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Import into mitochondria of precursors containing hydrophobic passenger proteins: pretreatment of precursors with urea inhibits import

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We have studied the import into isolated yeast mitochondria of three hydrophobic passenger proteins attached to the N-terminal cleavable presequence of mitochondrial ATPase subunit 9 from *Neurospora crassa*. One natural precursor (pN9) contained *N. crassa* subunit 9; two chimaeric precursors, N9L/Y8–1 and N9L/Y9–2, respectively contained yeast mitochondrial ATPase subunits 8 and 9. In the absence of urea, pN9 and N9L/Y8–1 are imported efficiently but N9L/Y9–2 is not imported. After pretreatment of precursors in 4 M urea, binding of pN9 to mitochondria is marginally affected while its import is substantially inhibited; the binding to mitochondria of chimaeric proteins, N9L/Y8–1 and N9L/Y9–2, is greatly enhanced but no import is observed. This behaviour of import precursors containing hydrophobic passenger proteins is contrasted with that of a hydrophilic chimaeric precursor pCOXIV-DHFR, whose binding and import are enhanced by pretreatment with a high concentration of urea (8 M). The import of N9L/Y8–1 is very sensitive to the presence of low concentrations of urea in the import reaction mixture, and is abolished above 0.5 M urea although precursor binding to mitochondria is increased. By contrast, neither the import nor binding of pCOXIV-DHFR is affected directly by urea up to 0.8 M. These deleterious effects of urea on import of the chimaeric precursors N9L/Y8–1 and N9L/Y9–2 are interpreted in terms of a non-productive binding of these precursors to mitochondria, brought about by exposure of their hydrophobic domains resulting from urea unfolding. The generalization that membrane translocation of mitochondrial import precursors is enhanced by their prior unfolding in urea thus does not apply in the case of these precursors containing hydrophobic passenger proteins.

Introduction

Most proteins imported into mitochondria are targeted to the organelle by means of an N-terminal cleavable presequence [1] which is specifically involved in protein translocation across the mitochondrial membranes [2]. Such presequences have been used to drive the import of proteins that are not normally imported into mitochondria, by fusion to the N-terminus of passenger proteins. For example, a protein that normally has a cytosolic location, mouse dihydrofolate reductase (DHFR), has been targeted into yeast mitochondria by fusion to the cleavable presequence of cytochrome oxidase subunit IV (pCOXIV) [3,4]. It has even been possible to import proteins that are normally synthesized within mitochondria, following expression in the nucleocytosolic system [5]. Thus, two hydrophobic proteins of the F_o sector of yeast mitochondrial ATP synthase (mtATPase), namely subunits 8 and 9 (Y8 and Y9, respectively), have been targeted into mitochondria following fusion to the cleavable presequence (N9L) of the naturally imported mtATPase subunit 9 of *Neurospora crassa* (N9) [6,7].

In characterizing the import properties of these chimaeric precursors, in vitro import systems utilising isolated mitochondria have played a pivotal role. The efficiency of import into isolated mitochondria of a chimaeric precursor containing a particular presequence is often less than that of the corresponding natural precursor protein from which the presequence was derived. Thus, pCOXIV-DHFR fusion proteins were reported to show a smaller extent of import than that of the authentic pCOXIV precursor [4]. Likewise, N9L-Y8 and N9L-Y9 fusions were imported to a reduced extent

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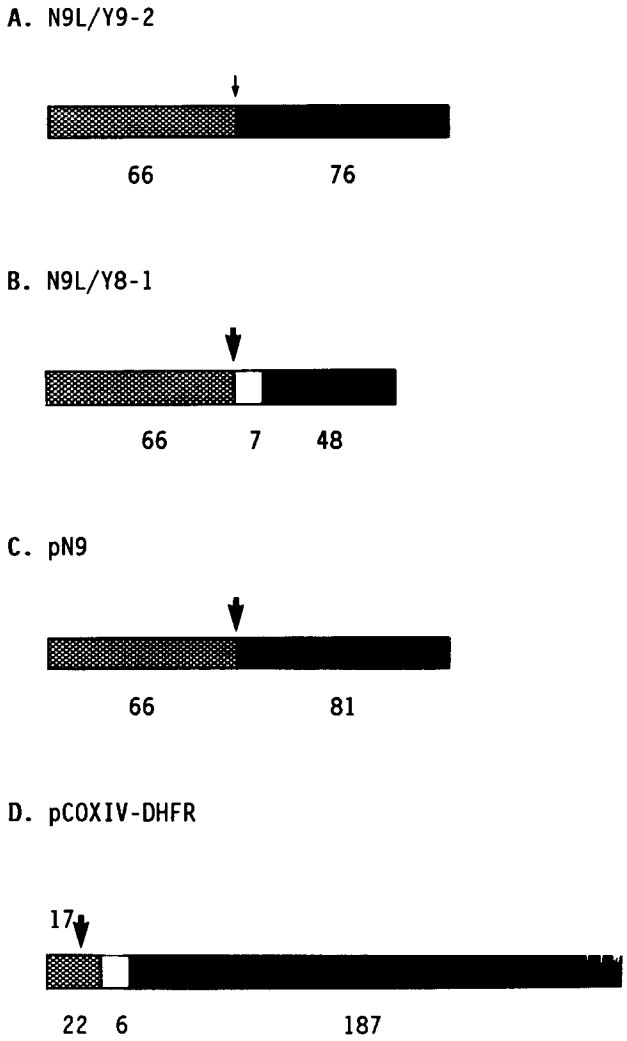

