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# The Amino Terminus of the $F_1$-ATPase β-Subunit Precursor Functions as an Intramolecular Chaperone To Facilitate Mitochondrial Protein Import

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**Mitochondrial import signals have been shown to function in many steps of mitochondrial protein import. Previous studies have shown that the $F_1$-ATPase β-subunit precursor (pre-$F_1$β) of the yeast *Saccharomyces cerevisiae* contains an extended, functionally redundant mitochondrial import signal at its amino terminus. However, the full significance of this functionally redundant targeting sequence has not been determined. We now report that the extended pre-$F_1$β signal acts to maintain the precursor in an import-competent conformation prior to import, in addition to its previously characterized roles in mitochondrial targeting and translocation. We found that this extended signal is required for the efficient posttranslational mitochondrial import of pre-$F_1$β both in vivo and in vitro. To determine whether the pre-$F_1$β signal directly influences precursor conformation, fusion proteins that contain wild-type and mutant forms of the pre-$F_1$β import signal attached to the model passenger protein dihydrofolate reductase (DHFR) were constructed. Deletions that reduced the import signal to a minimal functional unit decreased both the half-time of precursor folding and the efficiency of mitochondrial import. To confirm that the reduced mitochondrial import associated with this truncated signal was due to a defect in its ability to maintain DHFR in a loosely folded conformation, we introduced structurally destabilizing missense mutations into the DHFR passenger to block precursor folding independently of the import signal. We found that the truncated signal imported this destabilized form of DHFR as efficiently as the intact targeting signal, indicating that the primary defect associated with the minimal signal is an inability to maintain the precursor in a loosely folded conformation. Our results suggest that the loss of this intramolecular chaperone function leads to defects in the early stages of the import process.**

Although mitochondria have their own genome and the capacity to carry out protein synthesis, the great majority of mitochondrial proteins are encoded in the nuclear genome and translated on cytoplasmic ribosomes. Therefore, specific and efficient mechanisms to translocate these proteins into mitochondria must exist. Most mitochondrial proteins are synthesized on cytosolic polysomes as a larger precursor with an amino-terminal targeting signal that is removed following import. These mitochondrial import signals generally contain regularly spaced basic and hydroxyl-containing amino acids interspersed with apolar residues. While no specific primary amino acid homology has been found, the amphiphilic alpha-helical nature of these presequences is important for mitochondrial protein import (3, 37–39, 48).

Mitochondrial import signals have been implicated in many steps of mitochondrial import, including the interactions of precursors with cytosolic factors, import receptors, and components of the translocation complexes within the outer and inner mitochondrial membranes. The mitochondrial protein import receptor of *Saccharomyces cerevisiae* is a hetero-oligomeric complex composed of at least four different subunits assembled into two receptor subcomplexes, Tom37p-Tom70p and Tom20p-Tom22p (15, 20, 29). It has been proposed that the basic residues of mitochondrial import signals interact with the cytosolically exposed, acidic domains of Tom20p and Tom22p, as well as a second acidic domain of Tom22p following its penetration into the intermembrane space (4, 21, 22). After crossing the outer membrane, the presequence is inserted into the inner mitochondrial membrane in a ΔΨ-dependent manner (30, 43). The translocation channels within the inner membrane are gated by dimers of Tim23p. Upon presequence binding these dimers dissociate, allowing the channel to open and translocation to proceed (1).

Mitochondrial precursors must remain in a loosely folded conformation prior to their import. The importance of such an unfolded conformation is supported by a large body of data indicating that conditions that prevent folding stimulate the import of many precursors, while conditions that stabilize the native conformation of a precursor block its import (7, 8, 46, 47). The various abilities of different precursors to maintain import competence presumably reflect differences in their abilities to maintain an unfolded conformation. In addition to the problem of precursor folding, other processes, such as precursor aggregation, can also inhibit import (11). Molecular chaperones such as Hsp70 and Ydj1 are thought to facilitate mitochondrial protein import by maintaining precursors in an import-competent conformation prior to their interaction with the import machinery (5, 32). Another protein, called mitochondrial import-stimulating factor, has been reported to selectively bind mitochondrial precursors and deliver them to the Tom37p-Tom70p receptor complex (16, 17). Other cytosolic factors have also been proposed to participate in mitochondrial protein import (33, 34).

The β-subunit of the $F_1$-ATPase complex (pre-$F_1$β) has been used extensively to study the process of mitochondrial protein import. In a previous study, we demonstrated that reticulocyte lysate (RL) does not stimulate the initial rate of import of purified pre-$F_1$β but instead acts to extend the total time during which productive import can occur (18). This find-

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ing was consistent with the generally accepted model that molecular chaperones act to maintain mitochondrial precursors in an import-competent conformation. Pre-$F_1\beta$ has also been shown to contain a functionally redundant mitochondrial import signal at its amino terminus (2). In the present study, we examined the effect of two small nonoverlapping deletions on the function of the pre-$F_1\beta$ import signal. Our results indicate that the pre-$F_1\beta$ signal also acts to prevent precursor folding, in addition to its previously described roles in mitochondrial targeting and import. In addition, we show that the ability of this import signal to function as an intramolecular chaperone correlates well with the efficiency of precursor import into mitochondria.

## MATERIALS AND METHODS

**Construction of plasmids.** To construct the pET-3a derivative encoding wild-type (WT) pre-$F_1\beta$-dihydrofolate reductase (DHFR), the *Eco*RI-*Hin*dIII fragment containing the entire mouse DHFR gene was cloned from pDS5/2 (42) into pUC18. A 400-bp *Eco*RI-*Bam*HI fragment encoding the first 35 amino acids of WT pre-$F_1\beta$ (followed by a *Bam*HI site) was then PCR amplified from pDB288 (18) and cloned into the *Eco*RI-*Bam*HI site of pUC18/*DHFR* such that the pre-$F_1\beta$ import signal was in frame with the DHFR open reading frame. After verification of this construct by dideoxy sequencing (41), an *Nde*I-*Hin*dIII fragment encoding the complete fusion protein was subcloned into the *Nde*I and *Bam*HI sites of pET-3a, yielding the final WT pre-$F_1\beta$-DHFR expression plasmid pDB421. The same strategy was used to construct plasmids that expressed Δ1,2 pre-$F_1\beta$-DHFR (pDB423) and Δ1,2,3 pre-$F_1\beta$-DHFR (pDB424). (See Results for an explanation of the mutations.) To construct plasmids expressing WT pre-$F_1\beta$-DHFR* (pDB507), Δ1,2 pre-$F_1\beta$-DHFR* (pDB509), and Δ1,2,3 pre-$F_1\beta$-DHFR* (pDB510), a 268-bp *Bam*HI-*Sac*I fragment encoding DHFR containing the structurally destabilizing point mutations C7S, S42C, and D49C (47) was PCR amplified, sequenced, and used to replace the 268-bp *Bam*HI-*Sac*I fragments in pDB421, pDB423, and pDB424, respectively. Finally, the Δ1,2 pre-$F_1\beta$-spacer-DHFR plasmid (pDB537) was constructed by ligating a synthetic DNA fragment into the *Bam*HI site of pDB423. This fragment was made by annealing the oligonucleotides DB419 (5′ GATCCGCAGC GAACGCTAGC CTGGCGACCG 3′) and DB420 (5′ GATCCGGTCG CCAGGCTAGC GTTC GCTGCG 3′). The sequence of the final construct was confirmed by dideoxy sequencing (41).

