Mitochondrial presequences can induce aggregation of unfolded proteins

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Abstract **We have studied the interactions between various synthetic peptides and two model unfolded proteins, reduced α-lactalbumin and reduced and carboxymethylated α-lactalbumin. We found that mitochondrial presequences could induce aggregation of the unfolded α-lactalbumins but not of the native α-lactalbumin. The presequence-induced aggregation of unfolded α-lactalbumin was dependent on electrostatic interactions and on the amphiphilicity of the presequences. Since positive charge and amphiphilicity are necessary for the targeting function of mitochondrial presequences, presequence-induced aggregation may be responsible for the instability of mitochondrial precursor proteins and may need to be inhibited by binding factors in the cytosol.**

Key words: Mitochondrial presequence; Protein aggregation; Unfolded protein; Mitochondrial precursor protein

1. Introduction

Most mitochondrial proteins are encoded by nuclear genes, are synthesized on cytosolic ribosomes as precursor proteins, and are subsequently transported into the mitochondria. Mitochondrial precursor proteins carry, in most cases, amino-terminal presequences that direct the proteins to mitochondria and are cleaved by the processing protease within the mitochondrial matrix. The information responsible for the correct targeting of a precursor protein to mitochondria depends on the positive charge and amphiphilicity of the presequence [1]. Synthetic mitochondrial presequences, which exhibit little secondary structures in aqueous solutions, adopt amphiphilic helical structures upon binding to lipid bilayers or detergent micelles [2–4].

Many mitochondrial precursor proteins tend to aggregate when expressed in *E. coli* or when synthesized in a wheat germ cell-free system. For example, the precursor form of the β subunit of F_1-ATPase ($pF_1\beta$) is unable to be imported into isolated mitochondria when expressed in wheat germ extracts. Addition of purified yeast cytosolic hsp70 (Ssa1p/Ssa2p) and a yeast postribosomal supernatant, however, restores the ability of $pF_1\beta$ to be imported ([5]; Mitsui, S. et al., unpublished data). Hsp70 and the cytosolic factors presumably help to maintain the mitochondrial precursors in a translocation-competent state, although the molecular basis for these effects is still unclear.

In the present study, we have found that mitochondrial presequences can induce aggregation of unfolded proteins. The aggregation involves electrostatic interactions and depends on the amphiphilicity of the presequences. Since positive charge and amphiphilicity are necessary for the targeting function of mitochondrial presequences, the aggregation may occur normally as precursors are synthesized in the cytosol and may need to be inhibited by presequence binding factors in order for the precursors to be imported efficiently.

2. Materials and methods

2.1. Synthetic peptides

The synthetic peptides used in this study are listed in Fig. 1. The synthesis and characterization of the peptides were described previously [1]. The peptides were purified by reverse-phase HPLC, and their concentrations were determined by amino acid analyses.

2.2. Preparation of reduced and carboxymethylated α-lactalbumin (RCM-LA) and reduced α-lactalbumin (R-LA)

RCM-LA and R-LA were prepared from bovine α-lactalubumin (Sigma L-6010) as described previously [6]. RCM-LA was dissolved in HCl (pH 3) and stored at 4°C. R-LA was dissolved in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 30 mM DTT and 8 M urea and was stored at −80°C. Concentrations of RCM-LA and R-LA solutions were determined spectrophotometrically using an extinction coefficient at 280 nm of 28,500 M^{-1}.

2.3. Measurement of peptide-induced aggregation

R-LA or RCM-LA was diluted into 20 mM HEPES-KOH, pH 7.4, 50 mM KCl and 2.5 mM DTT (0.4 ml) except when indicated. After 1–5 min at room temperature, the synthetic peptides were added to the reaction mixture, and the increase in turbidity at 320 nm was monitored as a function of time.

