

# Effect of protein structure on mitochondrial import

Alexander J. Wilcox\*, Jason Choy†, Carlos Bustamante\*<sup>§¶</sup>, and Andreas Matouschek\*<sup>¶</sup>

\*Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208; and Departments of †Chemistry and ‡Physics and §Howard Hughes Medical Institute, University of California, Berkeley, CA 94720

Contributed by Carlos Bustamante, August 22, 2005

**Most proteins that are to be imported into the mitochondrial matrix are synthesized as precursors, each composed of an N-terminal targeting sequence followed by a mature domain. Precursors are recognized through their targeting sequences by receptors at the mitochondrial surface and are then threaded through import channels into the matrix. Both the targeting sequence and the mature domain contribute to the efficiency with which proteins are imported into mitochondria. Precursors must be in an unfolded conformation during translocation. Mitochondria can unfold some proteins by changing their unfolding pathways. The effectiveness of this unfolding mechanism depends on the local structure of the mature domain adjacent to the targeting sequence. This local structure determines the extent to which the unfolding pathway can be changed and, therefore, the unfolding rate increased. Atomic force microscopy studies find that the local structures of proteins near their N and C termini also influence their resistance to mechanical unfolding. Thus, protein unfolding during import resembles mechanical unfolding, and the specificity of import is determined by the resistance of the mature domain to unfolding as well as by the properties of the targeting sequence.**

protein unfolding | mitochondria | protein import | protein sorting | atomic force microscopy

**P**rotein unfolding is intimately associated with protein import into the mitochondrial matrix (1). Many mitochondrial proteins are synthesized in the cytosol as precursors containing an N-terminal targeting sequence or presequence followed by a folded mature domain. These proteins must be translocated across the two mitochondrial membranes to reach the matrix (2). During translocation, precursors are fully unfolded and threaded through the import channel amino acid by amino acid (1, 3). Mitochondria can import some precursor proteins many hundreds of times faster than these proteins can unfold spontaneously (4, 5), indicating that mitochondria can catalyze unfolding. Mitochondria accelerate the unfolding of synthetic precursors derived from the *Bacillus amyloliquefaciens* ribonuclease barnase by altering their spontaneous unfolding pathway to a mechanical one in which the mature domain is unraveled from its N terminus (4). Import and unfolding require both an electrical potential across the inner membrane and ATP hydrolysis by mitochondrial heat shock protein 70 in the matrix (2).

Fusing a mitochondrial targeting sequence to the N terminus of a cytosolic protein is usually sufficient to target it to mitochondria (6), and the efficiency of import is influenced by the amino acid sequence of the targeting signal. However, several observations suggest that targeting information is not limited to the presequences themselves. First, the targeting signal of the mitochondrial matrix protein superoxide dismutase induced efficient import of mouse dihydrofolate reductase but not yeast invertase when fused to these proteins (7). Second, the folding state of the mature domain can influence import efficiency. Precursors whose mature domains are destabilized by urea are imported better than native proteins *in vitro* (5, 8). Third, substituting the mitochondrial targeting signal of cytochrome oxidase subunit IV with randomly generated sequences resulted in one-quarter of the chimeric proteins still being targeted to mitochondria (9, 10).

Here, we investigate the effect of a mature domain's structure on precursor import. We find that the susceptibilities of proteins to unfolding by mitochondria, and thus their import efficiencies, are determined by their local structures adjacent to the targeting sequences. Specifically, mitochondria can unravel precursors much more effectively when the targeting sequence leads into a surface  $\alpha$ -helix than when it leads into a buried  $\beta$ -strand. The explanation of this observation appears to be that the extent to which the import machinery can modify the unfolding pathway of the targeted protein, lowering its activation energy, varies with the local structure first to encounter the entrance to the import channel.