To construct a yeast shuttle plasmid that expressed WT pre-$F_1\beta$ (pDB425), the 2,615-bp *Eco*RI-*Hin*dIII fragment containing the entire *ATP2* gene and its promoter was cloned into the *Eco*RI and *Hin*dIII sites of YCplac22 (12). To construct the Δ1,2 pre-$F_1\beta$ derivative of pDB425 (pDB427), the 1,539-bp *Hin*dIII fragment of pDB51 encoding Δ1,2 pre-$F_1\beta$ was used to replace the 1,575-bp *Hin*dIII fragment in pDB425.

**In vitro synthesis of pre-$F_1\beta$.** The plasmids used as DNA templates for in vitro transcription reactions were pDB95 (WT pre-$F_1\beta$), pDB45 (Δ1 pre-$F_1\beta$), pDB51 (Δ1,2 pre-$F_1\beta$), and pDB68 (Δ1,2,3 pre-$F_1\beta$). These plasmids each contained a 1.6-kbp *Hin*dIII fragment (2) encoding the indicated WT or mutant pre-$F_1\beta$ under SP6 promoter control in the vector pSP64. The in vitro transcription and RL translation of the precursors encoded by these templates were performed as described by the supplier (Promega).

**Expression and purification of recombinant proteins.** Mitochondrial precursors were expressed in the *Escherichia coli* strain BL21(DE3) as described previously (18). The final concentrations of purified urea-denatured precursor proteins were determined from Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels by using purified pre-$F_1\beta$ of a known concentration as a standard.

**Import assays.** Mitochondria were prepared from yeast strain D273-10B essentially as described previously (13). Import assays were performed as described before (18). The reaction was stopped by a 10-fold dilution into ice-cold SEM buffer (0.25 mM sucrose, 1 mM EDTA, 10 mM morpholinepropanesulfonic acid [MOPS] [pH 7.2]) supplemented with 0.5 μg of valinomycin, 20 μg of oligomycin, and 8 μg of antimycin, all per ml. If RL-translated forms of pre-$F_1\beta$ were used, parallel samples were treated with 0.1 mg of trypsin per ml for 10 min on ice, followed by the addition of 1 mg of trypsin inhibitor per ml. If purified forms of pre-$F_1\beta$-DHFR or pre-$F_1\beta$-DHFR* were used, the samples were treated with 0.1 mg of proteinase K per ml for 30 min on ice followed by the addition of 1 mM phenylmethylsulfonyl fluoride. The samples were analyzed by SDS-PAGE, and imaging and quantitation were performed with a PhosphorImager system (Molecular Dynamics). In control experiments, protease protection was not observed when 0.5 μg of valinomycin per ml and 20 μg of oligomycin per ml were present in the import reaction, or when Triton X-100 was added during protease treatment. This confirmed that the mitochondrially associated, protease-protected pre-$F_1\beta$-DHFR had undergone mitochondrial import.

**Binding experiments.** Mitochondria were preincubated with 0.5 μg of valinomycin, 8 μg of antimycin, and 20 μg of oligomycin, all per ml, for 5 min at 25°C. The precursor was then added and the binding reaction was carried out for 8 min at 25°C. An equal volume of ice-cold SEM (supplemented with 0.5 μg of valinomycin, 20 μg of oligomycin, and 8 μg of antimycin, all per ml) was then added, and the amount of each precursor bound to mitochondria was determined by SDS-PAGE followed by PhosphorImager analysis.

**Pre-$F_1\beta$-DHFR protease sensitivity assays.** To measure the protease sensitivity of pre-$F_1\beta$-DHFR, 0.5 mg of urea-denatured $^{35}$S-labeled pre-$F_1\beta$-DHFR per ml was diluted 40-fold in import buffer containing 50% RL and incubated at 30°C. At various times, two aliquots were withdrawn and diluted twofold into ice-cold dilution buffer (86 mM potassium acetate, 0.9 mM magnesium acetate, 10 mM MOPS [pH 7.2]). One of these duplicate aliquots was then treated with 50 μg of trypsin per ml for 30 min at 0°C. The proteolysis was terminated by the addition of a 20-fold excess of soybean trypsin inhibitor. Samples were analyzed by SDS-PAGE, followed by quantitation with a PhosphorImager system.

**Cell labeling and immunoprecipitation.** The *S. cerevisiae* strain YDB168 (*MAT***a** *leu2-3,112 ura3-52 trp1-Δ901 Δatp2::LEU2*), transformed with pDB425 or pDB427, was grown in synthetic medium (40) containing 2% glucose to a cell density of 0.5 to 0.7 $A_{600}$ units/ml at 30°C. The cells were resuspended in fresh medium to 4 $A_{600}$ units/ml and incubated for 15 min at various temperatures with shaking. The labeling reaction was initiated by the addition of 0.2 mCi of [$^{35}$S]EXPRESS protein labeling mix (DuPont NEN). The labeling reaction was terminated by the addition of 0.1 mg of cycloheximide per ml and 0.04 mg of methionine per ml, and incubation was continued during the chase period. To terminate the chase period, a 0.5-ml aliquot of cells was added directly to trichloroacetic acid (5% final concentration) and the mixture was incubated on ice for 30 min. Immunoprecipitations were carried out as described previously (2).