3. Results

Many mitochondrial precursor proteins tend to aggregate in the absence of cytosolic factors, but the molecular mechanism of this aggregation is not known. One obvious possibility is that the amino-terminal presequence induces aggregation of the precursors, since the mature proteins alone show less tendency to aggregate [7]. We thus asked if synthetic presequences could affect the aggregation states of unattached, unfolded proteins. We used R-LA and RCM-LA as models for the unfolded proteins. Native α-lactalbumin contains four disulfide bridges, and upon reduction and carboxymethylation, it can be used as a model unfolded polypeptide [6,8]. Neither R-LA nor RCM-LA fold significantly when diluted into aqueous solutions [6,8]. Various concentrations (1–4 μM) of the wild-type yeast cytochrome oxidase subunit IV presequence (WT-CoxIV) were incubated with RCM-LA (2 μM or 4 μM), and aggregation was followed by monitoring the increase in turbidity at 320 nm (Fig. 2). Incubation of WT-CoxIV with RCM-LA increased the tur-

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Abbreviations: RCM-LA, reduced carboxymethylated bovine α-lactalbumin; *E. coli*, *Escherichia coli*; pF_1b, the precursor form of the β subunit of F_1-ATPase; DTT, dithiothreitol; R-LA, reduced bovine α- lactalbumin; DHFR, mouse dihydrofolate reductase; PBF, a presequence-binding factor; MSF, mitochondrial import stimulation factor.

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bidity over a period of several minutes. As the concentrations of WT-Cox IV or RCM-LA were increased, the magnitude of the turbidity change was increased. Incubation of either WT-CoxIV or RCM-LA alone did not result in increased turbidity (not shown).

R-LA has 8 fewer negative charges than RCM-LA. Incubation of WT-CoxIV (4 μM) with R-LA (4 μM) caused a similar increase in turbidity to that observed with RCM-LA (Fig. 3), although the kinetics were somewhat slower. Incubation of the same concentration of WT-Cox IV with the native LA did not cause any increase in turbidity (Fig. 3). These results indicate that WT-CoxIV induces the aggregation of only the unfolded form of LA.

In order to gain insight into the molecular mechanism of the presequence-induced aggregation of unfolded proteins, we tested the effects of salt on the turbidity change. As shown in Fig. 4, the turbidity change of the solution of RCM-LA (4 μM) caused by WT-Cox IV was sensitive to the concentrations of KCl in the reaction mixture; increasing the concentration of KCl decreased the magnitude of the turbidity change. The circular dichroism spectra of RCM-LA alone under these conditions indicated a lack of secondary structure in the protein, and the spectra were affected only slightly by the different concentrations of salt (data not shown). These results therefore suggest that the interactions between the presequence peptide and the unfolded RCM-LA include an electrostatic component. Since WT-Cox IV possesses 5 positive charges and no negative charges, it is not surprising that electrostatic interactions of the positive charge of the presequence with negatively charged regions of unfolded proteins are important for the aggregation process. This interpretation is supported by the observation that the kinetics of the peptide-induced aggregation is slower for R-LA, which contains fewer negative charges, than for RCM-LA (see above). Besides the presequence failed to induce aggregation of the unfolded, but highly basic protein, apocytochrome *c* (data not shown).

We next compared the effects of various presequence analogues on the aggregation of RCM-LA. We tested four peptides, SynA2, SynB, SynC and Δ11,12-CoxIV (Fig. 1), whose structural features, interactions with lipid membranes, and abilities to target proteins to mitochondria have been studied in detail [1,9]. All the peptides have 5 or 6 positive charges and no negative charge, are rich in serine residues, and are similar

WT-CoxIV	MLSLRQSIRFFKPATRTLCSSRYLL-NH_2
Δ11,12-CoxIV	MLSLRQSIRFPATRTLCSSRYLL-NH_2
SynA2	MLSRLSLRLLSRLSLRLLSRYLL-NH_2
SynB2	MLSRQQSQRQSRQQSQRQSRYLL-NH_2
SynC	MLSSLLRLRSLSLLRLRLSRYLL-NH_2

Fig. 1. Amino acid sequences of the synthetic peptides. WT-CoxIV, wild-type presequence of yeast cytochrome oxidase subunit IV; Δ11,12-CoxIV, deletion mutant lacking residues 11 and 12 of WT-CoxIV; SynA2, SynB2, SynC, artificial presequences described in [1,8].

