the mitochondrial outer membrane may facilitate both unfolding and import (Eilers et al., 1989; Endo et al., 1989). The hydrolysis of ATP and (again with the exception of proteins of the outer membrane) the potential across the mito-

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[1]. *Abbreviations used in this paper:* AAC, ADP/ATP carrier; DHFR, dihydrofolate reductase; GIP, general insertion protein; STI, soybean trypsin inhibitor.

chondrial inner membrane ($\Delta\psi$) provide the energy necessary to drive import (Gasser et al., 1982; Schleyer et al., 1982; Pfanner and Neupert, 1985, 1986; Chen and Douglas, 1987a; Eilers et al., 1987).

The latter stages of the import process involve cleavage of the presequence, routing (sorting) of the protein to the proper intramitochondrial compartment, and refolding and assembly of the mature protein. Cleavage is accomplished by a now well-characterized metalloprotease located in the matrix space (Hawlitshek et al., 1988; Jensen and Yaffe, 1988), while refolding and assembly of several proteins involves a matrix-localized hsp60 complex (Cheng et al., 1989; Ostermann et al., 1989). However, the means by which proteins are sorted to specific mitochondrial compartments and the molecular signals that mediate the sorting process are, at present, poorly understood.

To more precisely understand how proteins are routed to the mitochondrial inner membrane we are analyzing the import of a well-studied inner membrane protein, subunit Va of yeast cytochrome c oxidase (Cumsky et al., 1985, 1987). Previously, we have shown that this protein is imported with unusual efficiency. It can be properly targeted to mitochondria with very short presequences (one as short as five amino acids) and with heterologous presequences that fail to target their cognate proteins (Glaser et al., 1988, 1990). Furthermore, we have shown that a hydrophobic stretch of amino acids in the COOH-terminal third of the protein is necessary for proper inner membrane localization of subunit Va; deletion of this region causes mislocalization of the protein to the mitochondrial matrix (Glaser et al., 1990).

In the present work, we have continued to examine the import of presubunit Va. We show that like the majority of mitochondrial precursor proteins studied, presubunit Va required the hydrolysis of ATP and a membrane potential for import. However, the levels of ATP required for import of subunit Va were lower than those required to import another mitochondrial protein, the β subunit of the F₁ ATPase (F₁β). Presubunit Va could also be imported into mitochondria at temperatures as low as 0°C, and import was not facilitated by denaturation of the precursor with urea. Furthermore, the precursor was efficiently imported into mitochondria that had been pretreated with high concentrations of trypsin or proteinase K. When taken together, these results support those of our previous studies, and strongly suggest that presubunit Va is not tightly folded in solution. They also suggest that a protease-sensitive surface receptor is not required for import of presubunit Va.

Materials and Methods

In Vitro Transcription and Translation

The plasmid pT7/Va was used to generate subunit Va mRNA in vitro. The construction of this vector has been described previously (Glaser et al., 1990). mRNA specifying the β subunit of the yeast F₁ ATPase (the product of the ATP2 gene) was synthesized in vitro from the plasmid pSP64-βwt, a generous gift of Drs. David Bedwell and Scott Emr (California Institute of Technology). In vitro transcription reactions were performed as described (Glaser et al., 1990).

To construct a fusion between subunit Va and mouse dihydrofolate reductase (DHFR), the plasmid pT7-Va was first linearized at the unique Bal I site (Glaser et al., 1990). A Bam HI-Eco RI fragment, derived from the plasmid pDS5/2 and encoding DHFR (Hurt et al., 1984), was then introduced using a synthetic Bgl II linker. The resulting plasmid, pT7-Va(1-118)/DHFR, directs the synthesis of a 318-amino acid fusion protein comprised of the

NH₂-terminal 118 amino acids of presubunit Va followed by the entire mouse DHFR sequence. Previous studies have shown that the subunit Va moiety, which is truncated by 35 amino acids at the COOH terminus, is efficiently imported and properly localized within isolated mitochondria (Glaser et al., 1990). The junction between the presubunit Va and DHFR sequences contains an additional arginine residue introduced by the linker.

RNA preparations were translated in vitro in the presence of [³⁵S]methionine using rabbit reticulocyte lysates purchased from Promega Biotec (Madison, WA) or Amersham Corp. (Arlington Heights, IL). Translation reactions were used immediately or stored frozen in aliquots at −70°C. In cases where the translations were stored, they were routinely centrifuged at 16,000 g for 30 min (at 4°C) before being used in in vitro import reactions. This procedure was necessary to remove aggregates of presubunit Va (bound to particulate matter in the lysate) which accumulate with storage of the translations. These aggregates are not efficiently imported unless dissociated first with urea (Miller, B. R., unpublished results).

In Vitro Import Reactions

Mitochondria were prepared from S. cerevisiae strain D273-10B (ATCC 24657) which had been grown to mid-log phase in YPGE medium (Sherman et al., 1986). The isolation procedure was essentially that of Daum et al. (Daum et al., 1982), using the modifications previously described (Glaser and Cumsky, 1990a,b).

In vitro import reactions (100 μl vol) were performed in TRB buffer (250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 20 mM 3-(N)-morpholino)-propanesulfonic acid [MOPS], pH 7.2, 3% BSA, 1 mM ATP, and 10 mM each K⁺-succinate and K⁺-malate). Each reaction contained 50 μg mitochondrial protein and 4-15,000 cpm radiolabeled precursor in reticulocyte lysate. Reactions were allowed to proceed for 10-30 min at the temperatures indicated in the figure legends, then terminated by the addition of valinomycin to 10 μg/ml. When protease sensitivity was to be determined, proteinase K was added to 200 μg/ml and the reactions digested for 30 min at 0°C. The protease digestions were terminated by adding PMSF to a concentration of 1 mM. Mitochondria were collected by centrifugation and prepared for SDS-PAGE as described (Glaser and Cumsky, 1990a; Glaser et al., 1990).