## RESULTS

**Mitochondrial import kinetics of pre-$F_1\beta$ mutants.** Pre-$F_1\beta$ contains a redundant mitochondrial import signal within its amino-terminal 34 amino acids. In a previous study, we found that any one of three nonoverlapping regions within this signal is sufficient to facilitate mitochondrial import in vivo (2). Since targeting signals consisting of only 9 to 12 amino acids can direct the import of many mitochondrial precursors (9, 23–26, 45), the physiological role of this extended pre-$F_1\beta$ import signal is unclear. In our previous study, we used three nonoverlapping deletions within the pre-$F_1\beta$ import signal to demonstrate that this region contains redundant mitochondrial import information (Fig. 1). Each deletion removed a different pair of basic residues; Δ1 removed amino acids 5 through 12, Δ2 removed amino acids 16 through 19, and Δ3 removed amino acids 28 through 34.

Our previous in vivo studies demonstrated that mutant forms of the pre-$F_1\beta$ targeting sequence containing any single deletion (Δ1, Δ2, or Δ3) or any pairwise combination of deletions (Δ1,2, Δ2,3, or Δ1,3) can still facilitate pre-$F_1\beta$ import to the extent that normal steady-state levels of $F_1\beta$ protein (and ATP synthase activity) are maintained in mitochondria (2). However, we found that constructs containing the Δ3 mutation were not processed normally upon import. Thus, to better understand the role of this redundant import information, we chose to examine the mitochondrial import kinetics of Δ1,2 pre-$F_1\beta$ in greater detail. This mutant precursor is missing two of the three redundant regions required for mitochondrial import, but it is still processed normally upon entry into the matrix.

In our previous study, we estimated the rate of Δ1,2 pre-$F_1\beta$ import at 30°C to be three- to fivefold lower than the rate of WT pre-$F_1\beta$ import. To determine whether a more severe import defect could be detected, we examined Δ1,2 pre-$F_1\beta$ import in cells growing at 15°C. Cells expressing either WT pre-$F_1\beta$ or Δ1,2 pre-$F_1\beta$ were pulse-labeled for 4 min with [$^{35}$S]methionine and then chased for various lengths of time in the presence of cycloheximide to block further protein synthesis. Under these growth conditions, we found that WT pre-$F_1\beta$ was still capable of rapid and efficient import, with 80% of the newly synthesized precursor already appearing as the mature form at the end of the 4-min labeling period (Fig. 2). This indicated that the half-time for import of WT pre-$F_1\beta$ at this

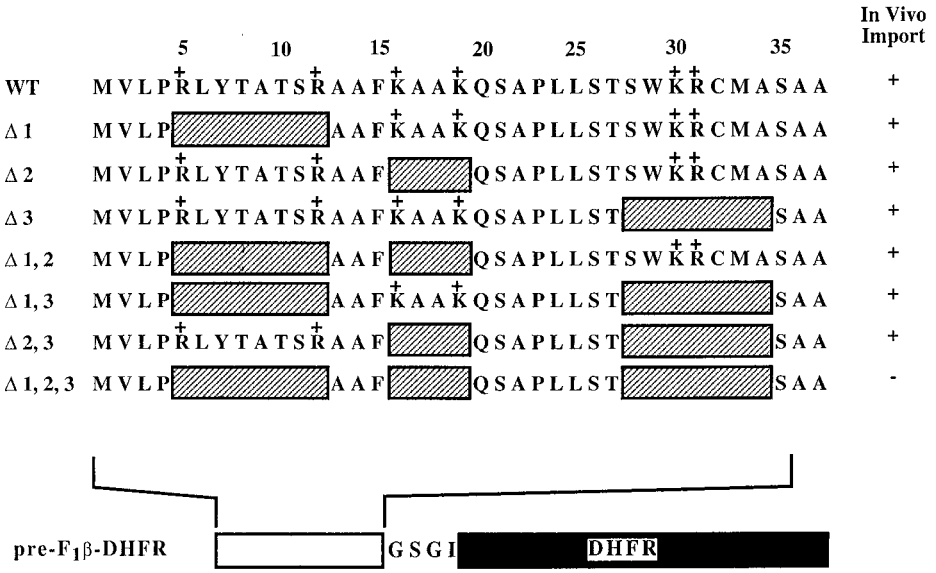


FIG. 1. Targeting signals present at the amino terminus of pre-$F_1\beta$ and pre-$F_1\beta$-DHFR precursors. Amino acid sequences of the amino termini of WT pre-$F_1\beta$ and derivatives containing various combinations of the Δ1, Δ2, and Δ3 mutations (2) as predicted from the nucleotide sequences. Deletions are designated by the hatched boxes. For the pre-$F_1\beta$-DHFR fusion proteins, the first 35 amino acids of WT pre-$F_1\beta$ (or the corresponding region of mutant pre-$F_1\beta$) was fused to mouse DHFR via the tetrapeptide GSGI. Basic residues are indicated by plus signs.

reduced temperature was still quite rapid (<3 min). However, Δ1,2 pre-$F_1\beta$ import was found to be much more severely compromised at 15°C. After an initial lag of 7 to 8 min, a linear rate of import was observed for roughly 20 min. During this period, 30% of the labeled precursor was imported, indicating a half-time for import of 30 to 35 min. Surprisingly, 20 min after the chase period was initiated, the rate of import decreased an additional threefold. While several interpretations are possible, this progressive decrease in the rate of import observed as a function of time after precursor synthesis could indicate that the Δ1,2 signal may be compromised in its ability to maintain the precursor in an import-competent conformation.

To further address this possibility, we next examined in greater detail the import of WT pre-$F_1\beta$ and Δ1,2 pre-$F_1\beta$ as a function of time after synthesis. Since the observed half-time of Δ1,2 pre-$F_1\beta$ import was significantly longer than that of WT pre-$F_1\beta$ import, it was possible that the WT precursor would also acquire an import-incompetent conformation if its import was similarly delayed. To test this possibility, we directly compared the abilities of WT and Δ1,2 pre-$F_1\beta$ to withstand a posttranslational delay in mitochondrial import. Cells expressing WT or Δ1,2 pre-$F_1\beta$ were pulse-labeled for 5 min at 30°C. During the labeling period, the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added either 40 s (for cells expressing WT pre-$F_1\beta$) or 4 min (for cells expressing Δ1,2 pre-$F_1\beta$) after the initiation of labeling. Because of the different initial rates of import of the two precursors, these times of CCCP addition allowed roughly 75% of each pre-$F_1\beta$ derivative to accumulate as a cytosolic, unimported species. The block in mitochondrial import was maintained for 30 min, and then β-mercaptoethanol (βME) was added to reverse the CCCP block, thus allowing the membrane potential to be reestablished (36). Control experiments confirmed that in the absence of βME, the import block was maintained for the duration of the experiment. However, the addition of βME allowed most of the remaining WT pre-$F_1\beta$ to reach the mitochondrial matrix during the next 45 min (Fig. 3). The initial lag observed presumably represented the time required to inactivate the CCCP and reestablish the potential. In contrast, only a small amount of the total Δ1,2 pre-$F_1\beta$ was imported during the first 30 min of incubation with βME, and no further import was observed thereafter. This severe import defect was not due solely to the incomplete restoration of the membrane potential, since the import of Δ1,2 pre-$F_1\beta$ synthesized after cells were treated in identical ways with CCCP and βME proceeded threefold more efficiently. These results indicate that a posttranslational import defect is specifically associated with Δ1,2 pre-$F_1\beta$, further supporting the model that the extended import signal present in WT pre-$F_1\beta$ acts to maintain the precursor in an import-competent conformation.