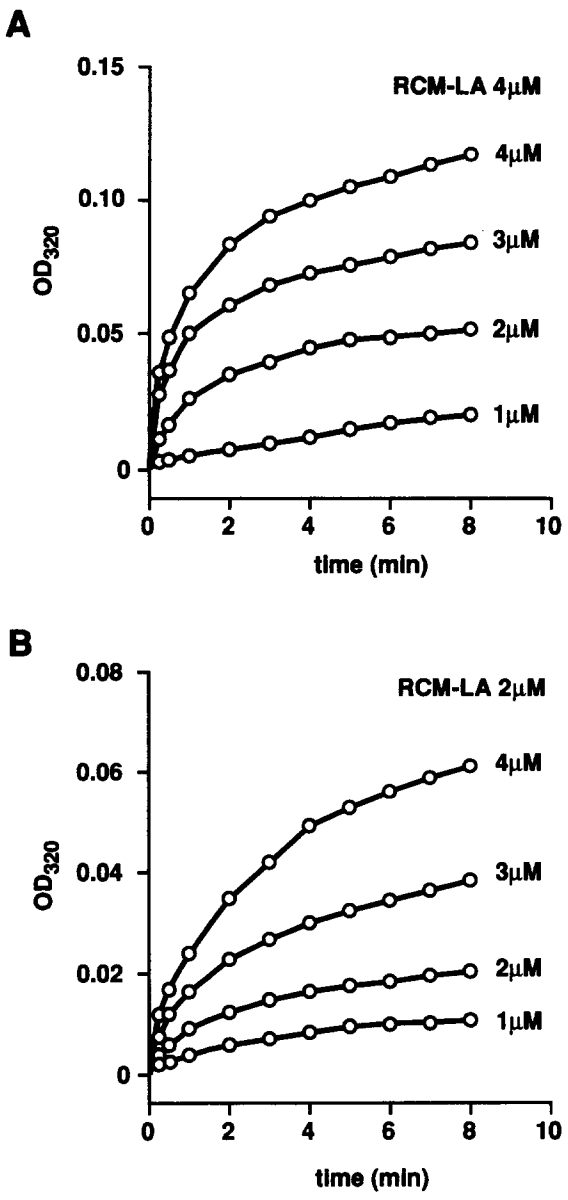

Fig. 2. Aggregation of RCM-LA in the presence of WT-Cox IV. R-LA (4 μM in A, 2 μM in B) was incubated with WT-CoxIV (1 μM, 2 μM, 3 μM or 4 μM) in buffer (20 mM HEPES-KOH, pH 7.4, 50 mM KCl, and 2.5 mM DTT) at room temperature. Formation of aggregates was followed by monitoring the absorbance at 320 nm.

in length. WT-CoxIV, SynA2, SynC, and Δ11,12-CoxIV can, when attached to passenger proteins, target the proteins to mitochondria, whereas SynB2 cannot [1,9]. The relative amphiphilicity of the presequences, as measured by monolayer insertion experiments, is SynA2 > SynC > WT-CoxIV > Δ11,12-CoxIV $\gg$ SynB2. This property is correlated with the ability of the presequences to target a passenger proteins to mitochondria [1].

As shown in Fig. 5, SynA2 and SynC (2 or 4 μM) increased the turbidity of a solution of R-LA (2 or 4 μM) much faster than WT-CoxIV. On the other hand, neither Δ11,12-CoxIV (4 μM) nor SynB2 (4 μM) caused aggregation of R-LA (4 μM). Since WT-Cox IV, SynA2, SynC, and SynB2 have the same charges (+6), electrostatic interactions alone cannot explain the

observed variation in the ability of the peptides to induce protein aggregation. Instead, the ability to cause aggregation of R-LA correlates well with the amphiphilicity of the peptides as measured by monolayer insertion experiments [1].