Preparation of mitoplasts and further fractionation of mitochondria was accomplished as described previously (Glaser and Cumsky, 1990b; Glaser et al., 1990). In the case of mitoplasts, control experiments using selected marker proteins demonstrated that the procedure resulted in release of the soluble components of the intermembrane space but retention of most of the outer membrane. As described in Results, the generation of the "m'" form of subunit Va also served as an internal control for the conversion of mitochondria to mitoplasts.

ATP Depletion

In vitro import reactions were performed under ATP-limiting conditions in two different ways. In the first, 25 μl of a postribosomal supernatant of reticulocyte lysate containing radiolabeled presubunit Va was diluted to 100 μl with H₂O (the postribosomal supernatant was generated by centrifuging an in vitro translation reaction containing radiolabeled presubunit Va for 60 min at 100,000 g). To remove ATP from the lysate, the entire mixture was then passed over two successive Sephadex G-25 spun columns (1 ml bed vol) that had been equilibrated with H₂O. Portions of the ATP-depleted lysate were then used in import reactions with mitochondria (in TRB lacking ATP) that had been pretreated with 10 units per ml of potato Apyrase (Grade VIII; Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. In certain cases, ATP was then added back to the reaction at a final concentration of 2 mM.

ATP was also depleted by adding 10 U of hexokinase per ml and 10 mM glucose to in vitro import reactions performed in TRB that lacked exogenously added ATP. When glucose and hexokinase were used the mitochondria were not pretreated with apyrase; the reticulocyte lysate used for the import reaction was, however, a postribosomal supernatant generated as described above.

Trypsin Pretreatment of Mitochondria

Mitochondria were suspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) at a concentration of 10 mg/ml and incubated for 10 min on ice. Trypsin (TPCK treated; Sigma Chemical Co.) was added to the concentrations indicated in the appropriate figure legend from a 10 mg/ml stock solution prepared in SEM. The mitochondria were

digested for 30 min on ice and the reactions terminated by the addition of 5 vol soybean trypsin inhibitor (STI) from a 20 mg/ml stock solution in SEM. After a further 10-min incubation on ice, mitochondria were reisolated by centrifugation at 10,000 g and resuspended in TRB supplemented with STI at 0.1 mg/ml. Mock-treated mitochondria were handled identically, except that the trypsin was not added to the buffer.

Proteinase K pretreatment of mitochondria was accomplished by adding proteinase K to 200 μg/ml from a 10 mg/ml stock solution in SEM. Digestions were allowed to proceed for 30 min at 0°C, then stopped by the addition of PMSF to 1 mM.

Urea Denaturation of Precursor Proteins

Presubunit Va was denatured by dilution of labeled reticulocyte lysate with 5 vol of 10 M urea. 1 μl or less (enough to give the required number of counts per minutes per reaction) was then added to an in vitro import reaction.

Miscellaneous Procedures

SDS-PAGE, fluorography, and densitometric analysis of fluorographed bands was performed as described previously (Glaser and Cumsky, 1990a).

Results

Mitochondrial Import of the Precursor to Yeast Cytochrome c Oxidase Subunit Va Requires a Membrane Potential and ATP

In recent years the use of a powerful in vitro assay (Gasser et al., 1982) has permitted a biochemical dissection of the process of protein import into mitochondria. Distinct steps on the import pathways of several different precursor proteins have been defined by trapping intermediates at different stages during the translocation process (Pfanner and Neupert, 1987; Pfanner et al., 1988; Hartl et al., 1989). To more thoroughly understand the pathway(s) by which proteins are routed to the inner mitochondrial membrane, we have begun to analyze the conditions required for efficient import of yeast cytochrome c oxidase presubunit Va using the in vitro assay.

In a previous study we demonstrated that the import of presubunit Va, as well as of several subunit Va derivatives lacking an internal localization signal, could be completely blocked by the K$^+$ ionophore valinomycin (Glaser et al., 1990; also shown in Fig. 5, below). Therefore, it is clear that the import of subunit Va is dependent upon a membrane potential (the $\Delta\psi$); in the absence of a potential the precursor remains outside mitochondria and is susceptible to complete digestion by even very low concentrations (5 μg/ml) of externally added proteinase K (not shown).

To determine whether ATP was also required for import, reticulocyte lysate containing radiolabeled presubunit Va was first depleted of ATP by gel filtration (see Materials and Methods), then diluted into import buffer (TRB) that lacked exogenously added ATP. The depleted lysate containing the precursor was added to mitochondria that had been pretreated with apyrase (an adenosine 5' di- and triphosphatase) to make sure that the intramitochondrial pools of adenine nucleotides were low (Hwang and Schatz, 1989). As shown in Fig. 1 A, this procedure effectively blocked the import of presubunit Va (column 3), but the block could be overcome by the readdition of ATP to the reaction (column 4; the removal or inactivation of apyrase is not necessary in this case since import of the precursor under these conditions is extremely rapid, see below). Thus, ATP is required for the import of presubunit Va.