To further examine this possibility, we next examined the import of mutant forms of pre-$F_1\beta$ into isolated mitochondria.

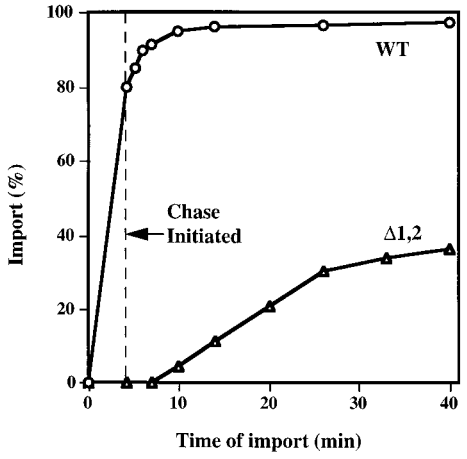


FIG. 2. Import kinetics of WT and Δ1,2 pre-$F_1\beta$ in vivo. Cells of yeast strain YDB168 expressing either WT or Δ1,2 pre-$F_1\beta$ were pulse-labeled for 4 min at 15°C, followed by a chase for the indicated times. Immunoprecipitations with pre-$F_1\beta$-specific antisera were carried out as described in Materials and Methods.

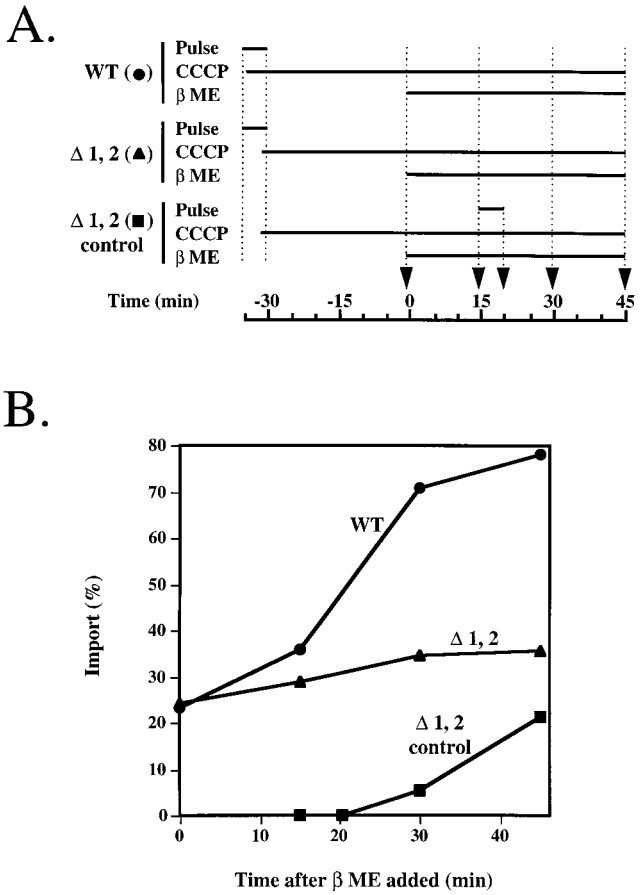


FIG. 3. Posttranslational import of WT and Δ1,2 pre-$F_1\beta$ in vivo. Cells of yeast strain YDB168 expressing pre-$F_1\beta$ derivatives were pulse-labeled for 5 min at 30°C. At 40 s (for cells expressing WT pre-$F_1\beta$) or 4 min (for cells expressing Δ1,2 pre-$F_1\beta$) after the initiation of labeling, CCCP (48 μM final concentration) was added to each labeling reaction mixture to block roughly 75% of each pre-$F_1\beta$ derivative as the precursor. Incubation with CCCP was continued for 30 min beyond the 5-min pulse-labeling period, and then βME was added to a final concentration of 1% to restore the membrane potential. Samples were removed for immunoprecipitation with pre-$F_1\beta$-specific antisera at the indicated times after βME addition. As a control, Δ1,2 pre-$F_1\beta$ was labeled for 5 min after βME addition to confirm that the membrane potential was sufficiently restored to support import of this precursor immediately after its synthesis. (A) Diagram of experiment. (B) Quantitation of data showing precursor import after the addition of βME. Symbols: circles, WT pre-$F_1\beta$; triangles, Δ1,2 pre-$F_1\beta$; squares, Δ1,2 pre-$F_1\beta$ control (labeled after βME treatment).

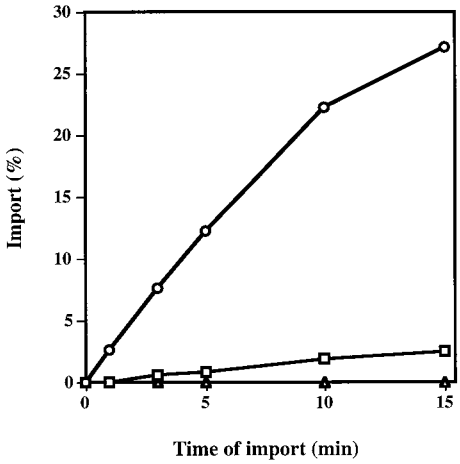


FIG. 4. Import of WT and mutant forms of pre-$F_1\beta$ into isolated mitochondria. The mitochondrial import of RL-translated WT pre-$F_1\beta$ (circles), Δ1 pre-$F_1\beta$ (squares), and Δ1,2 pre-$F_1\beta$ (triangles) was carried out at 25°C for the indicated times as described in Materials and Methods.

