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# Latent membrane perturbation activity of a mitochondrial precursor protein is exposed by unfolding

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**We have purified milligram amounts of an importable mitochondrial precursor protein [the presequence of yeast cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase (DHFR)]. This has made it possible, for the first time, to perform detailed studies on the conformation of a precursor protein and its interaction with lipid membranes. The precursor protein closely resembled authentic mouse DHFR with respect to secondary structure (measured by CD spectra) and stability towards urea (measured by tryptophan fluorescence and enzyme activity). With this precursor protein, the presequence thus does not significantly alter the folding of the attached 'passenger protein'. In contrast to the corresponding presequence peptide, the native precursor exhibited only weak ability to disrupt vesicles with a low mol% of negatively charged lipids, suggesting that the passenger protein masks the amphiphilic properties of the presequence. The membrane-perturbing properties of the precursor were greatly enhanced by increasing the vesicles' content of negatively charged lipid or by denaturing the precursor in 5 M urea. Interaction with vesicles rich in acidic phospholipid was accompanied by partial unfolding of the precursor, suggesting that such a conformational change may also be involved in the interaction of the precursor with the mitochondrial membranes.**

*Key words:* amphiphilicity/mitochondrial protein import/protein folding/phospholipids

## Introduction

There is rapidly mounting evidence that proteins must at least partly unfold in order to be translocated across a biological membrane. Indirect evidence had already suggested that some precursor proteins have a different conformation than the corresponding mature proteins (Fisher *et al.*, 1973; Giudice and Weintraub, 1979; Oxender *et al.*, 1980; Zimmermann and Neupert, 1980; Miyata and Akazawa, 1982; Chien and Freeman, 1986). More recently, precursor proteins were found to unfold partly before or during their transport across membranes (Schleyer and Neupert, 1985; Eilers and Schatz, 1986; Randall and Hardy, 1986). How this unfolding is achieved is still a mystery. Since import of precursors into mitochondria appears to be mediated by protein factors (Douglas *et al.*, 1986), one or more of these factors could participate in the unfolding. Alternatively, an amino-terminal presequence could, by itself, perturb normal folding of the attached 'mature' part of the precursor protein.

Chemically synthesized presequence peptides of mitochondrial precursor proteins bind to and perturb phospholipid monolayers and bilayers and adopt an amphiphilic secondary structure (Roise and Schatz, 1988). Does the 'mature' part of a mitochondrial precursor polypeptide modulate the amphiphilicity of the presequence? Conversely, does an interaction of the presequence with lipid bilayers perturb the conformation of the mature part? None of these questions has been directly investigated since sufficient amounts of a mitochondrial precursor protein have not been available.

In this study we have purified a mitochondrial precursor protein in amounts sufficient for physicochemical studies. The precursor is a fusion protein consisting of the presequence of yeast cytochrome oxidase subunit IV (pCOX IV, an imported mitochondrial protein) fused to mouse dihydrofolate reductase (DHFR, a cytosolic protein). This artificial precursor protein is efficiently imported and proteolytically processed by isolated yeast mitochondria, or by mitochondria in living yeast cells (Hurt *et al.*, 1984, 1985). We have analyzed the conformation of the fusion protein by CD, fluorescence spectroscopy and enzyme assays, and studied its interaction with phospholipid vesicles. The results show that the presequence does not significantly alter the conformation of the attached DHFR moiety and that the native precursor protein is not significantly amphiphilic. However, its amphiphilicity is unmasked when it unfolds at a lipid-water interface.

## Results

### *The purified precursor can be imported by isolated yeast mitochondria*

The purified precursor obtained by our scaled-up procedure was readily imported, and cleaved, by isolated yeast mitochondria (see also Eilers and Schatz, 1986): 30-40% of the added precursor was imported after 30 min incubation at 30°C provided that $<50$ $\mu$g precursor were added per mg mitochondrial protein. Efficiencies declined at higher precursor levels (not shown). Import was completely blocked by methotrexate (Eilers and Schatz, 1986).