In a separate experiment, ATP was again depleted from reticulocyte lysates containing radiolabeled presubunit Va. Here however, we used glucose and hexokinase, rather than gel filtration, to remove the nucleotide. When lysates depleted of ATP by this procedure were added to mitochondria that had not been pretreated with apyrase, we found that subunit Va was still imported at near wild-type levels (Fig. 1 B, columns 1 and 2). This was the case even if carboxyatractyloside was present in the reaction (data not shown; carboxyatractyloside is an inhibitor of adenine nucleotide exchange across the mitochondrial inner membrane via its
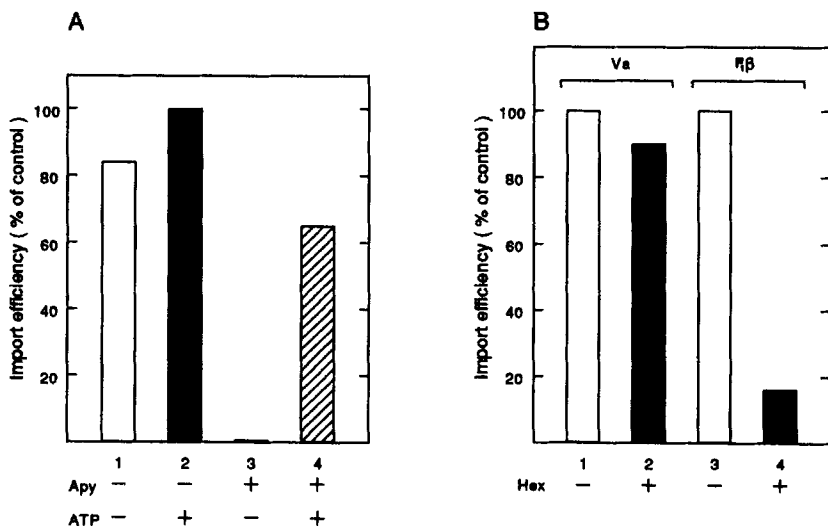
A

B

Figure 1. Import of yeast cytochrome c oxidase presubunit Va requires ATP. (a) Endogenous ATP was removed from a postribosomal supernatant of reticulocyte lysate containing radiolabeled presubunit Va by gel filtration (Materials and Methods). Approximately 1 μl of this lysate, containing 0.5–1.5 × 10^4 cpm of the radiolabeled precursor, was added to 50 μg mitochondria that had been pretreated with 10 U/ml apyrase (+ Apy) or left untreated (− Apy). Import reactions were carried out for 30 min at 30°C in 100 μl TRB which either lacked (columns 1 and 3) or was supplemented with 2 mM ATP (columns 2 and 4). The reactions were terminated with valinomycin, treated with 200 μg/ml proteinase K, and analyzed by SDS-PAGE and fluorography as described in Materials and Methods. The fluorographs were quantitated by densitometry, and import efficiencies calculated as the amount proteinase

K protected mature subunit Va relative to the control reaction (column 2). (b) Reticulocyte lysate containing 0.5–1.5 × 10^4 cpm of either radiolabeled presubunit Va (Va) or pre-F$_1\beta$ (F$_1\beta$), was supplemented with 10 mM glucose and 10 units per ml hexokinase (Hex) and added to 50 μg untreated mitochondria in TRB that lacked exogenously added ATP. The reactions (total vol 100 μl) were allowed to proceed for 30 min at 30°C, and the products analyzed after proteinase K digestion as described above. Import efficiencies are expressed as the percentage of protease protected mature protein relative to the untreated (−Hex) reactions in each case (lane 1 for Va and lane 3 for F$_1\beta$).
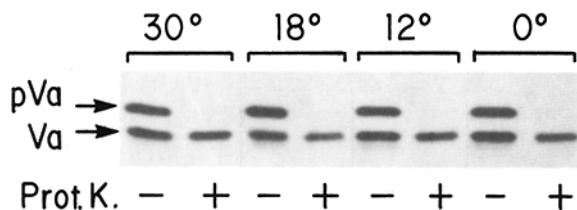
Figure 2. Import of presubunit Va is insensitive to temperature. Duplicate import reactions containing 1.5×10^4 cpm radiolabeled presubunit Va and 50 μg mitochondria in complete TRB (100 μl total vol) were performed at the indicated temperatures, taking care to ensure that the temperatures remained constant throughout the experiment. In the case of the 0°C sample, the reactions were performed on ice. After 10 min, the reactions were terminated by the addition of valinomycin to 10 μg/ml, and one of each pair of reactions treated with 200 μg proteinase K per ml for 30 min at 0°C. After the addition of PMSF to 1 mM, the reaction products were analyzed by SDS-PAGE and fluorography. The position of presubunit Va (*pVa*) and the mature subunit (*Va*) are indicated.

interaction with the ADP/ATP carrier [AAC]). However, when a different mitochondrial protein, the precursor to the β subunit of the F_1-ATPase (pre-$F_1\beta$), was analyzed in a similar manner, we found that import was almost completely blocked (Fig. 1 *B*, columns *3* and *4*). These results suggest that significantly less ATP is required for the import of presubunit Va than for the import of pre-$F_1\beta$. In addition, the results suggest, but do not prove, that ATP hydrolysis outside of the mitochondrion is not required for the import of presubunit Va.

Import of Presubunit Va Is Not Affected by Temperature

The import of presubunit Va was examined at a series of different temperatures, ranging from 30°C (the standard temperature) to 0°C (the reactions were performed on ice). Since temperatures lower than 20°C usually prevent (reversibly) the complete transfer of precursor proteins across the mitochondrial membranes, experiments of this nature have been used successfully to "trap" intermediates in the process of translocation (Schleyer and Neupert, 1985; Pfanner and Neupert, 1987; Eilers et al., 1988). As shown in Fig. 2, the import of presubunit Va did not appear to be affected by the temperature: the extent of import and processing of presubunit Va was virtually identical in each case. This result was in sharp contrast to what we and others observed for a control precursor, $F_1\beta$. As shown in Fig. 3, the import of pre-$F_1\beta$ was completely blocked at 0°C.

The results of the experiment presented in Fig. 2 suggested that the amount of subunit Va imported over a 20-min period was independent of the temperature at which the reactions were performed. However, the results could not rule out the possibility that the temperature affected the initial rate (but not the overall extent) of the subunit Va imported. Therefore, we examined the rate of import of presubunit Va at the two extreme temperatures, 30 and 0°C. The results of that experiment (*open circles* in Figs. 4, *A* and *B*) showed clearly that presubunit Va was imported at essentially the same rate at both temperatures. Thus, we conclude that the temperature does not affect the import of presubunit Va.