Radiolabeled mitochondrial precursors synthesized in RL translation systems are normally used as substrates to study mitochondrial protein import. Using such RL-translated precursors, we found that a precursor that retained two of the three redundant regions within the signal (Δ1 pre-$F_1\beta$) was still capable of limited mitochondrial import (Fig. 4), although its rate of import was reduced 15-fold relative to that of WT pre-$F_1\beta$ at 25°C. In contrast, Δ1,2 pre-$F_1\beta$, which retains only one of the three redundant regions, was capable of essentially no import in this in vitro system. These results again suggest that the redundant import signal is required to promote the efficient posttranslational import of RL-translated pre-$F_1\beta$ into isolated mitochondria.

**Import of DHFR fusion proteins.** We next wanted to address more directly whether the redundant targeting information of pre-$F_1\beta$ could influence the conformation of this precursor. However, because pre-$F_1\beta$ normally assembles into the oligomeric $F_1$-ATPase complex after import and processing, it was difficult to assess the ability of pre-$F_1\beta$ alone to fold into a native conformation by using a purified system. To circumvent this problem, we examined whether WT and mutant forms of the pre-$F_1\beta$ import signal could alter the conformation and import of the well-characterized, monomeric passenger protein mouse DHFR. This passenger was chosen because it has been used extensively to study mitochondrial protein import, particularly as a fusion containing the mitochondrial import signal of cytochrome oxidase subunit IV (pre-CoxIV-DHFR). In addition, three missense mutations within DHFR that destabilize its folded structure (C7S, S42C, and D49C) have been described. It was shown that a derivative of pre-CoxIV-DHFR containing these three destabilizing DHFR mutations exhibited an increased sensitivity to degradation by trypsin and an increased rate of import into isolated mitochondria, confirming that these mutations destabilized the conformation of the DHFR moiety (47).

To directly examine the influence of the pre-$F_1\beta$ import signal on precursor folding, constructs that fused the first 35 amino acids of WT pre-$F_1\beta$ (or the corresponding region of Δ1,2 and Δ1,2,3 pre-$F_1\beta$) to either DHFR or the destabilized form of DHFR (referred to hereafter as DHFR*) were prepared (Fig. 1). Each fusion protein was then expressed in *E. coli* and purified, and the mitochondrial import of each was examined as previously described (18). We found that purified Δ1,2 pre-$F_1\beta$-DHFR was capable of import into isolated mitochondria, although the rate and yield of Δ1,2 pre-$F_1\beta$-DHFR import were reduced four- to fivefold relative to those of WT pre-$F_1\beta$-DHFR (Fig. 5A). These results are consistent with our in vivo results indicating that this mutant targeting signal is capable of mediating import (although less efficiently than the WT signal) and also confirm that this signal can facilitate in vitro mitochondrial import. In contrast, the import of the conformationally destabilized Δ1,2 pre-$F_1\beta$-DHFR* was as efficient as that of WT pre-$F_1\beta$-DHFR* (Fig. 5B). As expected, fusions lacking all three redundant regions of the pre-$F_1\beta$ import signal (Δ1,2,3 pre-$F_1\beta$-DHFR and Δ1,2,3 pre-$F_1\beta$-DHFR*) were import incompetent, confirming that import was dependent upon a functional import signal. Since the DHFR and DHFR* constructs differ exclusively in the ability to acquire a tightly folded conformation, these results provide

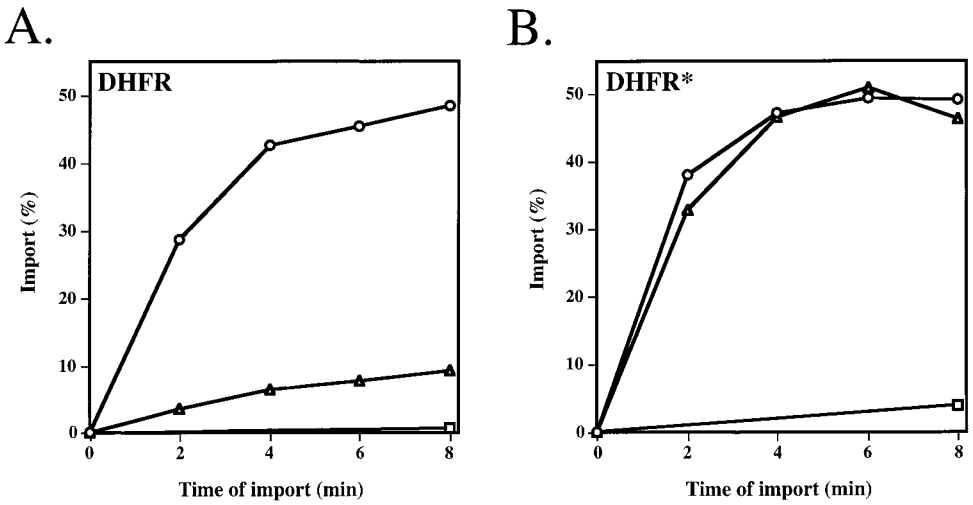


FIG. 5. Time course of import of pre-$F_1\beta$-DHFR and pre-$F_1\beta$-DHFR* and derivatives into isolated mitochondria. Symbols: circles, WT precursor; triangles, Δ1,2 precursor; squares, Δ1,2,3 precursor.

direct evidence that the primary import defect associated with the Δ1,2 import signal is its inability to maintain the attached passenger protein in an import-competent conformation.

**The pre-$F_1\beta$ targeting signal retards the folding of DHFR.** To confirm that different precursor conformations were responsible for the observed differences in the efficiency of mitochondrial import, we next used a protease sensitivity assay to probe the conformation of each pre-$F_1\beta$-DHFR derivative. Previous studies have shown that the DHFR portion of RL-translated DHFR fusion proteins can acquire a trypsin-resistant conformation, while attached mitochondrial import signals are degraded (7, 25). In preliminary experiments with purified pre-$F_1\beta$-DHFR derivatives, we found that neither of the fusion proteins tested (WT pre-$F_1\beta$-DHFR and Δ1,2 pre-$F_1\beta$-DHFR) was able to acquire a trypsin-resistant conformation following a rapid dilution from denaturant into buffer alone. However, a trypsin-resistant conformation was obtained if each of these unfolded proteins was diluted into buffer supplemented with RL, suggested that molecular chaperones within the RL assisted in folding these precursors. We therefore used this RL-assisted-folding assay to examine the ability of the targeting signal to influence DHFR folding. Each denatured fusion protein was diluted into buffer containing 50% RL (>50-mg/ml total RL protein concentration), and at various times aliquots were removed to determine the fraction of the total precursor that had acquired a trypsin-resistant conformation. We found that both the WT pre-$F_1\beta$-DHFR and Δ1,2 pre-$F_1\beta$-DHFR folded into a trypsin-resistant conformation in the presence of RL (Fig. 6A). However, the half-time for Δ1,2 pre-$F_1\beta$-DHFR folding into a trypsin-resistant conformation was roughly 8 min, while the half-time required for WT pre-$F_1\beta$-DHFR to fold into a trypsin-resistant conformation was 22

