Step-size Analyses of the Mitochondrial Hsp70 Import Motor Reveal the Brownian Ratchet in Operation*

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Newly synthesized mitochondrial precursor proteins have to become unfolded by the mitochondrial Hsp70 (mtHsp70) import motor to cross the mitochondrial membranes. To assess the mechanism of unfolding of precursor proteins by mtHsp70, we designed a system to measure step sizes of the mtHsp70 import motor, which are distances at which the motor system moves along polypeptide chains during a single turnover of ATP. We made a series of fusion proteins consisting of a mitochondrial presequence containing the first mtHsp70 binding site, a spacer sequence containing an Hsp70 avoidance segment followed by the second mtHsp70 binding site, and different folded mature domains. Analyses of the dependence of the import rates of those fusion proteins on the lengths of Hsp70 avoidance segments allowed us to estimate the step sizes, which differ for different mature domains and different lengths of the spacers. These results suggest that the mtHsp70 import motor functions at least as a molecular Brownian ratchet to unfold mitochondrial precursor proteins.

Protein molecular motors constitute essential cellular devices that convert the chemical energy such as ATP hydrolysis into movement and/or force to perform mechanical works in biological systems (1, 2). Hsp70s4 are a family of ubiquitous molecular chaperones of ~70 kDa with ATPase activities and conduct mechanical works by binding and releasing ~5-residue segments of unfolded protein substrates quickly or slowly in their ATP-bound or ADP-bound states, respectively (3). Several organellar Hsp70s function as import motors to drive vectorial transmembrane movement and/or concomitant unfolding of substrate precursor proteins through import channels (4). For secretary proteins, substrate trapping by Hsp70 or even antibodies in the endoplasmic reticulum lumen was shown to be sufficient to drive translocation of unfolded substrate proteins across the endoplasmic reticulum membrane (5). On the other hand, many folded proteins with a presequence can be imported into mitochondria in a post-translational manner, which requires unfolding of the folded protein domains by mitochondrial Hsp70 (mtHsp70) inside the organelles. However, the mechanism of active client protein unfolding by mtHsp70 has been a matter of longstanding debate (6–8).

Mitochondrial presequences penetrate through the import channels of the translocators in the outer and inner membranes to reach the matrix, where it is grasped by mtHsp70 that is bound to the inner membrane translocator, the TIM23 complex, via its subunit Tim44 (9, 10). Then a second mtHsp70 molecule cooperates with the first mtHsp70 to overcome the rate-limiting step of the import, active unfolding of the folded mature domain by the mechanism, which is still elusive. In the Brownian ratchet model, spontaneous unfolding of the mature domain allows translocation of the unfolded segment plus the presequence through the import channels by Brownian motions, once the first mtHsp70 dissociates from the TIM23 complex after hydrolysis of ATP (6, 7). In the power stroke or lever arm model, mtHsp70 tethered to the outlet of the import channel undergoes a conformational change upon ATP hydrolysis to exert a mechanical pulling force on the precursor protein, which drives unfolding of the mature domain (11). Next in both models, binding of the second mtHsp70 to the translocated segment in the matrix prevents backsliding and refolding, thereby leading to global unfolding of the mature domain for subsequent translocation toward the mitochondrial interior.

In the early 2000s, several new findings were reported in favor of the Brownian ratchet mechanism. Since mtHsp70 is locked on the substrate protein in the ADP form, but not in the ATP form, during its ATPase cycle, the peptide-ADP-mtHsp70 complex needs tethering to the TIM23 complex to generate a mechanical pulling force in the lever arm model, whereas the peptide-ADP-mtHsp70 complex has to be released from the TIM23 complex to allow forward movement of the substrate polypeptide in the Brownian ratchet model. Liu et al. (12) found that in the reconstitution system using purified mtHsp70, Mge1/Yge1, and Tim44, the peptide-ADP-mtHsp70 complex loses affinity for Tim44, which favors the latter situation. In the lever arm model, the distance between the binding sites for mtHsp70 cannot be larger than the one between the peptide binding site and the other end of the two-domain