### *The presequence does not prevent folding of the 'mature' part*

The conformation of the native precursor and its stability against urea-denaturation were compared with the corresponding properties of authentic mouse DHFR lacking a presequence by using CD and fluorescence spectroscopy. The CD spectrum of authentic mouse DHFR showed a negative CD band with mean residue ellipticity of $-4400°$ cm$^2$/dmol at $\sim$208 nm (Figure 1). The precursor exhibited a similar CD spectrum with a negative CD band at 208 nm: the mean residue ellipticity at the CD minimum was $-3450°$ cm$^2$/dmol. The close similarity in the CD spectra strongly suggests that both proteins adopt similar secondary structures in solution.

In order to compare the structural stabilities of the two proteins, unfolding by urea was followed by monitoring the change in intrinsic tryptophanyl fluorescence. Mouse DHFR contains three tryptophan residues at positions 25, 58 and 114; the presequence lacks tryptophan. The fluorescence spectrum of native mouse DHFR excited at 280 nm showed an emission maximum at 323 nm, which indicated that the three tryptophan residues were in relatively hydrophobic environments. The addition of 5 M urea shifted the emission maximum to 347 nm (not shown), suggesting that tryptophan residues were transferred to a more hydrophilic environment by denaturation. The fluorescence emission maximum of the precursor also shifted, from 323 nm to 347 nm, upon addition of 5 M urea. In Figure 2A mole fractions of the native form, which are calculated from the change in the fluorescence intensity at 320 nm, are shown as a function of urea concentrations for both proteins. The curves show a transition between 1 and 3.5 M urea. The midpoints of the denaturation were 2.2 ± 0.2 M urea for both mouse DHFR and the precursor. The analysis of the denaturation behavior on the basis of a two-state model (Pace, 1986) allowed us to estimate the free energy change of denaturation without denaturant, $\Delta G_D^{H_2O}$, of 4.4 ± 0.2 kcal/mol for mouse DHFR and 3.7 ± 0.3 kcal/mol for the precursor.

Stability against urea was also followed by assaying DHFR activity. The precursor showed ~60% of the DHFR specific activity ($1.3 \times 10^8$ U/mol) of authentic mouse DHFR ($2.2 \times 10^8$ U/mol). The relative DHFR activity was plotted against urea concentration as shown in Figure 2B. Both proteins were activated by a low concentration of urea as reported previously for DHFR (Duffy *et al.*, 1987). High urea concentrations caused loss of enzyme activity. The DHFR activity of the two proteins responded to urea in a very similar manner, suggesting that the DHFR moiety of the precursor is not significantly unfolded. These results and the CD spectra show that the precursor and authentic mouse DHFR have very similar conformations, and similar stabilities towards unfolding by urea.

This conclusion does not support the idea that the presequence by itself induces a conformation in the attached mature part that allows the precursor protein to be recognized by, and transported through its proper target membrane. Furthermore, the calculated free energy of denaturation predicts (Pace, 1986) that at most 0.2% of the precursor molecules are unfolded in the absence of urea, while up to 30–40% of the pure precursor can be imported into mitochondria (see above). Thus tight folding of a precursor protein does not irreversibly abolish its capacity to move across a biological membrane, provided that the precursor can be again unfolded before or during translocation (cf. above).

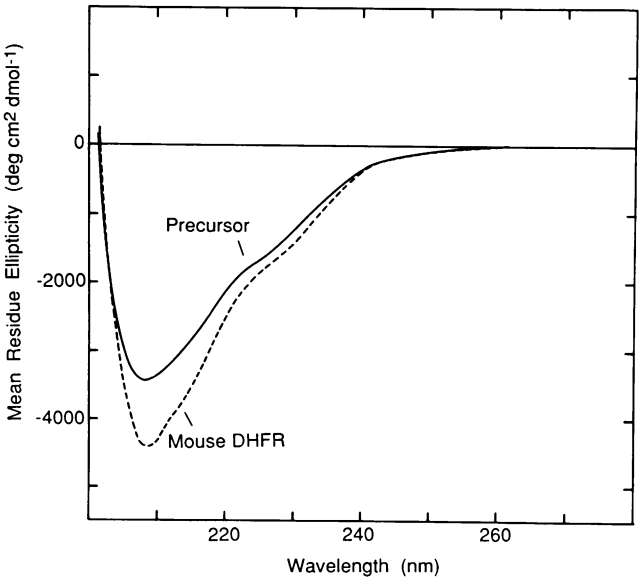


**Fig. 1.** CD spectra (200–280 nm) of the precursor (—, 20 μM) and of authentic mouse DHFR (---, 16 μM) at pH 7.0 and 26°C.