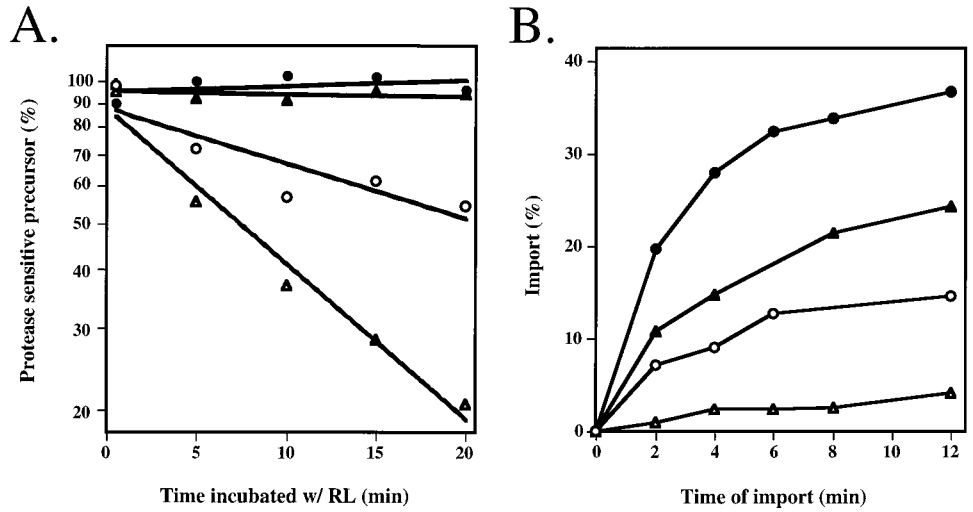


FIG. 6. Correlation between pre-$F_1\beta$-DHFR and pre-$F_1\beta$-DHFR* conformation and import of the proteins into isolated mitochondria. (A) Kinetics of precursor folding indicated by resistance to trypsin degradation. The denatured precursors were diluted into import buffer containing 50% RL. After incubation with RL at 30°C for the indicated time periods, the precursors were treated with trypsin and the percentage protease-sensitive precursor protein was determined. (B) Time course of mitochondrial import of precursors after predilution in 50% RL for 20 min. Symbols: open circles, WT pre-$F_1\beta$-DHFR; open triangles, Δ1,2 pre-$F_1\beta$-DHFR; closed circles, WT pre-$F_1\beta$-DHFR*; closed triangles, Δ1,2 pre-$F_1\beta$-DHFR*.

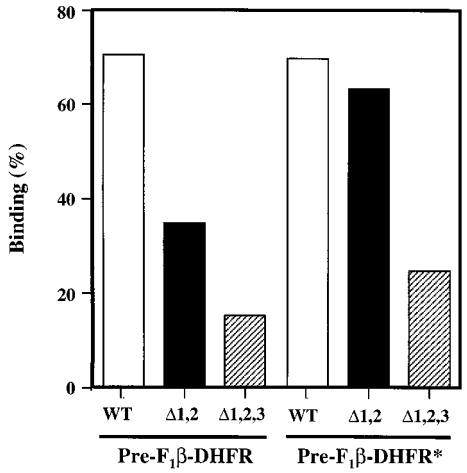


FIG. 7. Mitochondrial binding of Δ1,2 pre-$F_1\beta$-DHFR is reduced in a conformation-dependent manner.

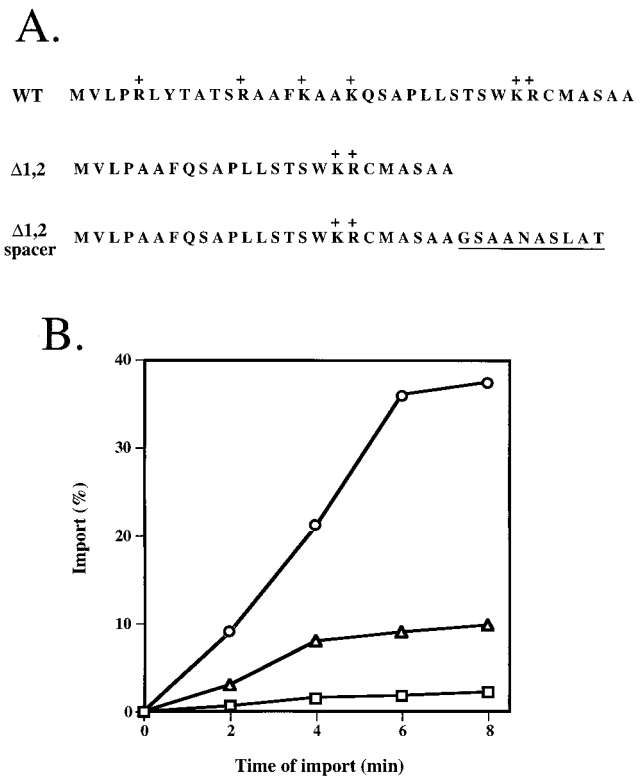


FIG. 8. The import defect associated with Δ1,2 pre-$F_1\beta$-DHFR is not due to the reduced length of the presequence. (A) Targeting signals of WT pre-$F_1\beta$-DHFR (WT), Δ1,2 pre-$F_1\beta$-DHFR (Δ1,2), and Δ1,2 pre-$F_1\beta$-spacer-DHFR (Δ1,2 spacer). The spacer sequence inserted between the Δ1,2 signal and DHFR is underlined. Basic residues are indicated by plus signs above the sequence. (B) Time course of mitochondrial import of denatured precursors. Symbols: circles, WT pre-$F_1\beta$-DHFR; triangles, Δ1,2 pre-$F_1\beta$-DHFR; squares, Δ1,2 pre-$F_1\beta$-spacer-DHFR.

min. These results confirm that the intact targeting signal can reduce the rate of folding of an attached passenger protein. In contrast, the destabilized WT pre-$F_1\beta$-DHFR* and Δ1,2 pre-$F_1\beta$-DHFR* both remained fully trypsin sensitive even after predilution for 20 min in RL, consistent with the inability of these precursors to attain a tightly folded conformation.