Fig. 1. Details of protein constructs. (A) N9L/Y9-2 is a direct fusion between the 66 amino acid presequence of N9L and the full length 76 amino acid sequence of Y9 [7,8]. (B) N9L/Y8-1 is a fusion between N9L containing the first five amino acid residues of the mature part of *N. crassa* subunit 9, plus two additional residues arising from gene fusion, and the full length 48 amino acid sequence of Y8 [6,8]. (C) pN9 is the natural precursor of *N. crassa* subunit 9 [26]. (D) pCOXIV-DHFR is a fusion between the 22 amino acid presequence of pCOXIV, plus six extra amino acids arising from gene fusion, and the full length 187 amino acid sequence of DHFR [3,4]. Stippled blocks, presequences; unshaded blocks, additional amino acids included at gene fusion points; filled blocks, passenger proteins; bold arrows, demonstrated sites of cleavage by matrix proteinase; thin arrow, anticipated site of cleavage by matrix proteinase (not experimentally demonstrated).

compared to that of the natural pN9 precursor [7]. In extreme cases, we have found combinations of presequences and passenger proteins that are unable to be imported. Examples include the presequence of cytochrome oxidase subunit VI fused to Y8 [6], and one particular fusion of N9L and Y9, denoted N9L/Y9-2 [8] in which the 66 amino acid N9L presequence is fused directly to the full length 76 amino acid sequence of Y9 (Fig. 1A). The incompetence of such chimaeric proteins for import may be related to their folded conformation, perhaps by interfering with the targeting function of the presequence [9].

One of our current objectives in investigating the assembly and function of the proton-pumping F_o sector is to achieve the import of Y9 and its assembly into mtATPase [7,8]. Adopting the premise that unfolding of a precursor is essential for its translocation across the mitochondrial membranes [10], we attempted to unfold N9L/Y9-2 with urea prior to its presentation to import-competent mitochondria. As reported here, while urea-treated N9L/Y9-2 binds more avidly to mitochondria, it is still not imported. This finding has led us to a more detailed assessment of the effects of urea on the import of a chimaeric precursor N9L/Y8-1 (Fig. 1B), which has been used extensively to achieve the import of Y8 and its assembly into mtATPase [5,6,8,11]. The deleterious effects of urea on the import of this precursor N9L/Y8-1 are directly contrasted with the behaviour of pCOXIV-DHFR (Fig. 1D), whose import is enhanced by prior denaturation in urea [12]. The results obtained here have general implications concerning the binding to mitochondria and membrane translocation of precursor proteins containing hydrophobic domains.

Materials and Methods

Structure and expression of protein constructs

The protein constructs used in this study are detailed in Fig. 1. Gene constructs encoding N9L/Y9-2, N9L/Y8-1 and pN9, having been cloned in plasmid pSP64T, were transcribed in vitro using SP6 RNA polymerase and translated to produce radiolabelled import precursors using rabbit reticulocyte lysate in the presence of [^{35}S]methionine as described [7]. Transcription of the gene construct encoding pCOXIV-DHFR, cloned in plasmid pDS5/2-1 which incorporates the phage T5 promoter, was effected in vitro using *E. coli* RNA polymerase [13] and was followed by translation of RNA in rabbit reticulocyte lysate to produce radiolabelled pCOXIV-DHFR.

Import into isolated mitochondria

Yeast mitochondria were prepared from *Saccharomyces cerevisiae* J69-1B [*rho*$^+$] and used immediately for import experiments with or without prior inactivation by an inhibitor cocktail essentially as described [7]. Except where indicated, each reaction mixture (100 μl) contained 20 mg/ml mitochondrial protein in import buffer [7], with a final urea concentration adjusted to 0.2 M by the addition of a 4 M urea stock. The incubation times for import experiments were 60 min for N9L/Y9-2, N9L/Y8-1 and pN9, and 30 min for pCOXIV-DHFR. The prolonged time for incubation of the former three hydrophobic precursors was chosen to maximize the opportunity to observe import,

potentially at low levels in this work. Control experiments on import of untreated N9L/Y8–1 and pN9 showed no evidence for degradation or loss of imported processed product. Methods for SDS-polyacrylamide gel electrophoresis and protein assay were as described [7].