### *Urea-denatured precursor, but not the native one, is amphiphilic*

The interaction of the purified precursor protein with lipid bilayers was investigated by three types of experiments using unilamellar phospholipid vesicles: release of vesicle contents; loss of electric potential across the membrane; and quenching of the fluorescence of precursor labeled with *N*-(7-dimethylamino)-4-methyl-3-coumarinyl maleimide (DACM). Figure 3 shows the effects of the precursor on the release of a self-quenching fluorescent dye, carboxyfluorescein, from unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-

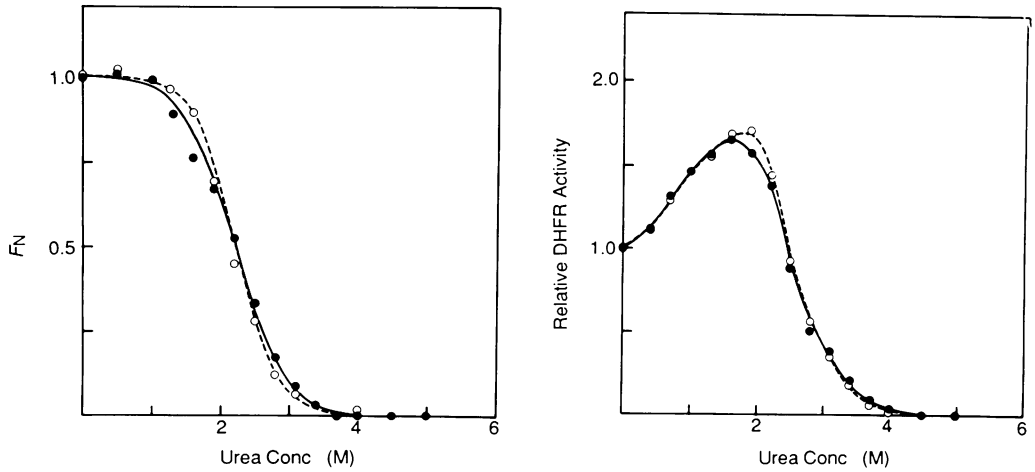


**Fig. 2.** Urea denaturation of authentic mouse DHFR (○-----○) and the precursor (●—●) monitored by fluorescence spectroscopy at pH 7.0 and 22°C (**A**) or by assaying DHFR activity (**B**). The precursor or mouse DHFR was preincubated with various concentrations of urea for 1 h at 22°C. The fraction of native protein, $F_N$, was calculated from the observed fluorescence intensity at 320 nm (excited at 280 nm,), $I_{obsd}$ , as $(I_{obsd} - I_D)/(I_N - I_D)$, where $I_N$ and $I_D$ are the fluorescence intensities at 320 nm for native and denatured forms of the protein. DHFR activity was normalized to that in the absence of urea.