We next examined the mitochondrial import kinetics of these precursors following a 20-min preincubation in buffer containing 50% RL to allow folding to occur (Fig. 6B). The prediluted WT pre-$F_1\beta$-DHFR* and Δ1,2 pre-$F_1\beta$-DHFR* remained capable of efficient import, although the initial rate of Δ1,2 pre-$F_1\beta$-DHFR* import was twofold lower. This reduced level of import suggests that the Δ1,2 pre-$F_1\beta$ import signal may be compromised in its ability to prevent even the limited folding of the destabilized DHFR* moiety. Interestingly, the rate of WT pre-$F_1\beta$-DHFR import is lower than the rate of either WT pre-$F_1\beta$-DHFR* or Δ1,2 pre-$F_1\beta$-DHFR* import. This suggests that unfolding of the DHFR passenger is relatively inefficient after predilution, even when the WT pre-$F_1\beta$ import signal is attached. Thus, precursor unfolding in the presence of the molecular chaperones present in RL may represent the rate-limiting step in the mitochondrial import of WT pre-$F_1\beta$-DHFR under these conditions.

**The Δ1,2 targeting signal causes defects early in the import pathway.** Since mitochondrial import signals participate in several stages of the import process, it is possible that the import defect associated with the Δ1,2 pre-$F_1\beta$ signal is caused by another defect besides its reduced chaperone activity. To test this possibility, we examined other stages of the import process that require a functional import signal. Since the targeting signal mediates the initial interaction between the precursor and the import machinery, we first compared the abilities of these precursors to bind to mitochondria (Fig. 7). Binding was carried out with mitochondria preincubated with antimycin, valinomycin, and oligomycin to prevent translocation of the precursor across the inner mitochondrial membrane. Under these conditions, we reproducibly found that Δ1,2 pre-$F_1\beta$-DHFR bound to mitochondria two- to threefold less efficiently than WT pre-$F_1\beta$-DHFR. This reduction in binding was dependent upon the conformation of the precursor, since the structurally destabilized Δ1,2 pre-$F_1\beta$-DHFR* bound to mitochondria as efficiently as either WT pre-$F_1\beta$-DHFR or WT pre-$F_1\beta$-DHFR*. These results suggest that increased folding in the cytosol reduces the ability of Δ1,2 pre-$F_1\beta$-DHFR to engage the import receptors.

It was also possible that the Δ1,2 pre-$F_1\beta$ signal is compromised in its ability to facilitate translocation across the inner membrane. Since the results shown in Fig. 5B indicate that the Δ1,2 pre-$F_1\beta$ signal can direct the import of the DHFR* passenger as efficiently as the WT pre-$F_1\beta$ signal, it appears that the ability of this signal to insert into the inner membrane in a potential-sensitive manner is not compromised. However, recent studies found that mitochondrial targeting signals that are unable to extend an adequate distance from the passenger protein are compromised in the ability to span the membranes and productively engage mitochondrial Hsp70 (mtHsp70) if the passenger protein is folded. This inability to properly engage the import machinery on the *trans* side of the inner membrane reduces the ability of the precursor to productively translocate into the matrix (14, 44). To determine whether the reduced length of the Δ1,2 signal also reduces its ability to span the membranes and productively engage mtHsp70, we made a fusion protein containing an additional 10 amino acids inserted between the Δ1,2 signal and the DHFR domain (referred to as Δ1,2 pre-$F_1\beta$-spacer-DHFR) (Fig. 8A). Since our objective was to increase the ability of the import signal to span the inner membrane without restoring either the chaperone or signal properties of the precursor, the spacer sequence was designed to have the following characteristics: (i) a lack of charged residues, (ii) a propensity to acquire an alpha-helical confor-

mation based upon Chou-Fasman rules (35), and (iii) a nonamphipathic nature. When the import of Δ1,2 pre-$F_1\beta$-spacer-DHFR was compared to the import of WT pre-$F_1\beta$-DHFR and Δ1,2 pre-$F_1\beta$-DHFR, we found that the addition of the spacer sequence did not restore import to the level observed with WT pre-$F_1\beta$-DHFR (Fig. 8B). In fact, the import of this fusion protein into isolated mitochondria was even less efficient than the import of Δ1,2 pre-$F_1\beta$-DHFR. This indicates that an inability of the signal to adequately span the membranes and engage mtHsp70 is not a major component of the import defect associated with the Δ1,2 pre-$F_1\beta$-DHFR. We conclude that the primary import defect associated with the Δ1,2 pre-$F_1\beta$ signal occurs prior to the membrane translocation step.

## DISCUSSION

Mitochondrial targeting signals generally vary in length from 20 to 40 amino acids. However, several studies have shown that a truncated signal of only 9 to 12 amino acids can direct the in vivo mitochondrial import of many precursors (9, 23–26). In one such study, it was found that the pre-$F_1\beta$ targeting sequence contains redundant import information that facilitates its import into mitochondria (2). In the present study, we found that the loss of this redundancy reduces the ability of pre-$F_1\beta$ to import in a posttranslational manner. This defect correlates with an increased rate of folding of the precursor and a defect in binding to mitochondria. Furthermore, we found that Δ1,2 pre-$F_1\beta$ DHFR* can import into mitochondria as efficiently as WT pre-$F_1\beta$ DHFR* when these urea-denatured precursors were diluted directly into the import reaction mixture. This indicates that the import defect is not attributable to an inability of the signal to directly engage the membrane machinery or insert into the inner mitochondrial membrane in a potential-sensitive manner. Recent studies have found that mitochondrial targeting signals below a certain length are compromised in the ability to extend across the inner membrane and productively engage mtHsp70. These observations led to two models to explain the role of mtHsp70 in translocation across the inner mitochondrial membrane. In the translocation motor model, mtHsp70 is proposed to actively pull the precursor across the membrane (14). In contrast, the Brownian ratchet model suggests that cycles of repeated binding and release by mtHsp70 act more passively to prevent the precursor from slipping back out of the translocation pore as it translocates into the matrix (44). However, the results obtained with the Δ1,2 pre-$F_1\beta$-spacer-DHFR construct indicate that a reduced signal length does not cause the import defect associated with Δ1,2 pre-$F_1\beta$-DHFR. Taken together, our results indicate that the major defect associated with the Δ1,2 pre-$F_1\beta$ import signal is an inability to maintain the precursor in a loosely folded conformation prior to membrane translocation. While a significant portion of this import defect appears to result from a conformation-dependent defect in the initial binding step, an inability to efficiently transfer the precursor to the translocation machinery may also be involved.