Urea pretreatment of import precursors

Urea pretreatment was carried out by dialyzing radiolabelled proteins against urea solutions of specified concentrations each containing 20 mM Tris-HCl/0.5 mM EDTA/250 mM KCl/1 mM dithiothreitol (pH 7.4), at 4°C for 16 h as described [12]. The urea-treated precursor was diluted into a prewarmed import mixture containing mitochondria and incubated under equivalent conditions to those used for the non-treated precursor.

Results and Discussion

Effect of urea pretreatment on import of N9L/Y9–2

The data in Fig. 2A (lanes 1 and 2) confirm our previous demonstration that in the absence of urea N9L/Y9–2 is not imported [8]. This fusion protein is seen to bind to either energized mitochondria (lane 2) that are competent for import of other precursors (see Fig. 2, B and C) or to inactivated mitochondria (lane 1) that were treated with an inhibitor mix to abolish the inner membrane energization required for import [2]. In neither case does processing occur of N9L/Y9–2 to material of the size of Y9.

Pretreatment of N9L/Y9–2 with 4 M urea does not lead to this fusion protein being imported into isolated yeast mitochondria (Fig. 2A, lanes 3 and 4). For this experiment, radiolabelled N9L/Y9–2 was pretreated with 4 M urea. The urea-treated N9L/Y9–2 was found to bind to both inactive and active mitochondria (lanes 3 and 4) to a greatly enhanced extent, but in neither case was observed any processing to material of the size of Y9. Quantitation of the recovery of urea-treated N9L/Y9–2 (data not shown) revealed more than 70% of added precursor to be associated with mitochondria.

Proteinase K treatment (data not shown) of the material in lanes 3 and 4 led to the disappearance of the N9L/Y9–2 precursor, indicating that it is not imported in an unprocessed form. A prominent band at the position of Y9 was, however, observed following proteinase K treatment of both inactive and active mitochondria. Moreover, this material was soluble in chloroform-methanol (data not shown), suggesting that it arose by embedding of the Y9 moiety of N9L/Y9–2 in the mitochondrial membrane.

Effect of urea pretreatment on import of other hydrophobic proteins targeted by the N9L leader

This behaviour of N9L/Y9–2 prompted us to ask whether its response to urea was typical of hydrophobic proteins fused to the N9L leader. Two further precursors were therefore studied under conditions as above, both of which can be imported into mitochondria in the absence of urea. The results show first, that urea pretreatment of N9L/Y8–1 (Fig. 2B) leads to a similar response to that of N9L/Y9–2 and second, that pN9 (Fig. 2C) is markedly less susceptible in this respect to the effects of the urea pretreatment.

Comparison of data in Fig. 2B (lanes 1 and 2), demonstrating the import of N9L/Y8–1 in the absence of urea pretreatment, with data in lanes 3 and 4 shows the greatly accentuated binding of urea-treated N9L/Y8–1 to both inactive (lane 3) and active (lane 4) mitochondria. As with N9L/Y9–2, the urea-treated N9L/Y8–1 bound almost quantitatively to mitochondria; for comparison, in a typical import experiment about 20% of the input untreated N9L/Y8–1 binds to mitochondria, approximately half of which is processed to Y8 (data not shown). In spite of the