4. Discussion

In the present study, we have examined the interactions between mitochondrial presequence peptides and model unfolded proteins. The results show that mitochondrial presequences can cause aggregation of unfolded proteins. The ability of the presequences to cause protein aggregation depends on the amphiphilicity and probably on the positive charge of the presequences. Since amphiphilicity and positive charge are necessary for the targeting function of mitochondrial presequences, it may be generally true that mitochondrial presequences can interact with the mature part of a precursor during the folding process and cause aggregation. Although it is not clear whether the interactions between the presequence and the mature part would be intermolecular or intramolecular, the latter possibility would be much more favorable in dilute solutions of precursors and in the presence of interfering proteins. In the experiments described here, the contacts between the presequence and the unfolded protein are clearly intermolecular. Depending on the rate-determining step for aggregation, a higher-order dependence of the rate on the concentrations of presequence and unfolded protein may result in a slower observed rate of aggregation than would occur with normal precursor proteins.

In addition to the positive charge and amphiphilicity of the presequence, the tendency of precursor proteins to aggregate may depend on the charge distribution of the mature part, the kinetics of folding of the mature part, and other properties of any intermediates that are formed during the folding process. For example, a fusion protein consisting of the presequence of CoxIV and mouse dihydrofolate reductase (DHFR; a cytosolic protein) can fold into a native conformation without aggregation [10], and the native fusion protein can subsequently be

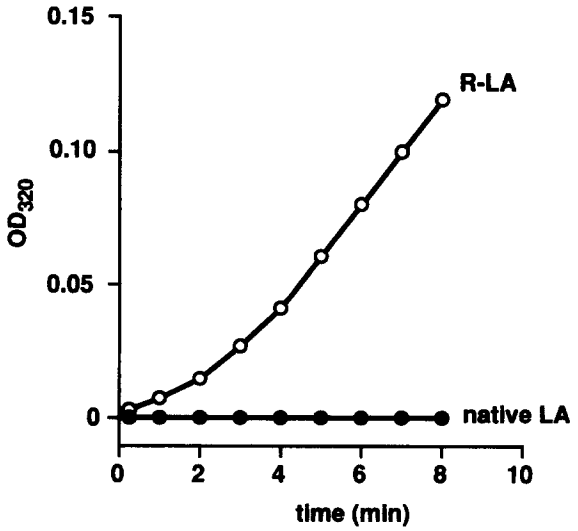

Fig. 3. WT-Cox IV induces aggregation of R-LA, but not of native LA. R-LA or native LA (4 μM) was incubated with WT-CoxIV (4 μM) in buffer (20 mM HEPES-KOH, pH 7.4, 50 mM KCl and 2.5 mM DTT) at room temperature. Formation of aggregates was followed by monitoring the absorbance at 320 nm.

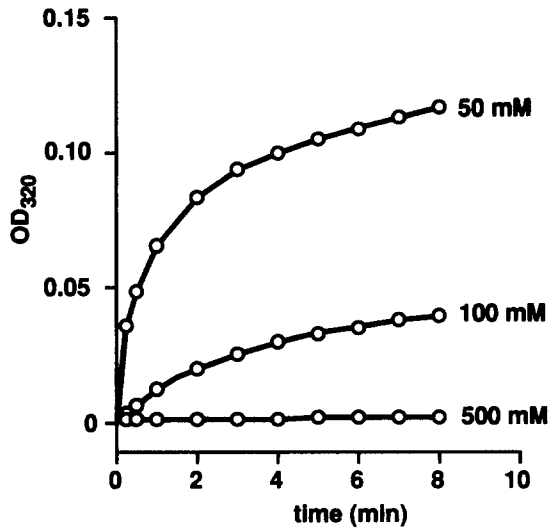

Fig. 4. WT-Cox IV-induced aggregation of RCM-LA depends on the concentration of salt. RCM-LA (4 μM) was incubated with WT-CoxIV (4 μM) in buffer (20 mM HEPES-KOH, pH 7.4, 2.5 mM DTT) containing 50 mM, 100 mM or 500 mM of KCl at room temperature. Formation of aggregates was followed by monitoring the absorbance at 320 nm.

imported into mitochondria in vitro without the aid of cytosolic chaperones. In this case, the DHFR moiety most likely folds into a correct conformation without interacting strongly with the presequence. Interestingly, other fusion proteins with DHFR can also be taken up by mitochondria without the aid of ATP-dependent cytosolic chaperones [11]. However, many other mitochondrial precursor proteins have a tendency to aggregate and require ATP-dependent cytosolic chaperones to maintain their competence for import into mitochondria [11].