The results of the experiments shown in Figs. 1, 2, and 4

are not typical of what has been observed for several other mitochondrial precursor proteins whose import has been studied in detail. However, the results are consistent with the view that in solution the subunit Va precursor is not tightly folded; in essence, presubunit Va behaves like a denatured protein. Denatured proteins are taken up by mitochondria more rapidly, efficiently, and at lower temperatures than their native counterparts (Eilers et al., 1988; Pfanner et al., 1988*b*), presumably because the unfolding required to achieve import competence is the rate limiting step in import (Eilers et al., 1988). The observation that pre-subunit Va required only low levels of ATP for import is consistent with this interpretation. Denatured or loosely folded proteins would not be expected to require the extensive hydrolysis of ATP outside the mitochondrion (Fig. 1) needed for unfolding and release from cytosolic hsp70 (Hartl and Neupert, 1990).

A prediction of the above model is that if presubunit Va is not tightly folded, pretreatment of the precursor with urea should not enhance its ability to be imported into mitochondria if ATP is present in the reaction. As shown in Fig. 5, we found that to be the case. While both the native and urea-denatured forms of the precursor required the Δψ for import (columns *2* and *4*), the same amount of each was imported into mitochondria over a short incubation period (5 min; columns *1* and *3*). We note that any significant change in the import efficiency of presubunit Va would be evident during these 5-min reactions (see Fig. 4 *A*, *open circles*).

Finally, it is noteworthy that two additional lines of experimental evidence also support the view that presubunit Va is loosely folded. First, as we have shown previously, subunit Va requires only minimal presequences for correct mitochondrial delivery (Glaser et al., 1988, 1990). Second, both the precursor and mature forms of subunit Va are extremely sensitive to proteases. Each was completely degraded by very low concentrations (0.5–1 μg/ml) of trypsin in 5 min at 4°C, and the urea-denatured and native forms of each protein showed the same protease sensitivity (Miller, B. R., and J. Foreman, unpublished results).

Import of Presubunit Va Is Not Dependent upon a Protease-sensitive Surface Receptor

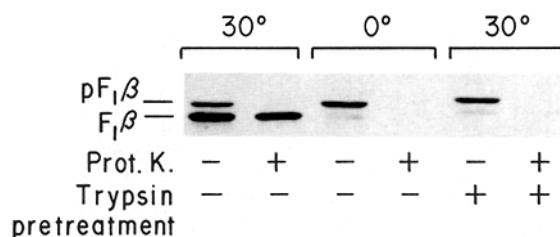Receptor-like proteins mediate the initial interaction be-

Figure 3. Import of pre-$F_1\beta$ is sensitive to temperature and trypsin pretreatment of the mitochondria. Duplicate import reactions containing 1.5×10^4 cpm radiolabeled pre-$F_1\beta$ and 50 μg mitochondria in complete TRB (100 μl vol) were performed at the indicated temperatures for 30 min, then terminated and the products analyzed as described in the legend to Fig. 2. One pair of reactions (as indicated) was performed on mitochondria that had been pretreated with 1 mg/ml trypsin as described in Materials and Methods. The positions of the precursor (pF$_1\beta$) and mature forms (F$_1\beta$) of the polypeptide are indicated.
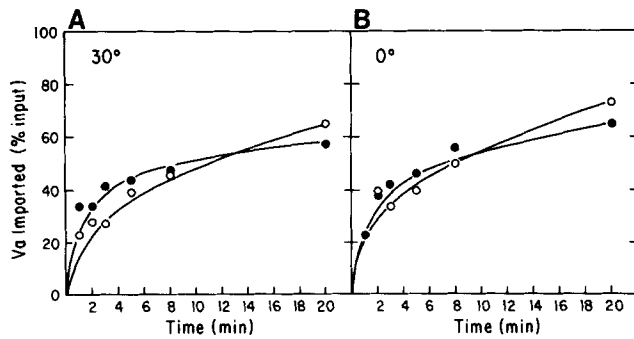
Figure 4. Rate of import of presubunit Va. (*a*) Mitochondria were pretreated with 1 mg/ml trypsin for 30 min on ice, or mock-treated as described in Materials and Methods. Import reactions containing 1.5 × 10⁴ cpm radiolabeled presubunit Va and 50 μg of either trypsin-treated (*closed circles*) or mock-treated (*open circles*) mitochondria were performed at 30°C in TRB supplemented with 0.1 mg/ml STI (100 μl total vol). At the indicated times individual reactions were terminated by the addition of valinomycin to 10 μg/ml, digested with proteinase K, and analyzed as described in the legend to Fig. 2. The data are expressed as the percentage of added presubunit Va converted into proteinase K-protected mature subunit Va, as determined by densitometric analysis of the fluorograph. (*b*) As in *a*, except that the import reactions were performed at 0°C.
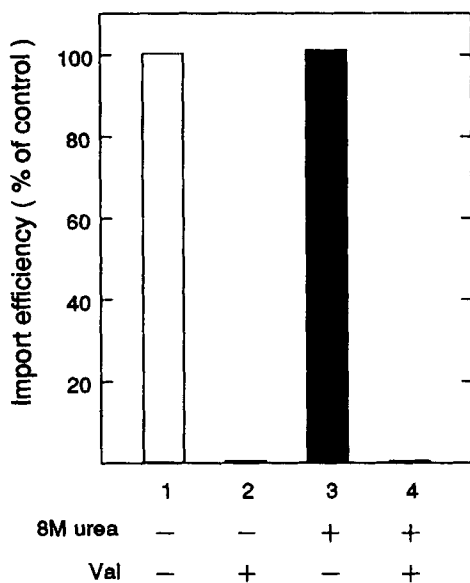