Lane	1	2	3	4
Precursor	native		denatured	
Inhibited	+	-	+	-

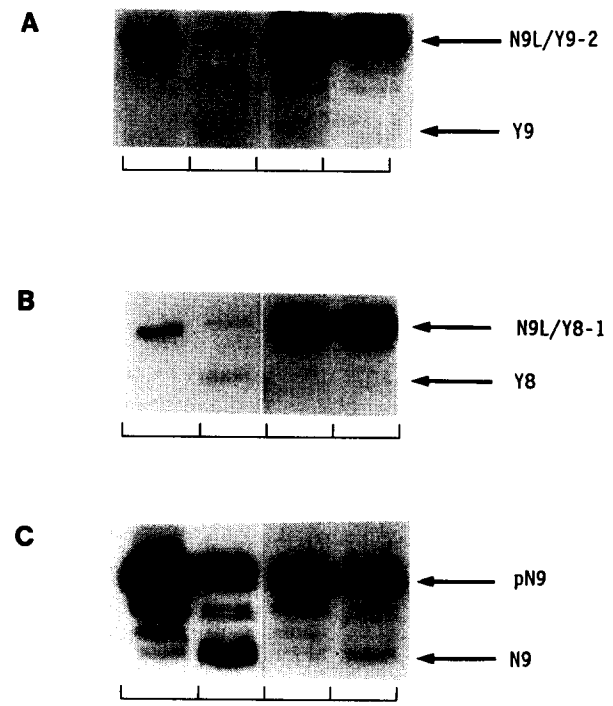

Fig. 2. Import of urea-pretreated hydrophobic proteins targeted by N9L leader. Each import mix contained inactive (lanes 1 and 3) or energized (lanes 2 and 4) mitochondria, which were incubated with native (lanes 1 and 2) or with 4 M urea-pretreated (lanes 3 and 4) radiolabelled precursors (approx. $2 \cdot 10^5$ dpm). The final concentration of urea in each import mix was adjusted to 0.2 M. Mitochondria were reisolated after import and proteins were analyzed by electrophoresis and fluorography. Mitochondrial import precursors: (A) N9L/Y9–2, (B) N9L/Y8–1, (C) pN9. Also indicated are the mobilities of Y9, Y8, and N9.

enhanced binding of N9L/Y8–1 after urea pretreatment, there is no evidence for processing of N9L/Y8–1 to a Y8-sized product by active mitochondria (lane 4), and this is indicative of a failure to import this urea-treated precursor. As with N9L/Y9–2, proteinase K treatment indicated that urea-treated N9L/Y8–1 was not imported in an unprocessed form, since N9L/Y8–1 was degraded leaving a prominent band of Y8 size (not shown here), which suggests a strong interaction between the hydrophobic Y8 moiety and the mitochondrial membrane (cf. Ref. 6).

The behaviour of pN9 (Fig. 2C) in these experiments is considerably different from that of the two chimaeric precursors. In the absence of urea pretreatment (lanes 1 and 2), pN9 binds strongly to mitochondria and shows extensive import and processing. Note the efficient binding (almost quantitative in lane 1) and import of this natural precursor relative to the untreated chimaeric precursor N9L/Y8–1 (Fig. 2B, lanes 1 and 2). Quantitation of binding and import of pN9 (data not shown) indicated that more than 90% of added pN9 was bound to inactivated mitochondria (lane 1). At least 60% of the bound precursor is processed to N9 by active mitochondria (lane 2). This is comparable to the efficiency of import of pN9 into *N. crassa* mitochondria reported by Pfanner et al. [14]. After urea pretreatment, the binding of pN9 appears to be little different (lanes 3 and 4) compared to untreated pN9 (lanes 1 and 2). In this case we observe a relatively small proportion of pN9 to be imported and processed to form a band of N9 size. This imported material is resistant to proteinase K digestion of mitochondria (data not shown). Urea-pretreated pN9 does not apparently become strongly embedded in mitochondrial membranes, since little radiolabelled material associated with the inactive mitochondria of lane 3 survives proteinase K digestion (not shown).

Precursors containing the N9L leader fused to each of three different hydrophobic passenger proteins, after pretreatment with urea, thus show a complete or partial inhibition of import. These phenomena are inconsistent with the reported facilitation of the import of pCOXIV-DHFR by urea pretreatment of this precursor [12]. To investigate this situation further, a more detailed comparative study was undertaken of the effects of urea on the import of N9L/Y8–1 and pCOXIV-DHFR.

Effect of urea pretreatment on import of N9L/Y8–1 and pCOXIV-DHFR

The foregoing experiments with N9L/Y8–1 and other precursors were carried out using a single concentration of urea for pretreatment, namely 4 M. A series of urea concentrations were utilized, in the range 0 to 6 M, to assess the impact of such pretreatments on the import of N9L/Y8–1 (Fig. 3A). As shown in lanes 1 and 2, the dialysis treatment as such does not have a substantial impact on the binding and import of pre-