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4 The abbreviations used are: Hsp70, heat-shock protein 70; mtHsp70, mitochondrial Hsp70; MPP, matrix-processing peptidase; DHFR, dihydrofolate reductase; HBD, heme binding domain of cytochrome b2; MTX, methotrexate; PK, proteinase K; MOPS, 4-morpholinepropanesulfonic acid; VS, varying spacer length; CS, constant spacer length.
Hsp70 structure (~115 Å) (13, 14), otherwise, whereas mtHsp70 pulls in the substrate precursor protein by the distance as large as 115 Å by using Tim44 as a fulcrum, the second mtHsp70 cannot bind to the incoming segment to prevent backsliding. Okamoto et al. (15) found that mitochondrial precursor proteins containing a 50-residue segment (~170 Å in an extended β-strand (16)) that cannot bind to mtHsp70 in front of folded domains can be imported into mitochondria, which is incompatible with the lever arm model.

However, subsequent identification of several new mitochondrial Hsp70-associated motor and chaperone (MMC) proteins, partners for mtHsp70 mediating mitochondrial protein import, has changed the situation (17, 18). Among them, Tim14/Pam18 is a J protein specific for mtHsp70 and forms a complex with another J-like protein Tim16/Pam17. Tim44, which functions as a tethering partner in the TIM23 complex for mtHsp70, is necessary to link the Tim14–Tim16 J complex to the TIM23 complex as well. This raised a possibility that nucleotide-dependent tethering to and release from the TIM23 complex for the peptide-mtHsp70 complex could be regulated by those additional partner proteins, which makes the findings by Liu et al. (12) not crucial for the Brownian ratchet model. It also raised a possibility of the multiprotein lever arm consisting of mtHsp70 with e.g. Tim44, Tim14, Tim16, and/or Pam17, which could undergo a complex conformational change or power stroke of >50 residues, rendering the findings by Okamoto et al. (15) not crucial for the Brownian ratchet model as well. Besides, the entropic pulling model (19) was proposed for the generation of a pulling force with the nucleotide dependence of mtHsp70 tethering to the TIM23 complex, compatible with the findings by Liu et al. (12). In this new model, the first mtHsp70 bound to the incoming precursor diffuses away from the TIM23 channel outlet to increase tumbling entropy in a more wide open space, which results in a mechanistic pulling force to achieve unfolding of the mature domain (8, 19). Therefore, it became open again which of those different models mainly contributes to active unfolding of mitochondrial precursor proteins by the mtHsp70 import motor system.

To assess the roles of mtHsp70 in unfolding of precursor proteins, we established here a system to characterize mtHsp70 as a molecular stepping motor, since the mitochondrial Hsp70 system can proceed on the track of an unfolded polypeptide. Since two molecules of mtHsp70 alternately interact with the incoming polypeptide at the outlet of the TIM23 channel in a hand-over-hand manner, at least one mtHsp70 always stays on the substrate polypeptide to prevent it from slipping off the import channels, thereby functioning as a “processive” import motor (9, 10, 20). The operation mechanism of a processive stepping motor can be accessed by the analyses of its “step size” (21). The step size of the mtHsp70 import motor is a distance at which the mtHsp70 motor system moves upon a single step, i.e. during a single turnover of ATP. Step sizes of the motor proteins can often be analyzed by single molecule tracking, which is yet difficult to apply to such complex membrane-based systems as the mitochondrial mtHsp70 motor machinery. We thus set up an alternative experimental system with an appropriate theoretical framework to estimate the step sizes of the mitochondrial mtHsp70 import motor in intact organelles. Employing this system, we measured step sizes for model mitochondrial precursor proteins with a different mature domain. The estimated step sizes differ for different mature domains and different lengths of the spacers between the precursor and mature domain, therefore indicating that the Brownian ratchet is a sufficient and minimal mechanism for mtHsp70 to drive unfolding of precursor proteins.