Figure 5. Denaturation of presubunit Va does not increase its import efficiency. Radiolabeled presubunit Va (in reticulocyte lysate) was either denatured by dilution into 8 M urea (Materials and Methods) or diluted similarly into water. Approximately 1 μl of each diluted lysate (containing 1.0 × 10⁴ cpm presubunit Va) was then added to 50 μg mitochondria in TRB (reactions shown in columns *1* and *3*), or to 50 μg mitochondria in TRB plus 10 μg/ml valinomycin (reactions shown in columns *2* and *4*). All reactions were allowed to proceed for 5 min at 30°C, stopped by the addition of valinomycin to 10 μg/ml (reactions *1* and *3*) and digested with proteinase K. The samples were then analyzed by SDS-PAGE and fluorography, and quantitated by densitometry. Import efficiency is expressed as the amount of proteinase K protected mature subunit Va relative to the control reaction (column *1*).
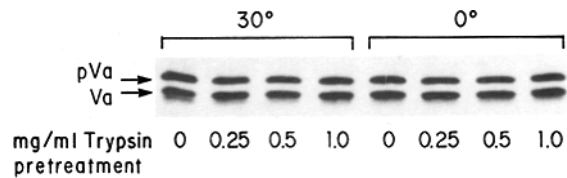

Figure 6. Trypsin pretreatment of mitochondria does not alter the import of presubunit Va. 50-μg samples of mitochondria that had been pretreated with the indicated concentrations of trypsin (pretreatment was as described in Materials and Methods) were assayed for their ability to import presubunit Va at either 30°C or 0°C. Each reaction contained 1 × 10⁴ cpm radiolabeled presubunit Va in TRB, and were allowed to proceed for 30 min at either temperature. The reactions were stopped by the addition of valinomycin, then analyzed directly by SDS-PAGE and fluorography without proteinase K digestion. The position of the precursor (*pVa*) and mature forms of subunit Va (*Va*) are indicated.

tween many precursor proteins and the mitochondrial surface (Pfaller et al., 1988, Söllner et al., 1989, 1990). To determine whether the import of presubunit Va was dependent upon the function of such proteins, import and processing of the precursor was assayed after pretreating mitochondria with trypsin. This procedure has been shown to abolish the ability of mitochondria to import several different precursor proteins, including $F_1\beta$, the ATP/ADP carrier (AAC), subunit IV of cytochrome *c* oxidase, a subunit IV-dihydrofolate reductase fusion protein, subunit 9 of the F_o ATPase, and porin (Ohba and Schatz, 1987; Pfaller et al., 1988, Söllner et al., 1990). As shown in Fig. 6, pretreatment of mitochondria with trypsin at concentrations as high as 1 mg/ml had no obvious effect on the import of presubunit Va, although as expected, a similar pretreatment completely blocked the import of pre-$F_1\beta$ (Fig. 3). As a more definitive test, the rate of import of presubunit Va, at both 30 and 0°C, was determined with mitochondria that had been pretreated with 1 mg/ml trypsin. The results presented in Figs. 4, *A* and *B* (*closed circles*) demonstrate that the trypsin pretreatment did not affect the rate of import at either temperature.

Presubunit Va could also be imported into mitochondria that had been pretreated with 200 μg/ml proteinase K (Fig. 7, lanes *1* and *2*) or 1 mg/ml chymotrypsin (not shown). These results suggest that the import of presubunit Va is resistant to general proteolysis of the mitochondrial surface, and not just to trypsin pretreatment. Because it has been shown that the requirement for a protease-sensitive receptor can be bypassed if the mitochondrial outer membrane is disrupted (Ohba and Schatz, 1987; Hwang et al., 1989), it is important to note that the results presented in Fig. 7 additionally demonstrate that the mitochondria used in our experiments were intact after the protease treatment. As seen in lanes *5* and *7*, presubunit Va could be imported into mitoplasts (mitochondria with a disrupted outer membrane) made from mitochondria that had been pretreated with either proteinase K or trypsin. Moreover, if the mitoplasts were digested with proteinase K after import, a new species which migrated faster on SDS-polyacrylamide gels was generated (lanes *6* and *8*). Recent studies have shown that the new species, which we call m', results from cleavage of the COOH-terminal portion of mature subunit Va (which faces the intermembrane space) by the externally added proteinase K
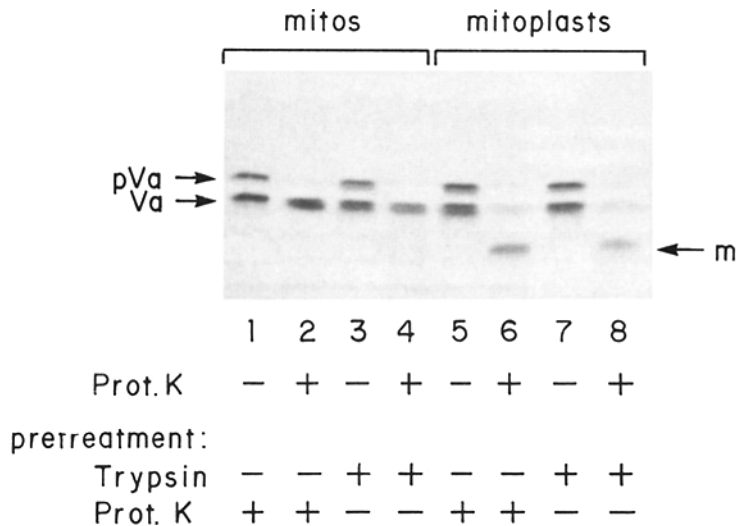
	mitos			mitoplasts				
	1	2	3	4	5	6	7	8
Prot. K	−	+	−	+	−	+	−	+
pretreatment:								
Trypsin	−	−	+	+	−	−	+	+
Prot. K	+	+	−	−	+	+	−	−