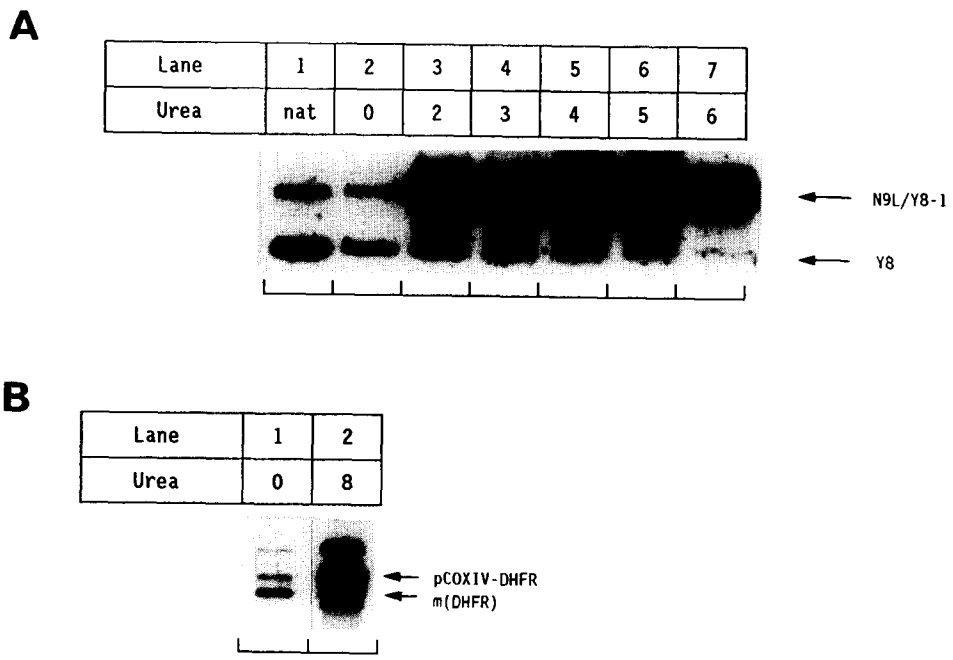

Fig. 3. Effect of urea pretreatment on the import of precursors N9L/Y8–1 and pCOXIV-DHFR. Radiolabelled precursors (approx. $3 \cdot 10^5$ dpm) were dialyzed against the indicated concentration of urea (M). (A) N9L/Y8–1, (B) pCOXIV-DHFR. Other experimental conditions and indications in panel A for N9L/Y8–1 were as for Fig. 2. Also indicated: nat, native precursor not dialyzed. In panel B the final concentration of urea for pCOXIV-DHFR was adjusted to 0.6 M; the mobilities of the precursor pCOXIV-DHFR, and the imported processed DHFR moiety m(DHFR) are indicated; additional visible bands in lane 2 have not been further investigated.

cursors. Although minor losses of radiolabelled proteins are evident, the ratio of precursor to processed Y8 is maintained at a consistent level. It is clear that urea pretreatment of N9L/Y8–1 by dialysis leads to a remarkably enhanced binding of this precursor to mitochondria over the entire range of urea concentrations tested, namely 2 M to 6 M urea. On the other hand, the efficiency of import (judged by the recovery of processed Y8) is gradually reduced over this concentration range, such that material at the position of Y8 is barely perceptible after 4 M urea pretreatment (lane 5), as was seen in Fig. 2B, lane 4.

By contrast, the import of pCOXIV-DHFR (Fig. 3B) was significantly enhanced by 8 M urea pretreatment. The data represented in lane 2 indicate both increased binding of the precursor pCOXIV-DHFR, as well as a greater quantity of DHFR imported, compared to those of lane 1. These results are essentially the same as those obtained by Eilers et al. [12], extending to the increased ratio of pCOXIV-DHFR to processed DHFR that is seen after urea pretreatment. This validates the methodology for urea pretreatment used here, the urea pretreatment being carried out on radiolabelled precursors prepared by in vitro translation using rabbit reticulocyte lysate. In contrast, the previous experiments with pCOXIV-DHFR [12] utilized this precursor prepared from recombinant bacterial cells [15].

We attempted to rationalize the effects of urea pretreatment of N9L/Y8–1 in terms of the putative unfolding of this precursor, by carrying out trypsin digestion of N9L/Y8–1 pretreated with different concentrations of urea (cf. Ref. 12). The results (data not shown) revealed complex changes in the susceptibility of N9L/Y8–1 to trypsin digestion, as a function of urea concentration. Thus, a slightly enhanced cleavage of N9L/Y8–1 by trypsin was evident after 2 M urea pretreatment. However, after 4 or 6 M urea pretreatment, this enhanced digestion by trypsin was no longer apparent, the treated precursor showing less digestion than the untreated control N9L/Y8–1 itself. The N9L/Y8–1 precursor may, after urea pretreatment, undergo initial unfolding events which lead to the subsequent acquisition of novel molecular configurations. These new structures may involve intramolecular refolding or intermolecular aggregation. The outcome in either case is that import of N9L/Y8–1 into mitochondria is blocked, while the binding of this precursor to mitochondrial membranes is greatly increased.

Import of N9L / Y8–1 is very sensitive to presence of urea

The possible influence of different urea concentrations present during the foregoing import experiments was avoided by adjusting the final urea concentration to 0.2 M in each case. As shown in Fig. 4A, this concentration of urea has no appreciable effect on the binding or import of N9L/Y8–1 (compare lanes 1 and 2), therefore validating the choice of 0.2 M urea as being of no discernable consequence to import behaviour. This final