**EXPERIMENTAL PROCEDURES**

**Model Fusion Proteins**—The genes for the dihydrofolate reductase (DHFR), barnase (with mutation H102A), and heme binding domain of cytochrome b2 (HBD) fusion proteins containing variable spacer sequences of n = 10–80 residues with different lengths (m) of the Gly repeat (Gly(m)) (see Fig. 2A) were constructed as follows. The DNA fragments (Gly(10)–N (5’-GGGAGGCGGTGAGGCGGTGAGGCGGGAGGCCCT-AGAGCTCATG-3’) and Gly(10)–C (5’-CATGAGCTTCTAGGCCTCGCCCTCCACCCTCCGCCCTCCC-3’)) for the 10-residue Gly repeat (Gly(10)); the DNA fragments (NA-N (5’-GGTATGCCACCGCTTCTTTGTCAAGGCTAGAGCTCATG-3’) and NA-C (5’-CATGAGCTTCTAGGCCGTGAGCAAGAGCGGTGAGGCGGTGAGGCGGTGAGGCGGGAGGCCCT-AGAGCTCATG-3’)) for the NA segment (GMANALLLTG); and the DNA fragments (NR-N (5’-GGATGCGCAAGACAGCTTCTGCTCAAGGCTAGAGCTCATG-3’) and NR-C (5’-CATGAGCTTCTAGCCCTGTGAGAAGTCTGTGGCCCATACC-3’)) for the NR segment (GMANRLLLTTG) were digested with SmaI and SacI and inserted into the SmaI/SacI sites of pBluescript II SK(−) to produce pBSK/Gly(10), pBSK/NA, and pBSK/NR, respectively. The DNA fragments for Gly(10), NA, and NR were cut out from pBSK/Gly(10), pBSK/NA, and pBSK/NR, respectively, with SmaI and SacI and were subsequently inserted into the Stul/Sacl sites of pBSK/Gly(10), resulting in the DNA fragments for Gly(20), Gly(10)–NA, and Gly(10)–NR. Similar procedures were taken to construct the cassette DNAs for Gly(20)–N, Gly(30)–N, Gly(40)–N, Gly(50)–N, Gly(60)–N, Gly(0)–NN, Gly(10)–NN, Gly(20)–NN, Gly(30)–NN, Gly(40)–NN, Gly(50)–NN, Gly(60)–NN, Gly(0)–NGNG, Gly(10)–NGNG, Gly(20)–NGNG, Gly(30)–NGNG, Gly(40)–NGNG, Gly(50)–NGNG, Gly(60)–NGNG, and Gly(70), where N indicates NA or NR and G indicates Gly(10). pBSK/Gly(70) linker DNA was subsequently amplified from pBSK/Gly(70) by PCR using the primers Gly-N (5’-GATATCGAATTCCTGCAGCCC-3’) and Gly(70)-C (5’-CGTCTGAGCAAGCTGTGGCCCATACC-3’) and inserted into the EcoRI/XhoI sites of pBluescript II SK(−). The plasmid bearing a DNA fragment for pb2(220)-DHFR (22) was digested with EcoRI and BamHI to obtain a DNA fragment for the first 167 residues of the cytochrome b2 precursor and inserted into the EcoRI/BamHI sites of pUC119. Then the DNA fragment for residues 43–77 of the cytochrome b2 precursor was replaced with the one with the Smal/Xhol site by PCR using the primer B2-40Sm2 (5’-CTCAAGCTTCCCGGGCTCGGAATAGATAGAAGGCCGA-3’) to yield pUC119/pb2(42). pBSK/Gly(70)+ linker was digested with Smal and Xhol and inserted into the Smal/Xhol sites of pUC119/pb2(42) to yield pUC119/pb2(42)-Gly(70)+ linker. pUC119/pb2(42)-Gly(70)+ linker was then digested with EcoRI.
and BamHI and inserted into the EcoRI/BamHI sites of pGEM2/pSu9(69)-HBD\textsuperscript{5} to yield the DNA fragment for the fusion protein consisting of the first 42 residues of the cytochrome \(b\_2\) precursor, Gly(70), the 10-residue linker segment (LELQLSRIDN), and the HBD. The DNA fragment for Gly(70) was replaced by PCR with the other cassette DNAs described above to obtain DNA fragments for the HBD fusion proteins with different spacer segments. The DNA fragment for the HBD was replaced with those for DHFR and barnase to obtain those for the DHFR and barnase fusion proteins.