Our results demonstrate that the pre-$F_1\beta$ targeting signal can maintain DHFR in a loosely folded conformation, indicating that the chaperone activity of this signal is not limited to a single passenger protein. This differs from the results of Endo et al. (10), who reported that the first 22 amino acids of pre-CoxIV was unable to prevent the folding of a pre-CoxIV-DHFR fusion protein. Their conclusion was based upon the finding that the folding state of this purified fusion protein was indistinguishable from native DHFR. This may indicate that not all mitochondrial import signals possess the chaperone-like function we have documented for the pre-$F_1\beta$ signal. However, the results of Endo et al. may also be attributable to the fact that the pre-CoxIV DHFR was expressed in a functional form in *E. coli* and purified by affinity chromatography, a method that would be expected to recover only a properly folded, functional protein population.

Many in vitro studies of protein folding and mitochondrial protein import have utilized RL to approximate a complex cytosolic environment containing molecular chaperones. Our results suggest that the WT pre-$F_1\beta$ targeting signal also helps chaperones maintain DHFR in a protease-sensitive, import-competent conformation more efficiently than the Δ1,2 pre-$F_1\beta$ signal. This stimulation of chaperone function could occur in two ways. The signal could indirectly increase chaperone binding by reducing the rate of precursor folding, thereby providing a longer period during which chaperones can productively bind. Alternatively, the signal could directly influence the recruitment of molecular chaperones to the precursor (possibly through a direct interaction between the signal and the chaperone). Interestingly, various mitochondrial import signals seem to influence chaperone function in different ways. For example, the amino-terminal 23 amino acids of rhodanese was reported to interfere with the chaperonin-mediated folding of that precursor (28), while the presequence of mitochondrial aspartate aminotransferase was reported to have only a minor role in influencing the folding rate of that protein (31). These various observations may result from subtle differences in the interactions between various mitochondrial import signals and their attached passenger proteins. For example, our observation that Δ1,2 pre-$F_1\beta$-spacer-DHFR imports less efficiently than Δ1,2 pre-$F_1\beta$-DHFR suggests that the spacing between the targeting signal and the passenger is one factor that influences the efficiency of chaperone activity within the targeting signal.

Our finding that the mitochondrial import signal of pre-$F_1\beta$ can influence precursor conformation in a manner that promotes import into mitochondria is a novel observation with regard to mitochondrial biogenesis. However, the role of a protein targeting signal as an intramolecular chaperone is not without precedent. For example, the leader peptide of the *E. coli* maltose-binding protein precursor (pre-MBP) also acts to slow precursor folding. Initially, it was observed that the export defect associated with some signal sequence mutations could be suppressed by a second mutation within the mature portion of pre-MBP (6). While the leader sequence mutations reduced the ability of the molecular chaperone SecB to bind pre-MBP, the destabilizing mutation within the mature portion of the precursor restored SecB binding (49). These results led to the model that the pre-MBP leader peptide slows precursor folding, which is necessary for SecB binding to occur. Once SecB is bound, it maintains MBP in a translocation-competent conformation until its export across the cytoplasmic membrane occurs. Since the final distribution of MBP along these alternate pathways depends upon the rate of MBP folding (or aggregation) relative to the rate of SecB binding, the efficiency of MBP export appears to be governed by a kinetic partitioning determined by the rate constants of these competing pathways (19, 27). Thus, the ability of the MBP leader sequence to slow precursor folding plays a crucial role in facilitating SecB binding. Our results suggest that a similar mechanism may be used by pre-$F_1\beta$ (and possibly other mitochondrial precursors) to facilitate mitochondrial import in a co- or posttranslational manner.

Our results indicate that the redundant pre-$F_1\beta$ import signal is capable of acting as an intramolecular chaperone to facilitate mitochondrial import. Based upon these findings, we

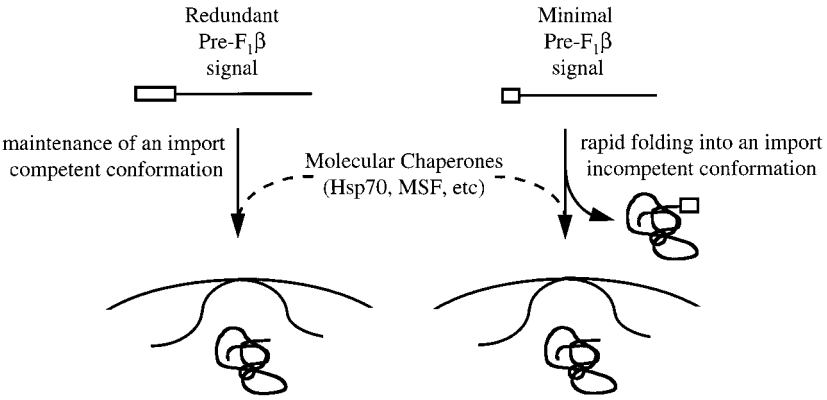


FIG. 9. Model of the cytosolic interactions of pre-$F_1\beta$ prior to mitochondrial import.

propose the model for pre-$F_1\beta$ shown in Fig. 9. In this model, one function of the redundant targeting signal of pre-$F_1\beta$ is to slow precursor folding. This intramolecular chaperone function of the signal provides a limited opportunity for the precursor to engage the mitochondrial protein import machinery. The chaperone function of the targeting signal is also capable of increasing the possibility of a productive interaction with molecular chaperones that allows the precursor to maintain an import-competent conformation for a more extended period of time. Overall, the combination of these processes allows the efficient import of precursors (such as pre-$F_1\beta$ and pre-$F_1\beta$-DHFR) that tend to fold rapidly. In contrast, when the targeting sequence is reduced to a minimal signal (such as the Δ1,2 pre-$F_1\beta$ signal), the precursor acquires an import-incompetent conformation much more rapidly (presumably either a tightly folded or an aggregated state). Once such a conformation is obtained, mitochondrial import becomes much less efficient. Thus, the rate of pre-$F_1\beta$ folding (as determined by the presence or absence of its redundant import signal) plays an important role in determining the overall efficiency of pre-$F_1\beta$ import.

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