## Materials and Methods

**Precursor Proteins.** Mitochondrial precursor proteins were constructed by fusing varying lengths of the N-terminal portion of precytochrome *b*<sub>2</sub> to the N termini of *B. amyloliquefaciens* ribonuclease barnase (5), the *E. coli* chemotaxis response regulator CheY (11), or circular permutants (CPs) of *E. coli* dihydrofolate reductase (DHFR) (12) in pGEM-3Zf(+) vectors. The CheY mutational analysis was conducted in F14N/V54T double mutant as pseudo wild type into which the mutations V11T, V33T, A42G, V83T, A98G, and A103G were introduced (11). All barnase precursors contained the inactivating mutation H102A. The effects of mutations on barnase import were taken from Huang *et al.* (4). The targeting sequences were mutated to prevent disulfide-bridge formation between targeting sequences (C14V), to prevent processing by the mitochondrial matrix processing protease (R30G) (13), and to inactivate the stop-transfer signal (L62P) (14), ensuring that this protein was targeted to the mitochondrial matrix. Radioactive precursors were expressed from a T7 promoter by *in vitro* transcription and translation in a rabbit reticulocyte lysate supplemented with [<sup>35</sup>S]methionine (Promega). Ribosomes and associated incompletely translated polypeptide chains were removed by centrifugation at 150,000 × *g* for 15 min. Precursor proteins were then partially purified by precipitation in 50% saturated ammonium sulfate for at least 1 h on ice, pelleted by centrifugation at 20,800 × *g* for 15 min, and resuspended in import buffer (0.6 M sorbitol/50 mM Hepes-KOH, pH 7.4/50 mM KCl/10 mM MgCl<sub>2</sub>/2 mM KH<sub>2</sub>PO<sub>4</sub>/5 mM unlabeled methionine/1 mg/ml fatty acid-free BSA). In the DHFR experiments, the buffer also contained the appropriate concentrations of methotrexate.

Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B (MAT $\alpha$ , American Type Culture Collection 2567) (15) and purified by centrifugation through a Nycodenz gradient (Nycomed, Oslo) (16).

**Import Experiments.** Import kinetics were determined as described in ref. 5. Thirty microliters of radiolabeled precursors

Abbreviations: AFM, atomic force microscopy; CP, circular permutant; DHFR, dihydrofolate reductase.

<sup>¶</sup>To whom correspondence may be addressed. E-mail: carlos@alice.berkeley.edu.

<sup>§</sup>To whom correspondence may be addressed at: Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3500. E-mail: matouschek@northwestern.edu.

© 2005 by The National Academy of Sciences of the USA

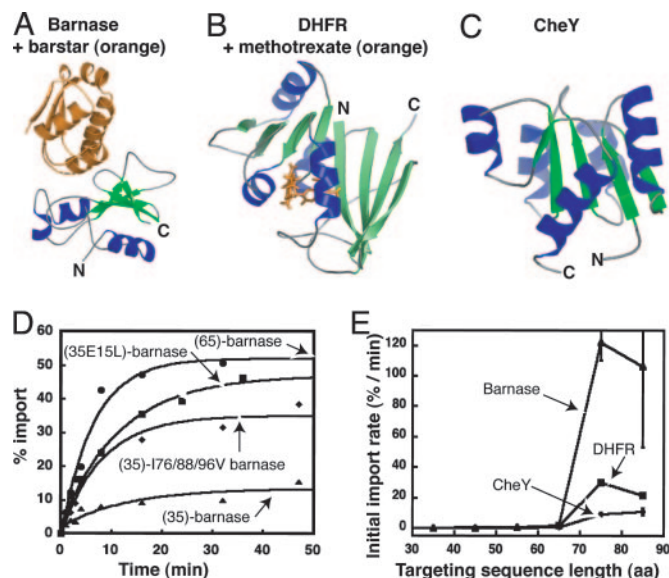
were added to 570  $\mu$ l of mitochondrial suspension at 0.5 mg of mitochondrial protein per ml in import buffer (described above) containing 4 mM ATP, 10.2 mM creatine phosphate, and 0.15 mg/ml creatine kinase. At defined time intervals, 50- $\mu$ l samples were transferred to 400  $\mu$ l of ice-cold mitoplasting buffer (20 mM Hepes-KOH, pH 7.4/1 mg/ml fatty acid-free BSA/0.1 mg/ml proteinase K/25 mM carbonylcyanide *p*-trifluoromethoxyphenylhydrazine). Proteinase K was inhibited with 1 mM PMSF after a 25-min incubation on ice. Mitochondria were reisolated by centrifugation at 20,800  $\times$  *g* and resuspended in SDS/PAGE sample buffer containing 2 mM PMSF. Samples were analyzed by SDS/PAGE, and the amount of imported protein was quantified by electronic autoradiography (Instant Imager, Packard). The extent of import was plotted as a percentage of the total amount of modified precursor in the import reaction. The import kinetics were analyzed with KALEIDAGRAPH (Abelbeck Software, Reading, PA) assuming a first or second order process.