\textit{In Vitro Import}—\textit{In vitro} protein import into isolated mitochondria was performed as described previously (23, 24). Briefly, the fusion proteins were synthesized in rabbit reticulocyte lysate by coupled transcription/translation in the presence of \(^{[35]}\text{S}\)methionine. The radiolabeled fusion proteins were incubated with isolated yeast mitochondria (0.5 mg protein/ml) in import buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2, 80 mM KCl, 2.5 mM KPi, 2 mM methionine, 5 mM dithiothreitol, 5 mM MgCl\(_2\), 2 mM ATP, 2 mM NADH, 1% bovine serum albumin) at 16 or 30 °C. The import reactions were stopped by adding valinomycin to 10 \(\mu\)g/ml. The samples were halved, and one aliquot was treated with 50 \(\mu\)g/ml proteinase K (PK) for 20 min on ice. After the addition of 1 mM phenylmethylsulfonyl fluoride, the mitochondria were resuspended by centrifugation and were washed once with SEM buffer (250 mM sucrose, 5 mM EDTA, and 10 mM MOPS-KOH, pH 7.2). Proteins were analyzed by SDS-PAGE and radioimaging with a Storm 860 image analyzer (Amersham Biosciences).

\section*{RESULTS AND DISCUSSION}

Utilization of the \(\text{mtHsp70}\) Avoidance Segment between \(\text{mtHsp70}\) Binding Sites to Measure Step Sizes of the Import Motor—Since a step size of the processive import motor can be operationally measured as a maximal distance between the binding sites for the first and second \(\text{mtHsp70}\) molecules, we designed a series of model mitochondrial precursor proteins containing an Hsp70 avoidance segment that cannot bind to Hsp70 tightly. Assume that various lengths of the Hsp70 avoidance segment (\(m\)-residues) are inserted between the first and second \(\text{mtHsp70}\) binding sites (10 residues each) in the presequence and following the spacer sequence (\(l\)-residues), respectively, in front of the folded mature domain in model mitochondrial precursor proteins (Fig. 1A). After binding of \(\text{mtHsp70}\) to the first (N-terminal) binding site, the second \(\text{mtHsp70}\) binding site is mechanically pulled in (the lever arm and entropic pulling models) or is allowed to move forward by Brownian motions after spontaneous and transient unfolding of the folded domain outside mitochondria (the Brownian ratchet model) in the import channels by a distance of the step size (Fig. 1B, \textit{left panels}). However, if the \(\text{Hsp70}\) avoidance segment is longer than the step size, the second binding site for \(\text{mtHsp70}\) located downstream of the \(\text{Hsp70}\) avoidance segment is not accessible from the matrix so that backsliding and refolding of the precursor protein will prevail. Therefore, when we systematically change the length of the \(\text{Hsp70}\) avoidance segment \(m\), we can determine the step size of the \(\text{mtHsp70}\) motor as a function of the \(\text{Hsp70}\) avoidance segment length (\(\text{mc}\_x\)) of the spacer sequences, respectively. The distance across two mitochondrial membranes corresponds to 50 residues. \(x\) is the length (in residues) of the segment that enters the matrix by spontaneous unfolding (the Brownian ratchet model) or by \(\text{mtHsp70}\) pulling (the lever arm and entropy-pulling models). In the Brownian ratchet model, the unfolding size (\(y\)) is constant over different lengths (\(l\)) of the spacer sequences, whereas in the lever arm and entropy-pulling models, the pulling distance (\(x\)) remains constant.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{How to measure the step size of the mitochondrial import motor. \(A\), fusion proteins used for step-size estimation. \(l\) is the length (in residues) between the presequence and the folded mature domain, \(m\) is the length (in residues) of the \(\text{Hsp70}\) avoidance segment (blue) sandwiched by the two 10-residue \(\text{Hsp70}\) binding sites (\(y\)). \(B\), the \textit{left panels} show \(y\) residue unfolding of the fusion protein by the Brownian ratchet mechanism (\textit{upper panel}), lever arm mechanism (\textit{central panel}), and entropy-pulling mechanism (\textit{lower panel}), and the \textit{right panels} show possible dependences of import rates on the lengths of Gly repeats and the step size (\(m\_x\) in residues) as the Gly repeat length at which the import rate drops. \(m\_1\), \(m\_2\), and \(m\_3\) are step sizes for the \(l\_1\), \(l\_2\), and \(l\_3\) residue spacer sequences, respectively. The distance across two mitochondrial membranes corresponds to 50 residues. \(x\) is the length (in residues) of the segment that enters the matrix by spontaneous unfolding (the Brownian ratchet model) or by \(\text{mtHsp70}\) pulling (the lever arm and entropy-pulling models). In the Brownian ratchet model, the unfolding size (\(y\)) is constant over different lengths (\(l\)) of the spacer sequences, whereas in the lever arm and entropy-pulling models, the pulling distance (\(x\)) remains constant.}
\end{figure}

\textsuperscript{5} M. Esaki, T. Kanamori, S. Nishikawa, and T. Endo, unpublished results.