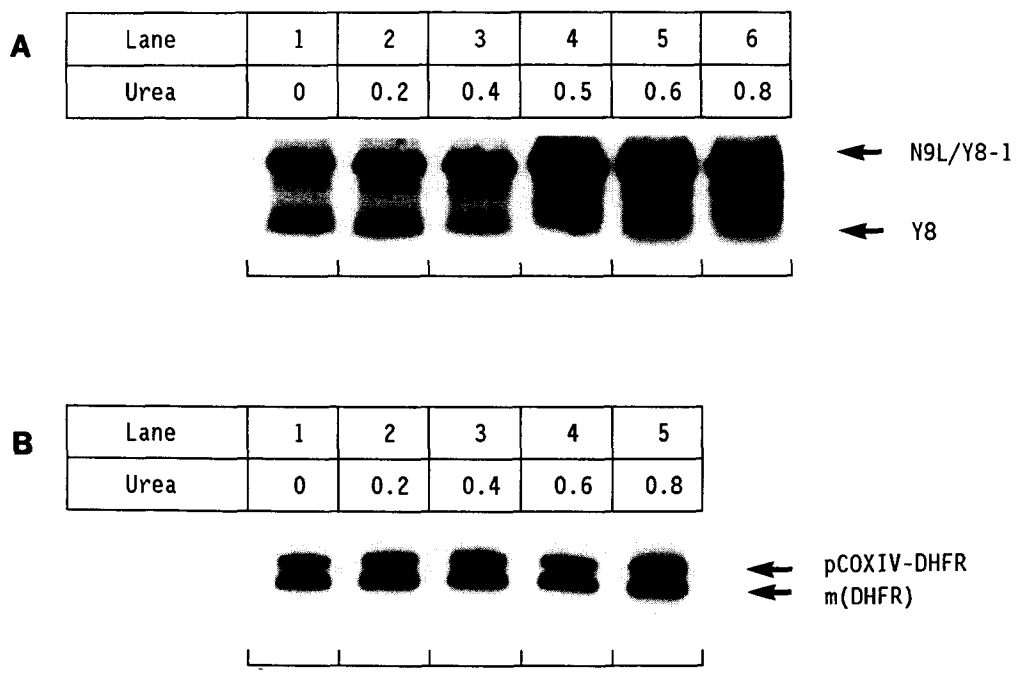

Fig. 4. Direct effect of urea on the import of N9L/Y8–1 and pCOXIV-DHFR. Import reaction mixtures (30 μl) containing mitochondria (60 mg protein) were assembled on ice with radiolabelled precursors: (A) N9L/Y8–1 ($7.5 \cdot 10^4$ dpm), (B) pCOXIV-DHFR ($2.5 \cdot 10^4$ dpm). Immediately following the addition of 4 M urea to the indicated final concentration (M), the mixtures were warmed to 27°C under import conditions. Indications are as for Fig. 3.

urea concentration of 0.2 M in the import mixes has also been adopted by Ostermann et al. [16] for use with a chimaeric import precursor construct containing N9L and DHFR, but is rather less than that used by Eilers et al. [12] for import studies on pCOXIV-DHFR, namely 0.4 to 0.6 M.

The effect of further increasing the urea concentration directly added to the import mix is also shown in Fig. 4, lanes 3 to 6. In the presence of 0.4 M urea, import of N9L/Y8–1 is markedly reduced (Fig. 4A, lane 3). At higher urea concentrations (up to 0.8 M) the binding of this precursor is enhanced, although import is completely inhibited (lanes 4 to 6). By contrast, neither binding nor import of pCOXIV-DHFR is affected by the presence of urea in the range 0 to 0.8 M (Fig. 4B), which emphasizes the differences in response to urea of hydrophilic and hydrophobic proteins, and is consistent with the use of higher final urea concentrations by Eilers et al. [12].

At first sight, there appears to be an inconsistency in the urea concentrations effective in impeding import of N9L/Y8–1 when urea is administered directly into an import mix (0.4 M and above; Fig. 4A) as opposed to when precursors are previously dialyzed against urea. Thus, we have already shown that pretreatment of this precursor with 2 M urea still allowed an appreciable extent of import, although, as discussed below, this did occur along with an increased binding of precursor to mitochondria (Fig. 3A, lane 3). This apparent contradiction results from the different experimental design. In the latter case, the urea treatment occurs during dialysis and affects only the precursor. The dialysate is then diluted into an import mix, yielding a final urea concentration of 0.2 M, which manifestly has no effect on import of N9L/Y8–1 as such (Fig. 4A, lane 2). In these experiments, any effect on import thus results from irreversible (or slowly reversible) changes in the structure of N9L/Y8–1. On the other hand, increasing urea directly in the import mixes (above 0.2 M) potentially influences not only the precursor but also the mitochondria, as well as the interaction between these two participants in the import process. For example, one might envisage that a relatively mild degree of structural change in the precursor N9L/Y8–1 (maintained in the presence of urea) may cause the observed enhanced binding that concomitantly is not productive in terms of import. One possibility as discussed in more detail in the next section, is that this direct urea effect may involve enhanced hydrophobic interaction between the Y8 moiety and the lipids of the outer membrane. Note that direct administration of urea into the import mix does not appear to compromise the bioenergetics of the import apparatus as such, because import of pCOXIV-DHFR is not affected up to 0.8 M urea (Fig. 4B).