Figure 7. Import of presubunit Va into proteinase K-pretreated mitochondria or mitoplasts derived from them. Mitochondria were suspended at 10 mg/ml in SEM buffer and digested on ice with either 1 mg/ml trypsin (lanes *3, 4, 7,* and *8*) or 200 μg per ml proteinase K (lanes *1, 2, 5,* and *6*) for 20 min. The proteolysis reactions were terminated by the addition of 5 vol ice-cold SEM buffer containing STI (10 mg/ml) and PMSF (1 mM), and held on ice for an additional 10 min. Mitochondria were reisolated by centrifugation and resuspended at 10 mg/ml in SEM containing 10 mg per ml STI (10 mg/ml STI was also present in all subsequent steps). Each mitochondrial preparation (trypsin- or proteinase K-pretreated) was then divided; one half was diluted 10-fold with SEM (reactions *1* and *2* for the proteinase K-pretreated mitochondria, reactions *3* and *4* for the trypsin-pretreated mitochondria) or diluted 10-fold with ME buffer (20 mM MOPS, 1 mM EDTA, pH 7.2) to make mitoplasts (reactions *5* and *6* for the proteinase K-pretreated mitochondria, reactions *7* and *8* for the trypsin-pretreated mitochondria). The diluted samples were incubated on ice for 20 min with occasional mixing, reisolated by centrifugation, then resuspended in TRB to 500 μg/ml for import assays. The mitoplasting procedure results in the release of the contents of the intermembrane space but retention of the majority of the outer membrane as determined by immunoblotting against representative marker proteins (data not shown, see text). Import reactions were performed in duplicate using 1.5 × 10⁴ cpm radiolabeled presubunit Va (in reticulocyte lysate) and 50 μg of mitochondria or mitoplasts. The reactions were allowed to proceed for 10 min at 30°C, then stopped by the addition of valinomycin to 10 μg/ml. One of the duplicate reactions (lanes *2, 4, 6,* and *8*) was digested with proteinase K as described in the legend to Fig. 2, then all products analyzed by SDS-PAGE and fluorography. The positions of presubunit Va (*pVa*) and mature subunit Va (*Va*) are indicated. The m′ fragment is generated by cleavage of a COOH-terminal portion of mature subunit Va (which is exposed to the intermembrane space) and is observed only when mitoplasts are digested with proteinase K after performing an import reaction (see text). The m′ fragment therefore serves as a reliable indicator of the integrity of the outer membrane since it is never observed when intact mitochondria are used in our import reactions.

(Miller, B. R., unpublished results). Most importantly, the m′ form serves as a reliable marker of mitochondrial integrity. It is never observed when the mitochondria are intact, and it is always observed with mitoplasts (Miller, B. R., unpublished results). Therefore, it is clear that the protease pretreatment procedures used in our experiments leave the mitochondrial outer membrane intact, and that the ability of presubunit Va to be imported into protease-treated mitochondria cannot be explained on the basis of bypass import. From the combined results of Figs. 4, 6, and 7 therefore, we conclude that the import of presubunit Va is not dependent upon the function of a protease-sensitive receptor.

Subunit Va Is Properly Sorted after Import at Low Temperature or into Protease-treated Mitochondria

Was subunit Va properly sorted to the inner membrane after import into mitochondria at reduced temperature or after proteolysis of the outer membrane? To address this question, import assays were performed with untreated mitochondria at 30°C (standard conditions), with untreated mitochondria at 0°C, and with trypsin-treated mitochondria at 30°C. The mitochondria were then digested with proteinase K, reisolated, and separated into total membrane and soluble fractions. We found that the vast majority of subunit Va fractionated with the membranes (Fig. 8), which was consistent with previous results and reflected proper inner membrane localization of the protein (Glaser et al., 1990; Glaser and Cumsky, 1990*b*). We also found that the intramitochondrial distribution of subunit Va was essentially the same for each experimental condition. Thus, presubunit Va was routed correctly when imported at low temperature or into protease-

treated mitochondria. We conclude, therefore, that presubunit Va follows its authentic import pathway under these conditions.

Presubunit Va Is Imported via Translocation Contact Sites

Because presubunit Va was imported so rapidly, we could not identify translocation intermediates by performing import reactions at low temperature. Therefore, in the hope of slowing down the rate of presubunit Va import, sequences specifying the NH₂-terminal 118 residues of the precursor were cloned in front of those encoding a well-studied passenger protein, mouse dihydrofolate reductase (DHFR). This particular construct was built because first, we had shown that the subunit Va moiety (the NH₂-terminal three-fourths of the precursor protein, 118/153 amino acids) was imported normally into isolated mitochondria (Glaser et al., 1990). Second, several laboratories had shown that the import of artificial precursor proteins using DHFR as the passenger were reversibly blocked by low temperature (Eilers et al., 1988; Ostermann et al., 1989).