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FIGURE 1. How to measure the step size of the mitochondrial import motor. A, fusion proteins used for step-size estimation. \(l\) is the length (in residues) between the presequence and the folded mature domain, \(m\) is the length (in residues) of the \(\text{Hsp70}\) avoidance segment (blue) sandwiched by the two 10-residue \(\text{Hsp70}\) binding sites (\(y\)). \(B\), the \textit{left panels} show \(y\) residue unfolding of the fusion protein by the Brownian ratchet mechanism (upper panel), lever arm mechanism (central panel), and entropy-pulling mechanism (lower panel), and the \textit{right panels} show possible dependences of import rates on the lengths of Gly repeats and the step size (\(m\_x\) in residues) as the Gly repeat length at which the import rate drops. \(m\_1\), \(m\_2\), and \(m\_3\) are step sizes for the \(l\_1\), \(l\_2\), and \(l\_3\) residue spacer sequences, respectively. The distance across two mitochondrial membranes corresponds to 50 residues. \(x\) is the length (in residues) of the segment that enters the matrix by spontaneous unfolding (the Brownian ratchet model) or by \(\text{mtHsp70}\) pulling (the lever arm and entropy-pulling models). In the Brownian ratchet model, the unfolding size (\(y\)) is constant over different lengths (\(l\)) of the spacer sequences, whereas in the lever arm and entropy-pulling models, the pulling distance (\(x\)) remains constant.

Evidently, step sizes depend on the folded mature domains differently in the different models. In the lever arm model, a conformational change of the lever involving \(\text{mtHsp70}\) mechanically pulls in the presequence by the distance of the linkage of the \(\text{Hsp70}\) avoidance segment (\(m\_x\)) at which the import rate significantly decreases (Fig. 1B, \textit{right panels}).
step size. In the entropic pulling model, tumbling entropy drives a mechanical pulling force, which depends only on the distance of mtHsp70 from the outlet of the import channel, which is as large as 30 Å (19). Therefore, the step size as a maximal distance between the two mtHsp70 binding sites is constant for different mature domains in those two models (Fig. 1B, central and lower left panels). On the other hand, in the Brownian ratchet model, the maximal distance between the first Hsp70 that had bound to the presequence and the second Hsp70 that binds to the incoming unfolded segment depends on the size of spontaneous unfolding of the folded mature domain. The step size thus differs for different mature domains (Fig. 1B, upper left panel).

Further consideration of the schematic models (Fig. 1B) reveals that step sizes depend on the total spacer lengths (l) differently in different models as well. Since 50 residues are long enough to span the two mitochondrial membranes (9, 25, 26), (l-50) residues at the C terminus of the spacer sequence in front of the folded mature domain remain outside the import channels in an unfolded state in the beginning (the initial states in the three models in Fig. 1B, left panels). Then in the Brownian ratchet model, if the N-terminal y residue segment in the mature domain becomes spontaneously unfolded, x or (y+l-50) residues of the fusion protein outside the mitochondria can enter the import channels by Brownian motions once the first mtHsp70 locked onto the fusion protein dissociates from the TIM23 complex in the matrix (Fig. 1B, upper left panel). In the lever arm and entropic pulling models, mtHsp70 directly pulls in the fusion protein by the distance of the conformational change of the mtHsp70 fulcrum or the entropy-driven diffusion (x residues), which leads to N-terminal unfolding of y or (x-l+50) residues in the mature domain (Fig. 1B, central and lower left panels).