Nevertheless, we must explain, why after 2 M urea pretreatment of N9L/Y8–1 we observe the enhanced binding of this precursor, coincident with an extent of import similar, or slightly greater, to that which occurs in the absence of urea (Fig. 3A, lanes 2 and 3). It may be that after 2 M urea pretreatment, the structure of only a proportion of the N9L/Y8–1 precursor molecules is irreversibly changed to generate non-importable molecular configurations. The remainder is either unchanged or, subsequent to an initial structural change, refolded to an import-competent state upon dilution into the import mix. At higher urea concentrations during dialysis, the residual level of import-competent N9L/Y8–1 molecules becomes vanishingly small.

General aspects of import and unfolding of proteins

The present data concerning the response to urea of mitochondrial import precursors in terms of their binding and subsequent membrane translocation, should be evaluated in the context of relevant features of the mitochondrial import process. In general [2], import of a protein into mitochondria occurs at contact sites between the inner and outer membranes through a specific proteinaceous import apparatus; the translocation of the precursor through this apparatus is driven by an electrochemical potential ($\Delta\psi$) across the inner membrane. The positively charged cleavable presequence, which plays a key role in membrane translocation, also has the general function of targeting the precursor to the organelle, mediated primarily by specific external proteinaceous receptors [14]. Other soluble external factors (such as members of the heat shock protein family (hsp70) and *N*-ethylmaleimide-sensitive factors) are also found to contain import stimulating activities [17,18].

What is germane to the data in the present paper are the events occurring prior to translocation, namely the binding of the precursor to the outer membrane and the steps which lead to protein unfolding. Thus, it has been recognized that precursor proteins containing hydrophobic domains bind to mitochondria more efficiently than those lacking such domains, and such hydrophobic interactions accelerate the import process [14]. Additionally, unfolding of precursors plays a key role in the lead-up to membrane translocation [10,19] to the extent that if unfolding is prevented, no import into mitochondria occurs [12,15,20]. Factors suggested to be involved in naturally facilitating such unfolding include ATP-dependent proteins with unfolding activities, such as hsp70 proteins [21]. A further suggestion is that unfolding of the precursor prior to translocation may be facilitated by the interaction of the presequence with acidic phospholipids of the outer membrane [22,23].

The special properties of chimaeric precursors with hydrophobic domains

The canonical view of the effects of deliberately unfolding a mitochondrial import precursor has been

developed largely through studies on pCOXIV-DHFR, a soluble and generally hydrophilic fusion protein [10]. Thus, as confirmed here, unfolding pCOXIV-DHFR by urea pretreatment enhances both its binding and import, presumably by making the presequence more readily available to the import apparatus [9] and by circumventing the initial ATP-dependent steps in the import process [12], which may include protein-mediated changes in the conformational state of the precursor [24].

As outlined above, the chimaeric precursor N9L/Y8–1 that contains at least one strongly hydrophobic domain, has binding properties different from those of pCOXIV-DHFR. Thus, the Y8 moiety binds to the mitochondrial membrane in its own right, even in the absence of $\Delta\psi$ [6]. In general, such an interaction involves hydrophobic regions and has been shown to facilitate import [14]. Yet when N9L/Y8–1 is pretreated with urea, import is completely abolished, although binding of the precursor is greatly increased (Figs. 2B and 3A). We propose that this enhanced binding represents an exaggerated version of the hydrophobic interaction between the Y8 moiety and the mitochondrial membrane. This non-productive membrane embedding possibly involves interactions with phospholipids, and could thus be considered an aberration of the natural interactions of precursors and membrane lipids that are considered to play an important role in import [23].

The failure of the fusion protein N9L/Y9–2 to be imported in the absence of urea could be explained in terms of two possibilities. First, it may be tightly folded so that, while it binds to mitochondria in the absence of $\Delta\psi$ (possibly via the hydrophobic Y9 moiety), the N9L presequence may be constrained from gaining access to the import apparatus. Second, the N9L leader may interact initially with the import apparatus, but the binding of the Y9 moiety to the membrane may be too strong to allow subsequent protein translocation to occur. The unfolding of N9L/Y9–2 with urea leads to the same outcome as seen with urea-pretreated N9L/Y8–1. No import at all occurs in spite of enhanced precursor binding, again probably representing non-productive membrane embedding through the exposed hydrophobic regions of the Y9 moiety. The proteinase-resistance of the Y9 portion of the bound urea-treated N9L/Y9–2 (see above) supports this view.