It has been pointed out that the average pI values of mitochondrial proteins are more basic by about 1.4 units than those of the corresponding cytosolic isoproteins [12]. The evolutionary selection for positive charge in mitochondrial isoproteins may be related to the possible lower tendency of the basic proteins to interact with presequences. Any interactions between the presequence and the mature part of a precursor would presumably hinder the ability of the precursor to be imported by the mitochondria.

Several cytosolic factors that bind specifically to mitochondrial presequences have been identified in mammalian cells. A presequence-binding factor (PBF) binds to the presequence of several mitochondrial precursor proteins, prevents the aggregation of the precursors, and, in cooperation with cytosolic hsp70, stimulates the import of the precursors into mitochondria [13,14]. Another cytosolic factor, mitochondrial import stimulation factor (MSF), also binds to the presequence of mitochondrial precursor proteins, prevents aggregation of the precursors at the expense of ATP hydrolysis, and facilitates the targeting of the precursors to mitochondria [15]. Hsp70 has also been shown to bind to the presequence of mitochondrial precursor proteins ([16,17]; Endo, T. et al., in preparation). Therefore, PBF, MSF, and cytosolic hsp70 may stabilize the import-competent state of the mitochondrial precursor proteins, at least in part, by a common mechanism, binding to the presequences of precursor proteins.

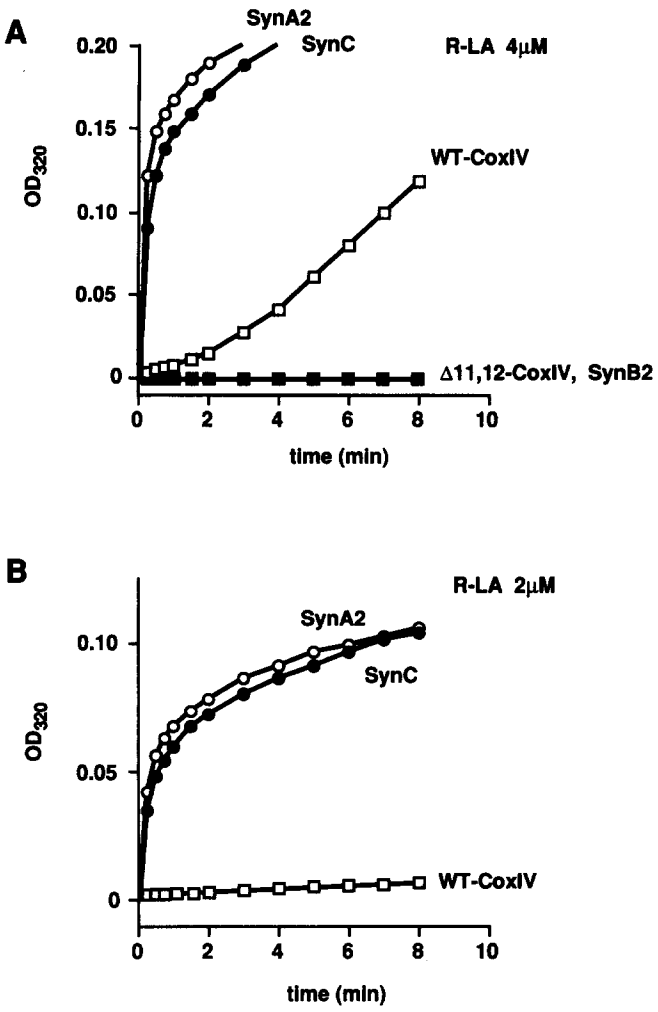

Fig. 5. Aggregation of R-LA in the presence of other presequences. R-LA (4 μM in A, 2 μM in B) was incubated with WT-CoxIV, Δ11,12-CoxIV, SynA2, SynB2 or SynC (4 μM in A, 2 μM in B) in buffer (20 mM HEPES-KOH, pH 7.4, 50 mM KCl, and 2.5 mM DTT) at room temperature. Formation of aggregates was followed by monitoring the absorbance at 320 nm.

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