In all those three models, the distance between the binding sites for the first and second mtHsp70 molecules, x, and the size of the local unfolding in the mature domain, y, are related to each other by the equation

$$y = x - l + 50 \quad \text{(Eq. 1)}$$

The size of local unfolding, y, directly linked to the step size, can be thus estimated as (m_r - l + 50). Evidently, although the induced unfolding size (y), but not the step size (m_r), depends on the lengths of the spacer sequences (l) in the lever arm and entropic pulling models (Fig. 1B, central and lower right panels), the Brownian ratchet model predicts that the step size (m_r) for the same size of local unfolding (y) of the mature domain may well depend on the lengths of the spacer sequences (l) (Fig. 1B, upper right panel). Therefore, dependence of the step size (m_r) on the length of mtHsp70 avoidance segment can be used to estimate the contribution of the Brownian ratchet mechanism in unfolding of the folded mature domains. Since the Hsp70 avoidance segment length dependence curves of the import rates differ for different spacer lengths l in any of the three models (Fig. 1B, right panels), a step size m_r can be estimated only within a series of fusion proteins with the same spacer sequence length l, irrespective of the operating model.

Model Fusion Proteins with an Hsp70 Avoidance Segment—
We made a series of model fusion proteins containing a 43-residue mitochondrial matrix-targeting presequence (the N-terminal 42 residues of the cytochrome b2-presequence plus one additional residue), spacer sequences of different numbers of 10-residue Gly repeat units (blue and mtHsp70 binding units (yellow); NA, GMANALLLTG; NR, GMANRLLLTG), a 10-residue linker segment, and the mature domain (DHFR, barnase, or the HBD). AA, amino acids. B, the structures of DHFR, barnase, and the HBD. α-Helices and β-strands are indicated in red and blue, respectively. The atomic coordinates were taken from the Protein Data Bank (1DHF, 1B27, and 1FCB, respectively).
dependence curve of the import rates of the fusion proteins with the same spacer sequence length (Fig. 1B), we also prepared a CS (constant (spacer length) series of fusion proteins with a constant length \( l = (n + 10) \) residues of the spacer segment that consists of the \( m \)-residue Gly repeat \((m = n-40, n-30, n-20, \) or \( n-10 \)) followed by an \((n-m)\)-residue alternate sequence of the 10-residue NR or NA segment and a 10-residue Gly repeat and the 10-residue linker segment (Fig. 2A).

We first confirmed that the presequence and spacer segments of the fusion proteins contain a high affinity binding site for mtHsp70 and an mtHsp70 avoidance segment, respectively, as expected. We incubated the CS DHFR fusion proteins with a 50-, 60-, 70-, or 80-residue spacer sequence \((n = 40, 50, 60, \) or \( 70 \)) containing a 30-residue Gly repeat and the NR segment as a binding site for mtHsp70 (Fig. 2A) with isolated mitochondria in the presence of methotrexate (MTX). Since a DHFR domain stabilized by its ligand MTX cannot go across the outer membrane, a presequence-containing DHFR fusion protein complexed with MTX forms a two-membrane spanning intermediate. The MTX-stabilized intermediate becomes resistant against PK only if mtHsp70 is tightly associated with the segment of the intermediate that is exposed to the matrix since tight binding of mtHsp70 blocks backsliding of the intermediate through the import channel so that the DHFR domain is tightly apposed to the outer membrane to prevent access of the added protease to the spacer segment (6, 28).

Now we found that the presequence-cleaved mature forms of the fusion proteins with a 50- or 60-residue spacer sequence were PK-resistant, whereas those with a 70- or 80-residue spacer sequence were PK-sensitive (Fig. 3). This indicates that the DHFR domains of the fusion proteins with a 50- or 60-residue spacer, but not of those with a 70- or 80-residue spacer, are closely apposed to the outer membrane. A previous study showed that \(-52\) amino acid residues in front of a folded protein domain are sufficient to span both mitochondrial membranes and to allow the interaction with mtHsp70 in the matrix (9). Therefore, mtHsp70 binds to the C-terminal part (residues 33–43) of the presequence after cleavage of the N-terminal part by MPP so that the fusion protein is prevented from backsliding in the import channels. On the other hand, since the DHFR domain of the fusion proteins with a 70- or 80-residue spacer is not closely apposed to the outer membrane, the Gly repeat of those with a 70- or 80-residue spacer that is exposed to the matrix by Brownian motions is not capable of binding to mtHsp70 so that the fusion proteins can backslide in the import channels for access to the added protease. Therefore, the C-terminal part (residues 33–43) past the MPP cleavage site of the presequence provides a high affinity binding site for mtHsp70, whereas a Gly repeat sequence functions as an mtHsp70 avoidance segment.