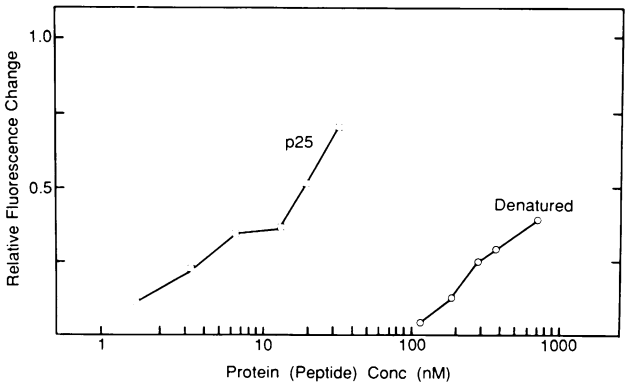


**Fig. 3.** Release of carboxyfluorescein from cardiolipin-free phospholipid vesicles. Leakage of vesicle contents was measured according to Roise *et al.* (1986), adapted from Weinstein *et al.* (1977). Vesicles were prepared from POPC and POPG (mol ratio 4:1) using a buffer containing 50 mM potassium carboxyfluorescein, 75 mM potassium tartrate, 10 mm Hepes–KOH (pH 7.2) and freed from untrapped dye by passage through a Sephadex G-75 column. The vesicle suspension was diluted 1:250 into the assay mixture (150 mM tartrate, 10 mM Hepes–KOH, pH 7.2). The urea-denatured precursor (denatured, ○—○; final urea concentration <0.2 M) or the presequence peptide (p25) (□—□) was added and fluorescence (excited at 490 nm) was monitored at 520 nm 100 s after initiating the reaction. One hundred percent values were measured upon addition of Triton X-100 to 0.1%. The final concentration of lipids was 15 μM.

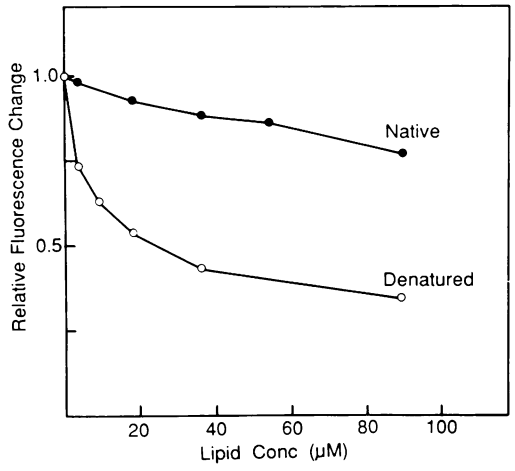


**Fig. 4.** Quenching of fluorescence of the DACM precursor (native, ●—●; denatured, ○—○) by lipid vesicles containing NBD-PE (POPC:POPG:NBD-PE = 8:2:0.25; NBD-PE was from Avanti Polar Lipids, Inc., USA). Vesicles were added to a 180 nM solution of the native DACM precursor (in 150 mM KCl, 10 mM Hepes–KOH, pH 7.2, 0.4 mg/ml bovine serum albumin), or the urea-denatured DACM precursor was diluted into the buffered vesicle suspension (final urea concentration <0.08 M). Fluorescence quenching was measured as the relative fluorescence change of DACM at 470 nm (excited at 400 nm) 100 s after initiating the reaction, compared with the fluorescence intensity of a solution lacking NBD-PE vesicles.

glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (4:1). For comparison, we also measured vesicle disruption by a chemically synthesized peptide (p25) corresponding to the entire presequence of cytochrome oxidase subunit IV (Figure 3). It has been reported that chemically synthesized presequence peptides of imported mitochondrial proteins are amphiphilic (Roise and Schatz, 1988). Indeed, lysis of the vesicles was observed at >30 nM of the presequence peptide. Unexpectedly, however, the native precursor caused no dye release from these vesicles even at 0.78 μM (not shown). The folded DHFR domain appears to shield the presequence sterically from the phospholipid bilayer.

This view was supported by the observation that denaturation by 5 M urea for 1 h at room temperature enhanced the precursor's ability to disrupt vesicles (Figure 3). At comparable concentrations, neither native nor denatured authentic mouse DHFR induced release of vesicle contents (not shown). The amphiphilicity of the denatured precursor is, thus, caused by the presequence rather than by some sequence normally buried within the folded DHFR moiety.

Inability of the native precursor to disrupt lipid vesicles could reflect an inability of its presequence to insert into a lipid membrane or an inability of the presequence to form oligomeric aggregates in a lipid environment. Either mechanism would render the presequence operationally non-amphiphilic. The finding that only the denatured precursor, but not denatured authentic DHFR, disrupts vesicles could merely reflect the fact that the attached presequence delays refolding, thereby prolonging the time during which any amphiphilic sequence normally buried within the folded DHFR moiety could interact with the vesicles upon lowering the urea concentration. However, when authentic DHFR and the precursor were diluted from 5 M urea in the absence of vesicles, both proteins refolded with half-times of 40–50 s as measured by tryptophanyl fluorescence (not shown). Yet another possibility would be that the native precursor forms aggregates in which the presequence is masked; urea would unmask the presequence by dissociating such aggregates. However, gel filtration experiments did not reveal the presence of large aggregates (unpublished results). We therefore favor the view that the urea-induced amphiphilicity of the precursor is caused by unfolding of the protein.