Initial experiments demonstrated that temperatures <12°C prevented complete import of the presubunit Va/DHFR fusion protein (pVa-DHFR), and that the import of the precursor was dependent upon the Δψ. When radiolabeled pVa-DHFR was imported into isolated mitochondria at 8°C, a fraction of the added precursor became trapped at regions where the inner and outer membrane were closely apposed (Fig. 9). That is, the NH₂-terminal portion of the protein was exposed to the matrix space (the presequence had been removed by the matrix metalloprotease), while the rest of the
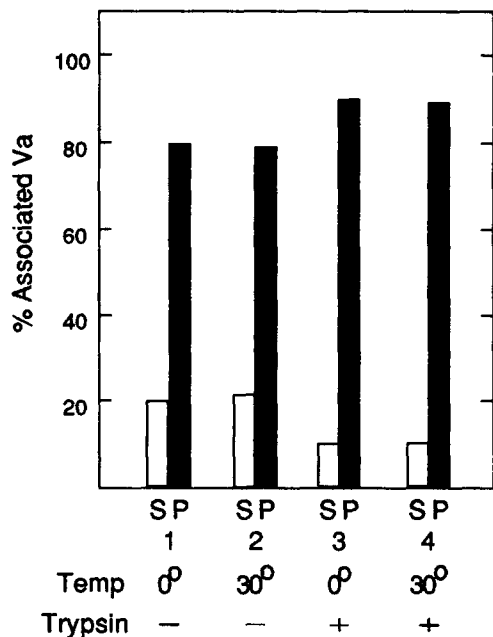
Figure 8. The submitochondrial localization of subunit Va is unaffected by proteolysis of the outer membrane or by low temperature. Import assays (containing 3.0×10^4 cpm radiolabeled presubunit Va in reticulocyte lysate) were performed at the indicated temperatures with mitochondria (100 μg per reaction) that were mock-treated (reactions *1* and *2*), or pretreated with 1 mg/ml trypsin (reactions *3* and *4*) as described in Materials and Methods. After 10 min the reactions were terminated by the addition of valinomycin to 10 μg/ml and digested with 200 μg/ml proteinase K for 30 min at 0°C. The proteinase K digestion was stopped by the addition of PMSF to 1 mM and the mitochondria reisolated and separated into total soluble (*s*) and membrane (*p*) fractions as previously described (Glaser and Cumsky, 1990b; Glaser et al., 1990). Each fraction was then analyzed by SDS-PAGE and fluorography, and the resulting fluorograph quantitated by densitometry. Values reported represent the percentage of the total mature subunit Va that was found in each fraction.

protein remained outside mitochondria in a position that rendered it susceptible to complete digestion by externally added proteinase K (Fig. 9, lanes *9–12*). Importantly, the formation of this intermediate was dependent upon a membrane potential (lanes *7* and *8*). When the same experiment was

performed at room temperature, we found that the precursor was imported normally: the majority had been processed and was resistant to proteinase K digestion (Fig. 9, lanes *3–6*). Thus, we conclude that presubunit Va is normally imported through translocation contact sites.

Discussion

We report here the results of studies designed to elucidate the conditions under which the precursor to yeast cytochrome c oxidase subunit Va is imported into, and localized within, isolated mitochondria. When taken together, the combined results of the experiments presented here, and those of our earlier studies, suggest that presubunit Va is efficiently imported into mitochondria via a pathway that is clearly unusual: while import was dependent upon an NH$_2$-terminal presequence (Glaser et al., 1988), the $\Delta\psi$, ATP, and translocation contact sites, it was independent of a protease-sensitive surface receptor and the temperature. A schematic diagram, illustrating our working model of the subunit Va import pathway, is presented in Fig. 10.

It is clear that the initial interaction between presubunit Va and the mitochondrial outer membrane is not mediated by the yeast equivalents of either MOM 19, a general receptor apparently used by many precursors, or MOM 72, the receptor apparently used by AAC (Söllner et al., 1989, 1990). Therefore, presubunit Va must either associate directly with the outer membrane and/or the contact sites, or utilize a "receptor" that is not sensitive to degradation by high concentrations of protease. Consistent with this view, we have found that presubunit Va does not bind specifically to mitochondria (Pfaller et al., 1988). That is, if the presubunit Va is bound to mitochondria that have been deenergized by treatment with antimycin A and oligomycin, it cannot be "chased" into the organelle after restoration of the $\Delta\psi$ with ascorbate and $N,N,N'N'$-tetramethylphenylenediamine (TMPD; Miller, B. R., unpublished results). Under identical conditions, we have been able to "chase" other precursor proteins into mitochondria (Glaser and Cumsky, 1990a; Glaser, S. M., unpublished results).

It is known that the uptake of apocytochrome c is also receptor-independent (Nicholson et al., 1988; Stuart et al., 1990); however, it seems likely that the mechanism of inser-
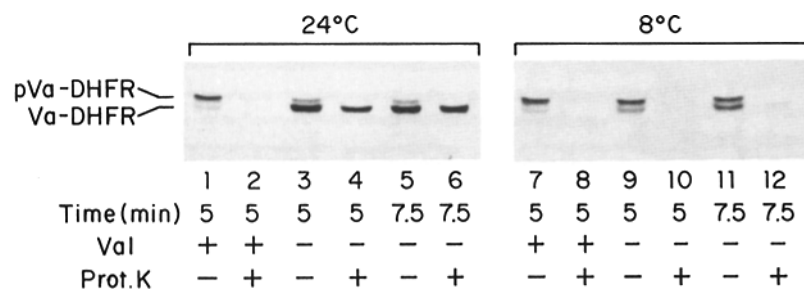

Figure 9. Presubunit Va is imported through translocation contact sites. A fusion between sequences specifying the NH$_2$-terminal 118 residues of presubunit Va and those encoding mouse DHFR was constructed (Materials and Methods), and the resulting protein synthesized in vitro by coupled transcription/translation as was described for authentic presubunit Va. Mitochondria (100 μg) were preincubated for 5 min in 200 μl of TRB at either 8°C or room temperature (24°C) in either the presence (reactions *1, 2, 7,* and *8*) or absence