In Vitro Import of the Fusion Proteins Depends on Gly Repeat Lengths and Folded Mature Domains—The radiolabeled DHFR, HBD, and barnase fusion proteins with a spacer sequence containing different lengths of the Gly repeat were synthesized in reticulocyte lysate. Protease treatment of the fusion proteins revealed that folding of the mature domains is not significantly affected by the attachment of the presequence and spacer sequences (data not shown). The fusion proteins were incubated with isolated yeast mitochondria at 16 or 30 °C in the presence of ATP. Amounts of protease-protected, imported mature forms were plotted against incubation time, and import rates were determined from the initial slopes for VS and CS fusion proteins (Fig. 4). Under the present import conditions, unfolding of the folded mature domain is rate-limiting in the entire import process (29). We found first that both VS and CS series of DHFR, HBD,
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**A VS series**

![Graphs showing initial import rate vs. Gly repeat length for DHFR, NA, 16°C, DHFR, NR, 16°C, HBD, NA, 16°C, HBD, NR, 16°C, barnase, NA, 16°C, barnase, NR, 16°C.

**B CS series**

![Graphs showing initial import rate vs. Gly repeat length for DHFR, NA, 30°C, DHFR, NR, 30°C, DHFR, NA, 16°C, DHFR, NR, 16°C, HBD, NA, 16°C, HBD, NR, 16°C, barnase, NA, 16°C, barnase, NR, 16°C.

**FIGURE 4. Import of model fusion proteins into isolated mitochondria.** Radiolabeled VS (A) and CS (B) fusion proteins were incubated with isolated yeast mitochondria at 16 or 30 °C. Imported and protease-protected proteins were analyzed by SDS-PAGE and radioimaging, and import rates were plotted. The amounts of radiolabeled proteins added to each reaction are set to 100%. Error bars represent S.D. from three independent experiments. aa, amino acids.

...and barnase fusion proteins with the NR segments behind the Gly repeats exhibited higher import rates than those with NA segments (Fig. 4). This points to the important role of binding of the second mtHsp70 to the incoming unfolded segment, likely in preventing the backsliding and refolding of the remaining part of the substrate protein. Second, we found that the Gly repeat length dependence curves or step sizes of CS series of the fusion proteins differ for different lengths of the spacer sequences (Fig. 4). This points to the important role of binding of the second mtHsp70 to the incoming unfolded segment, likely in preventing the backsliding and refolding of the remaining part of the substrate protein.

The suggested unfolding sizes or sizes of the unfolding structural units for the HBD, barnase, and DHFR are consistent with their structures (Fig. 2B). In the HBD and barnase, residues 12–17 and residues 7–17, respectively, form helical structural units, the opening of which (~20 residues) appears to involve disruption of the local hydrophobic cores, and its trapping by the second mtHsp70 may efficiently trigger global unfolding. On the other hand, both the N-terminal segment and the C-terminal segment of DHFR are involved in the same β-sheet struct...
ture, and the N-terminal β-strand is further sandwiched by the two α-helices. Therefore, although the N-terminal 10-residue β-segment plus 10-residue loop may spontaneously unfold (~20 residues) and trigger global unfolding, its unfolding is likely inefficient and comparable with that of more global unfolding (~40 residues).

Here we consider the case in which unfolding of the revealed N-terminal structural units take place in a highly cooperative manner. In such a case, unfolding could be triggered by not only spontaneous and transient structural breathing (the Brownian ratchet model) but also partial disruption of the structural elements by a mechanical pulling force. Then the unfolded segments can further enter the matrix by Brownian motions if the bound first mtHsp70 is released from the TIM23 complex (in the Brownian ratchet and entropy-pulling models but not in the lever arm model) and can be trapped by the second mtHsp70. Therefore, if positions, sizes, and stabilities of the N-terminal structural units are sufficiently appropriate, the entropy-pulling mechanism is logically not mutually exclusive with the Brownian ratchet mechanism.