This dramatic effect of urea denaturation on the vesicle–precursor interaction was confirmed by fluorescence-quenching experiments using the DACM-labeled precursor and vesicles containing *N*-(nitrobenzoxa-diazoyl) phosphatidyl ethanolamine (NBD-PE). Precursor specifically labeled with a fluorescent DACM group at the cysteine residue in position 19 of the presequence was prepared and quenching of the fluorescence of the protein-bound DACM group by the NBD-PE-containing vesicles was examined.

When a constant amount of urea-denatured, DACM-labeled precursor was mixed with increasing amounts of vesicles containing the quencher NBD-PE, the fluorescence of the protein-bound DACM group was quenched (Figure 4) and the fluorescence of the quencher increased correspondingly (not shown). Quenching of DACM fluorescence was partly relieved by adding a 2-fold excess of vesicles containing normal 1-palmitoyl-2-oleoyl glycero-3-phosphoethanolamine (POPE) instead of NBD-PE (not shown). Thus at least part of the quenching was caused by a reversible binding of the precursor to the bilayer. Quenching was also found with DACM-labeled native precursor, but its extent was much less than that with the urea-denatured precursor (Figure 4).

### *Partial unfolding at a lipid–water interface renders the native precursor amphiphilic*

The amphiphilicity of the native precursor can also be unmasked by exposing the protein to cardiolipin(CL)-rich vesicles. Figure 5 shows the effect of cardiolipin on the vesicle-disrupting ability of the precursor as monitored by

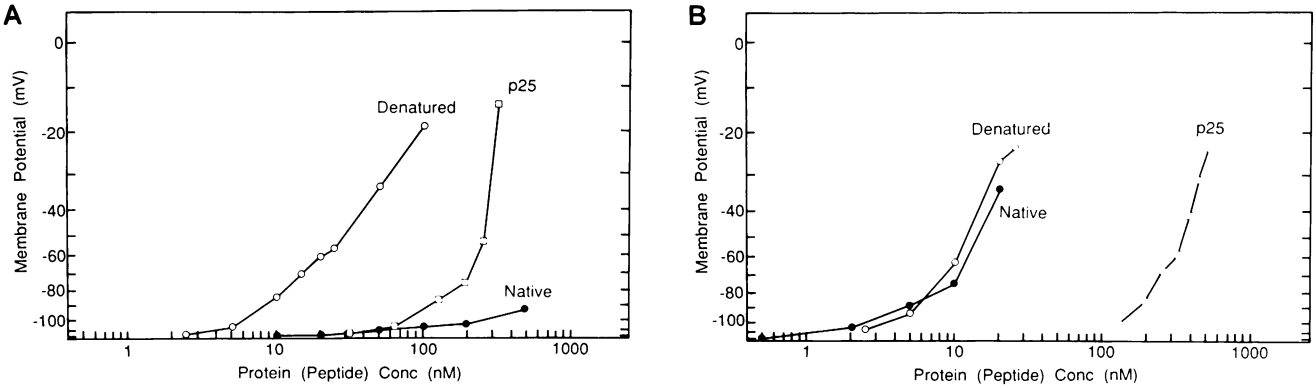


**Fig. 5.** Cardiolipin greatly enhances the membrane potential-collapsing effect of the precursor. Vesicles were prepared from POPC, CL and POPE [8:1:0.25 (**A**) or 6:2:0.25 (**B**)] using a buffer containing 10 mM Hepes–KOH (pH 7.2), 150 mM KCl. They were treated with 10 μM valinomycin and diluted 1:125 into buffer containing $Na^+$ instead of $K^+$, followed by addition of the potential-sensitive dye, diS-$C_3$-(5) (final concentration 2 μM), and, subsequently, of the precursor or the presequence peptide p25. The final concentration of lipids was 30 μM and the final concentration of urea was <0.04 M. Decay of the membrane potential was measured 50 s after addition of the precursor (native, ●—●; denatured, ○—○) or the presequence peptide (p25, □—□) to the vesicle suspension (Sims *et al.*, 1974; Roise *et al.*, 1986).