Endo et al. [23] have suggested that chimaeric precursors tend to be more tightly folded than natural precursors. This property of chimaeras would reflect the apposition of polypeptide domains not normally covalently linked or not usually available to undergo such physical interaction. The observed diminished efficiency of import of chimaeric precursors compared to their natural counterparts may indeed reflect a greater unfolding 'effort' that is required from the import apparatus and associated soluble factors in order to overcome any intramolecular adhesions or precursor-membrane interaction. (In these terms, N9L/Y9–2 could be considered to be very tightly folded). Paradoxically, in the case of hydrophobic fusion proteins such as those used here, the chemically assisted unfolding by urea promotes such strong membrane attachment that import is completely prevented. It is moreover feasible that the direct inhibitory effects of urea at low concentration (Fig. 4A) arise because urea might disturb interactions between these hydrophobic fusion proteins and other proteins with molecular chaperone activities [25]. One function of such proteins, in addition to their maintenance of precursors in relatively unfolded states, is to prevent precursor aggregation or membrane interactions of an inappropriate nature, prior to membrane translocation of the precursor.

Presumably, these considerations apply in general to hydrophobic proteins that are transported around cells and are targeted to specific organelles. Yet the natural precursor pN9, targeted from cytosol to mitochondria, is observed to be relatively resistant to those effects of urea which were seen with N9L/Y8–1 (Fig. 2B and C). We infer that the presequence (N9L) has co-evolved with the hydrophobic passenger protein (N9), to generate a 'streamlined' precursor structure in pN9. This precursor probably has a looser structure [23] than those of N9L/Y8–1 or N9L/Y9–2 and folds such that the N9L presequence can effectively interact with the import apparatus, the specific binding efficiency being boosted by the membrane affinity of the hydrophobic domains in N9 [14]. Moreover, little non-productive embedding of pN9 appears to occur even after urea pretreatment (see above). Such properties of a mitochondrial import precursor, that maximize the specific membrane interactions, are likely to be important in preventing misallocation of such hydrophobic membrane proteins which must be targeted within the cell to a specific organelle. The action of molecular chaperones probably also plays a role in minimizing non-specific interactions of hydrophobic import precursors that could lead to misallocation in vivo.

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References

1 Hurt, E.C. and Van Loon, A.P.G.M. (1986) Trends Biochem. Sci. 11, 204–207.

2 Hartl, F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45.
3 Hurt, E.C., Pesold-Hurt, B. and Schatz, G. (1984) FEBS Lett. 178, 306–310.
4 Hurt, E.C., Pesold-Hurt, B., Suda, K., Oppliger, W. and Schatz, G. (1985) EMBO J. 4, 2061–2068.
5 Nagley, P. and Devenish, R.J. (1989) Trends Biochem. Sci. 14, 31–35.
6 Gearing, D.P. and Nagley, P. (1986) EMBO J. 5, 3651–3655.
7 Farrell, L.B., Gearing, D.P. and Nagley, P. (1988) Eur. J. Biochem. 173, 131–137.
8 Law, R.H.P., Farrell, L.B., Nero, D., Devenish, R.J. and Nagley, P. (1988) FEBS Lett. 236, 501–505.
9 Verner, K. and Lemire, B.D. (1989) EMBO J. 8, 1491–1495.
10 Eilers, M. and Schatz, G. (1988) Cell 52, 481–483.
11 Nagley, P., Farrell, L.B., Gearing, D.P., Nero, D., Meltzer, S. and Devenish, R.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2091–2095.
12 Eilers, M., Hwang, S. and Schatz, G. (1988) EMBO J. 7, 1139–1145.
13 Hurt, E.C., Pesold-Hurt, B. and Schatz, G. (1984) EMBO J. 3, 3149–3156.
14 Pfanner, N., Muller, H.K., Harmey, M.A. and Neupert, W. (1987) EMBO J. 6, 3449–3454.
15 Eilers, M. and Schatz, G. (1986) Nature 322, 228–232.
16 Ostermann, J., Horwich, L.A., Neupert, W. and Hartl, F.-U. (1989) Nature 341, 125–130.
17 Chirico, W.J., Waters, M.G. and Blobel, G. (1988) Nature 332, 805–810.
18 Randall, S.K. and Shore, G.C. (1989) FEBS Lett. 250, 561–564.
19 Meyer, D.I. (1988) Trends Biochem. Sci. 13, 471–474.
20 Chen, W.-J. and Douglas, M.G. (1987) J. Biol. Chem. 262, 15605–15609.
21 Deshaies, R.J., Koch, B.D. and Schekman, R. (1988) Trends Biochem. Sci. 13, 384–388.
22 Eilers, M., Endo, T. and Schatz, E. (1989) J. Biol. Chem. 264, 2945–2950.
23 Endo, T., Eilers, M. and Schatz, G. (1989) J. Biol. Chem. 264, 2951–2956.
24 Wickner, W. (1989) Trends Biochem. Sci. 14, 280–283.
25 Ellis, R.J. and Hemmingsen, S.M. (1989) Trends Biochem. Sci. 14, 339–342.
26 Viebrock, A., Perz, A. and Sebald, W. (1982) EMBO J. 1, 565–571.