We also note in Fig. 4 that the import rates of the CS barnase fusion proteins, but not their VS versions or the HBD fusion proteins, exhibited peculiar reverse V-shaped dependence on the Gly repeat length; import rates are smaller for the CS fusion proteins with \( m = n = 40 \) than those with \( m = n = 30 \) (e.g. compare fusion proteins with \((m, n) = (0, 40), (10, 50), \) and \((20, 60)\) with those with \((m, n) = (10, 40), (20, 50), \) and \((30, 60)\), respectively). This reverse V-shaped dependence of the import rates on the Gly repeat length can be also explained by the framework of the Brownian ratchet model. Fig. 5 shows schematic representation of the second mtHsp70 binding to the CS barnase fusion proteins with transient unfolding of the N-terminal 10-, 20-, or 30-residue region. Since CS fusion proteins have only a 10-residue NR or NA segment as the second mtHsp70 binding, CS fusion proteins with \( m = n = 40 \) and \( m = n = 30 \) allow the transiently opened species with N-terminal unfolding of ~10 or ~30 residues and ~20 residues, respectively, in the mature domains to be trapped by the second mtHsp70 if we ignore larger (~40 residues) unfolding for simplification (crosses indicate that the second mtHsp70 cannot bind to the incoming NR or NA segment). According to the structure of barnase (Fig. 2B), residues 7–17 of barnase form an amphiphilic helix on the periphery of the molecule, which may well form a cooperative structural unit to become detached transiently from the rest of the molecule through ~20-residue N-terminal unfolding. On the other hand, 10-residue N-terminal unfolding of barnase does not seem to take place frequently because it is achieved only by partial, but not entire, disruption of the amphiphilic helix (residues 7–17). Thirty-residue unfolding also seems to take place infrequently because hydrophobic interactions between Leu-20 and Tyr-24 likely prevent further large (~30-residue) unfolding after detachment of the amphiphilic helix. Thus for the barnase fusion proteins, 20-residue unfolding may well take place most frequently, and trapping of 20-residue unfolding is the most efficient in inducing global unfolding so that the fusion proteins with \( m = n = 30 \) were imported most efficiently (Fig. 4B).

**Step-size Analyses of Mitochondrial Import Motor**

![Image of Step-size Analyses of Mitochondrial Import Motor](image)

**FIGURE 5. Interpretation of the peculiar Gly repeat length dependence of the import rates of barnase fusion proteins.** A schematic representation of the second mtHsp70 binding to barnase fusion proteins in the matrix is shown. mtHsp70 binds to the incoming segment of the CS fusion proteins with a 50-residue spacer containing a 10-, 20-, or 30-residue Gly repeat with different sizes of N-terminal unfolding (\( y = 10, 20, \) or 30 residues). The N-terminal white box, the following blue boxes, the yellow boxes, the white boxes, and the gray box represent the presequence or the first mtHsp70 binding site, the 10-residue Gly repeat units, the 10-residue NR or NA units, and the 10-residue linker segment, respectively.

**Conclusions**—In the present study, we have established a novel method to determine the step sizes of the protein import motor for mitochondrial precursor proteins. Estimated step sizes of the mtHsp70 processive import motor differ for different mature domains and depend on the lengths of the spacers between the presequence and mature domain. These results suggest that the Brownian ratchet is a sufficient and minimal mechanism to explain active unfolding of precursor proteins by two mtHsp70 molecules for protein import, although additional (likely minor) contribution of the lever arm and/or entropic pulling mechanism, depending on the mature domains, presequences, etc., is not ruled out. In other words, the amplitude and frequency of spontaneous N-terminal unfolding of the mature domain are sufficient, if harvested efficiently by the Brownian ratchet trapping, to promote rate-limiting global unfolding of the mature domain. This also means that the analyses of the step sizes of the mitochondrial mtHsp70 motor could reveal detailed dynamic aspects of spontaneous local unfolding around the N-terminal region of the folded proteins, which could complement the information obtained by e.g. NMR hydrogen-deuterium (H-D) exchange experiments. The proposed method to estimate step sizes of the unfolding motor is readily applied to other protein unfolding machineries that are too complex to use single-molecule tracking techniques, including translocators in other membranes.
and ATP-dependent proteases, by introducing appropriate segments for motor binding as well as motor avoidance into substrate polypeptides as a track for the motor system.

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