the loss of electric potential. The experiments were done with vesicles containing either 10 or 25 mol% cardiolipin. The concentration of peptide causing collapse of the membrane potential was >10-fold higher than that required to cause leakage of POPC/POPG vesicles (see above). On the other hand, collapse by the urea-denatured precursor became already evident at concentrations of 5–10 nM (Figure 5A,B) which were 10-fold lower than those causing release of carboxyfluorescein from POPC/POPG vesicles. Urea-denatured or native authentic mouse DHFR at comparable concentrations did not affect the potential of these vesicles (not shown).

The most dramatic effect of cardiolipin was seen with the native precursor. Whereas this protein had been essentially inactive towards POPC/POPG vesicles (see above), it showed a weak, but significant, collapsing effect with vesicles containing 10 mol% cardiolipin (Figure 5A); when the cardiolipin concentration was raised to 25 mol% the native precursor was as active as the denatured one. The enhanced interaction of the native precursor with lipid vesicles did not specifically require cardiolipin since cardiolipin could be replaced by 60 mol% POPG (not shown). When the native precursor was stabilized with 50 μM methotrexate, its potential-collapsing effect on cardiolipin-containing vesicles was almost completely blocked (not shown, but see Figure 6 below).

Assaying the release of vesicle contents using cardiolipin-rich vesicles (Figure 6) gave results similar to those described above; increasing the concentration of cardiolipin in the vesicles greatly enhanced the vesicle-disrupting ability of the native precursor. Preincubation of the native precursor with 50 μM methotrexate blocked its ability to cause release of carboxyfluorescein from vesicles. The effect of the urea-denatured precursor on release of vesicle contents (or on the membrane potential) was not affected by methotrexate. The release of the vesicle contents by the native precursor was also inhibited by lowering the temperature from 25°C (the normal assay temperature) to 12°C. In contrast, the effects of the urea-denatured precursor or the presequence peptide were actually slightly increased at the lower temperature (Figure 6).

In contrast, comparable concentrations of the precursor do not uncouple intact mitochondria. The reason for this is unknown; however, mitochondria continuously energized by

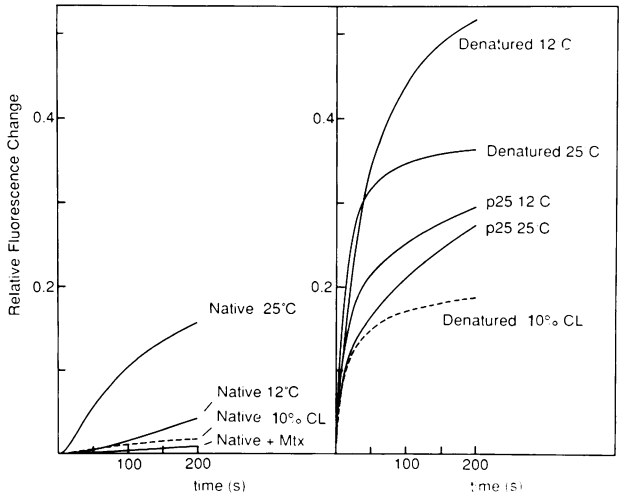


**Fig. 6.** Effects of methotrexate and low temperature on carboxyfluorescein release from vesicles by the precursor and the presequence peptide p25. For methods, see Figure 3. (——): experimental traces for the native precursor (100 nM) at 25°C and 12°C, the native precursor preincubated with 50 μM methotrexate (Mtx) (final concentration of methotrexate in the assay mixture, 0.2 μM), the urea-denatured precursor (25 nM) at 25°C and 12°C, and p25 (200 nM) at 25°C and 12°C, using vesicles (POPC:CL:POPE = 6:2:0.25). (---): traces for the native precursor (100 nM, 25°C) and the urea-denatured precursor (25 nM, 25°C) using vesicles (POPC:CL:POPE = 8:1:0.25). The final lipid concentration was 15 μM.

ATP or respiration may have the ability to stabilize the potential across their inner membrane against precursor-induced perturbation of the lipid phase.