(reactions *3–6, 9–12*) of 5 μg/ml valinomycin Radiolabeled pVa-DHFR in reticulocyte lysate (1–2 × 10^4 cpm in 2 μl) was then added and the reactions allowed to proceed at the indicated temperatures. After either 5 or 7.5 min, the reactions were terminated by the addition of 10 μg/ml valinomycin, divided in half, and placed on ice. One-half of each reaction (100 μl) was then digested with 200 μg/ml proteinase K. After 20 min the digestions were terminated by the addition of PMSF to 1 mM and the mitochondria reisolated and analyzed as described in previous figure legends. The positions of the unprocessed (*pVa-DHFR*) and processed (*Va-DHFR*) forms of the fusion protein are indicated.
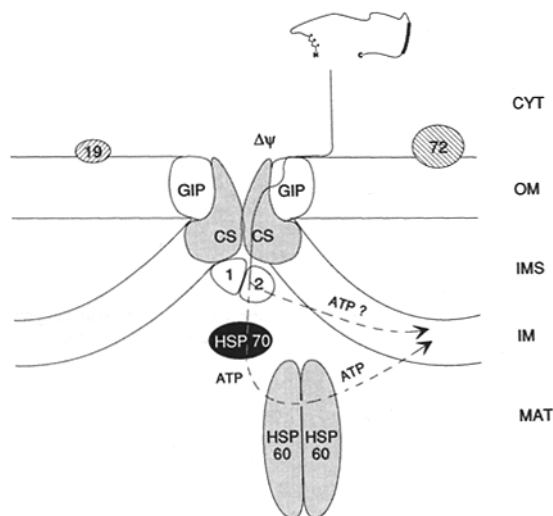
Figure 10. Working model of the subunit Va import pathway. Presubunit Va is drawn as loosely folded; the positively charged region at the NH₂ terminus represents the presequence, the bold region near the COOH terminus of the protein depicts the putative membrane localization domain. In the presence of a membrane potential (Δψ) presubunit Va associates directly with components of the outer membrane or contact sites but not with the yeast equivalents of either MOM 19 or MOM 72 (represented as surface proteins *19* and *72*). It is then transported through the contact sites in a Δψ-dependent fashion to the inner membrane with concomitant processing by the matrix metalloprotease (proteins labeled *1* and *2* correspond to the subunits of the protease, the *MAS1* [PEP] and *MAS2* [MPP] products, respectively). At present the precise route followed by subunit Va after processing is not clear; the two likely possibilities are indicated by the dashed lines and depict subunit Va partitioning directly within the inner membrane (without completely crossing it) or following the "conservative sorting" pathway of Hartl and Neupert (Hartl and Neupert, 1990). Steps suggested as ATP-dependent are indicated. *CS,* contact sites; *CYT,* cytoplasm; *GIP,* general insertion protein; *HSP 60,* heat shock protein 60; *HSP 70,* mitochondrial heat shock protein 70; *IM,* inner membrane; *IMS,* intermembrane space; *MAT,* mitochondrial matrix; *OM,* outer membrane.

tion into and across the outer membrane is different for apocytochrome *c* and presubunit Va. Apocytochrome *c* is transported across the outer membrane in a manner that does not rely on the Δψ but requires the function of cytochrome *c* heme lyase (Nicholson et al., 1988; Stuart et al., 1990). Presubunit Va, on the other hand, appears to follow the outer membrane protein (GIP)-contact site pathway used by many other precursor proteins (Fig. 10).

From the contact sites the route followed by presubunit Va is not yet clear. Because presubunit Va is processed by the matrix metalloprotease (Miller, B. R., and S. M. Glaser, unpublished results), we know that the NH₂-terminus of the precursor must be at least transiently exposed to the matrix space. However, we do not know whether the protein crosses the inner membrane completely and is reinserted into the bilayer (referred to as conservative sorting; Hartl and Neupert, 1990), or whether it is directly assembled into the holoenzyme complex without leaving the membrane. Despite several attempts, we have not yet been able to conclusively distinguish between these two possibilities. We have shown that removal of a hydrophobic domain from the

COOH-terminal portion of subunit Va causes the protein to be mislocalized to the matrix (Glaser et al., 1990). In addition, we have recently demonstrated that the same membrane-spanning region of subunit Va can redirect a matrix protein to the inner membrane (Miller, B. R., and M. G. Cumsky, manuscript in preparation). Interestingly, what appears to be the essential region of the domain is a hydrophobic stretch of 16 amino acids bracketed on both sides by positively charged residues (Glaser et al., 1990). Thus, it could act as a "stop-transfer" sequence that prevents the complete translocation of presubunit Va across the inner membrane, but still allows the NH₂ terminus to be exposed to the matrix and processed by the metalloprotease. On the other hand, the overall structure of the domain is also reminiscent of a bacterial export sequence (Lee and Beckwith, 1986), and therefore not inconsistent with the conservative sorting model (Hartl and Neupert, 1990). At present, we are performing experiments we hope will further define the subunit Va localization signal, and also provide information on its mode of action.

The results presented here also support the conclusion that presubunit Va is not tightly folded in solution, and that the loose conformation of presubunit Va contributes to the efficiency with which it is imported into mitochondria. It is appropriate to ask, then, whether the characteristics we observed for the import of presubunit Va are unique, or whether other precursors might have similar properties and follow the same receptor-independent pathway. Although the appropriate data are not yet available, we predict that what we have found for presubunit Va will be true for other mitochondrial proteins. We believe that the relatively small size of subunit Va (the precursor protein is 17 kD), and that it is somewhat hydrophobic, contribute significantly to its import efficiency. We note that many other proteins of the inner membrane, including other polypeptide subunits of the cytochrome oxidase complex, have similar physical properties.

Currently, we are attempting to further define the subunit Va import pathway. We are especially interested in determining whether the precursor completely crosses the inner membrane, and whether we can identify an hsp60-subunit Va complex. We would also like to investigate whether other proteins of the inner membrane follow an import pathway similar to that of presubunit Va, and at what step on this putative pathway ATP hydrolysis is specifically required. Hopefully, the successful completion of these and other studies ongoing in our laboratory will provide a more detailed picture of how proteins are correctly delivered to the mitochondrial inner membrane.

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