Interaction of the native precursor with vesicles rich in acidic phospholipids thus involves a methotrexate- and temperature-sensitive conformational change in the DHFR moiety. Such a conformational change, or a partial unfolding, of the DHFR moiety is also confirmed by the observation that, on addition of 30 μM cardiolipin-containing vesicles (POPC:CL:POPE = 5:2.5:0.25), the emission maximum of tryptophanyl fluorescence of the precursor (1.3 μM), but not of authentic mouse DHFR (1.3 μM), was red-shifted by 8–9 nm, which indicated the transfer of tryptophan residues to a more hydrophilic environment. Since a hydrophobic membrane environment might alter the fluorescence properties of tryptophan residues, we cannot tell whether the red shift of only 9 nm reflects partial or extensive unfolding of

the precursor protein. Most probably, the enhanced interaction of the positively charged presequence with an acidic bilayer involves concomitant partial unfolding of the DHFR moiety. In other words, the acidic bilayer may bind the partially accessible, positively charged presequence of the native precursor strongly enough to accumulate the precursor at the lipid–water interface. This may then cause partial unfolding of the mature part, complete exposure of the presequence and subsequent insertion of the presequence into the bilayer.

## Discussion

This study provides the first detailed structural comparison between a mitochondrial precursor polypeptide and the corresponding presequence-free protein. While the results obtained with this artificially created precursor may not apply to all naturally occurring mitochondrial precursors, they do show that a functional mitochondrial presequence does not necessarily prevent normal folding of the attached 'mature' moiety of the precursor. *In vivo*, however, a presequence may well impair folding indirectly, e.g. by binding cytosolic proteins that interfere with folding of the precursor polypeptide.

Do the present data have any bearing on the situation *in vivo*? While import of precursors into mitochondria appears to be mediated by proteins (Douglas *et al.*, 1986), it is striking that the interaction of the native precursor with acidic phospholipid vesicles resembles the initial stages of its import into isolated mitochondria which are described in the accompanying paper (Eilers *et al.*, 1988): both processes are inhibited by methotrexate or low temperature, are facilitated by urea-denaturation, require a mitochondrial presequence, are ATP independent and involve at least partial unfolding of the protein. Thus interaction of the precursor with a lipid bilayer, accompanied by partial unfolding of the mature moiety, may represent a key aspect of protein import into mitochondria.

## Materials and methods

### *Purification of the precursor protein*

The artificial fusion protein pCOX IV-DHFR was purified from an *Escherichia coli* strain harboring the expression plasmid pKK-pCOX IV-DHFR essentially as described by Eilers and Schatz (1986) but with some modification to allow scale-up. The transformed *E.coli* cells were grown to the late logarithmic phase on LB medium, induced for 2 h at 37°C with 1 mM isopropyl-β-thiogalactoside, harvested, osmotically shocked to release most of the periplasmic contents and suspended in 150 mM KPi (pH 8.0), 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol (DTT) and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF). The shocked cells were gently lysed by adding lysozyme to 0.4 mg/ml and, after 30 min, octylpolyoxyethylene (a gift from Dr J.Rosenbusch, Biocenter) to 1%. The protease inhibitors macroglobin (0.025%), aprotinin (0.25 U/ml), leupeptin (0.0003%), antipain (0.00015%), chymostatin (0.0003%), elastatinal (0.0002%), pepstatin (0.0004%) and phosphoramidon (0.0001%) were also added to the indicated final concentrations to suppress proteolytic degradation. The lysate was diluted 4-fold with 50 mM KPi (pH 5.6), 2 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.5 mM PMSF, mixed with protamine sulfate (final concentration 2.5 mg/ml), stirred for 15 min and centrifuged at 10 000 *g* for 10 min. The supernatant was applied to a methotrexate–agarose affinity column (Sigma). The column was washed with low-salt (0 M KCl) and high-salt (1 M KCl) buffers (20 mM Tris–HCl, pH 7.0, containing 1 mM DTT and 1 mM EDTA), and the precursor was eluted with low-salt buffer containing 1 mM dihydrofolate (Sigma). The eluted fractions were pooled and directly applied to a CM-Sepharose CL6B column which had been equilibrated with 20 mM Tris–HCl (pH 7.0), 1 mM DTT and 1 mM EDTA. After washing the column extensively with 20 mM Tris–HCl (pH 7.0), 1 mM DTT and 1 mM EDTA to remove dihydrofolate, the intact precursor was eluted separately from its degraded forms by a linear KCl gradient (0–500 mM). The precursor fractions were identified by DHFR activity measurements and SDS–PAGE, pooled, dialyzed extensively against 20 mM Tris–HCl (pH 7.0), 50 mM KCl or NaCl and 1 mM DTT and, finally, concentrated by ultrafiltration using an Amicon YM10 membrane. The precursor contained no detectable residual dihydrofolate as judged by UV absorption at 350 nm. It was stored at −70°C in dialysis buffer containing 10% glycerol. A 2-l culture of *E.coli* gave ~1 mg of the chemically pure precursor. Authentic mouse DHFR was a generous gift from Dr R.L.Then (Hoffmann-La Roche & Co., Basel).

### *Preparation of lipid vesicles*

Vesicles were prepared from a mixture of synthetic POPC and POPG or gradient (0–500 mM). The precursor fractions were identified by DHFR activity measurements and SDS–PAGE, pooled, dialyzed extensively

### *Analysis of denaturation by urea*

Urea-denaturation curves were analyzed on the basis of a two-state model. The apparent free energy change of unfolding, $\Delta G_D$, was calculated from the experimental data using the equation $\Delta G_D = -RT \ln (F_D/F_N)$, where $F_D$ and $F_N$ represent the fractions in the denatured (D) and native (N) states. By extrapolating the linear dependence of $\Delta G_D$ on the urea concentration, observed in the transition region, to zero concentration, the free energy change of denaturation in the absence of urea ($\Delta G_D^{H_2O}$) is calculated by fitting the data according to the equation, $\Delta G_D = \Delta G_D^{H_2O} - m[\text{urea}]$, where $m$ is a parameter describing the co-operativity of the transition. This approach gives a minimal estimate of $\Delta G_D^{H_2O}$ (Pace, 1986).

### *Assay of DHFR activity*

The assay mixture (Gupta *et al.*, 1977) contained 50 mM Tris–HCl (pH 7.0), 100 mM KCl, 1 mM 2-mercaptoethanol, 50 μM NADPH, 50 μM dihydrofolate and 0.04 μM precursor or mouse DHFR (22°C).

### *Preparation of fluorescently labeled precursor protein*

The purified precursor was labeled with the fluorescent dye *N*-(7-dimethylamino)-4-methyl-3-coumarinyl maleimide (DACM, Sigma). The precursor in 20 mM Tris–HCl (pH 7.0), 50 mM KCl and 1 mM EDTA was reacted with 1.2 molar equivalents of DACM for 5 min at 0°C. The reaction was quenched by addition of a large excess of *N*-acetylcystein and the reaction mixture was dialyzed against 20 mM Tris–HCl, 50 mM KCl and 1 mM EDTA. Assay of free thiol groups (Kortt and Liu, 1973) in the labeled protein revealed that 0.8 mol equivalents of DACM had been introduced. Since authentic mouse DHFR was not labeled with DACM under these conditions, the precursor appeared to be specifically labeled with a DACM group at the cysteine residue in the presequence. DACM-labeling of the precursor did not alter its ability to collapse the membrane potential of lipid vesicles (not shown).

### *Import assays*

Import of the precursor into isolated yeast mitochondria was assayed as described by Eilers and Schatz (1986) except that import was assayed by immune blotting (Haid and Suissa, 1983) using antiserum to mouse DHFR. Immune blots were quantified with a Camag TLC scanner II.

### *CD and fluorescence measurements*

CD spectra were recorded on a Cary 60 Circular Dichrograph. Fluorescence measurements were carried out with a Schoeffel RRS 1000 fluorescence spectrometer.

### *Other assays*

The concentrations of pure precursor and mouse DHFR were determined by UV absorption at 280 nm, using the molar extinction coefficient of 21 000 of authentic mouse DHFR. The molar extinction coefficient had been determined by measuring protein by the BCA method described by Pierce Chemical Co. (USA).

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