**Single-Molecule Force Spectroscopy.** Force vs. extension curves were obtained as previously described (17) by stretching single protein polymers between a silicon nitride cantilever and a gold surface in a custom-built atomic force microscopy (AFM) apparatus. The genes for the multidomain proteins were constructed by standard molecular biology techniques so that their expression in *E. coli* yielded polypeptides in which the C terminus of each DHFR or CP P25 DHFR moiety was connected to the N terminus of the next by a 15-aa linker. The N terminus of the full-length polyprotein contained the 95 C14V amino acid mitochondrial targeting sequence, and the C terminus contained a 10-aa linker followed by two cysteines. The AFM was constructed by using a Digital Instruments (Santa Barbara, CA) Nanoscope II detector head mounted over a closed-loop single axis piezoelectric positioner (NPS-Z-15B, Queensgate Instruments, Torquay, U.K.). The detector head was retrofitted with a fiber-coupled laser source (Schäfter+Kirchhoff, Hamburg). Data acquisition and instrument control were performed via a data-acquisition board (PCI-6052E, National Instruments) and custom LABVIEW software (National Instruments, Austin, TX). The spring constants of each AFM cantilever (cantilever A MLCT-AUNM, Digital Instruments) were measured in solution before each experiment by using the equipartition method (18). Unfolding forces were extrapolated to a loading rate of 135,000 pN/s.

## Results

**Properties of the Targeting Sequence and Mature Domain Affect the Efficiency of Mitochondrial Protein Import.** To investigate the substrate specificity of the mitochondrial matrix import machinery, we constructed a series of precursor proteins whose mature regions were derived from barnase, DHFR, and CheY (Fig. 1 A–C) (see *Materials and Methods*). The proteins were targeted to the mitochondrial matrix by N-terminal targeting sequences of varying lengths derived from cytochrome *b*<sub>2</sub>.

Import of barnase precursor proteins with a 35-aa-long targeting sequence [(35)-barnase] into mitochondria was inefficient, with only  $\approx$ 12% of the protein having been transported after  $\approx$ 50 min (Fig. 1D). However, import of barnase could be improved through three separate modifications of the precursors. First, increasing the positive charge density of the targeting sequence with the mutation E15L increased the extent of import approximately 4-fold [Fig. 1D, (35 E15L)-barnase]. The higher positive charge density in the targeting sequence presumably enhanced its interaction with the membrane potential, facilitating its threading through the import channel (19). Second, lengthening the targeting sequence from 35 to 65 amino acid residues without changing its linear charge density increased the extent of import 5-fold [Fig. 1D, (65)-barnase]. The longer



**Fig. 1.** Dependence of import efficiency on targeting sequence and mature domain. The color diagrams represent barnase (A), DHFR (B), and CheY (C), showing the N and C termini and the ligand-binding sites. (D) The dependence of mitochondrial import of (35)-barnase on the targeting sequence charge density, (35 E15L)-barnase, targeting sequence length, (65)-barnase, and mature domain's thermodynamic stability, (35)-176/88/96V barnase. The percent of precursor imported is mapped over time. (E) The dependence of the initial import rate on the targeting signal length of precursors with barnase, DHFR, and CheY as the mature domains.

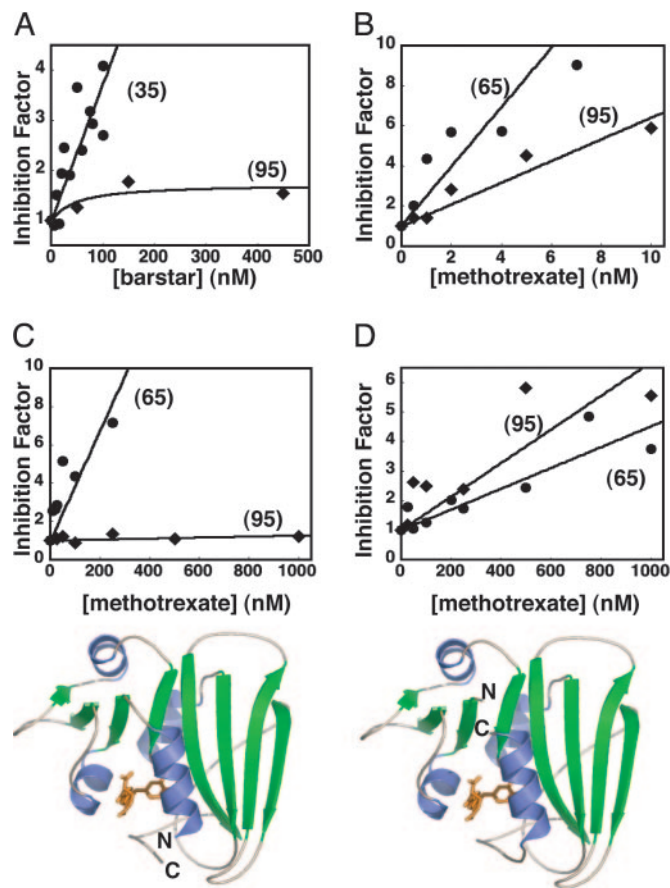
targeting sequence probably allowed precursors to engage the mitochondrial unfolding machinery more effectively (4, 5, 19). Finally, removing three methyl groups in the hydrophobic core of the protein (176/88/96V barnase), which destabilizes the protein against unfolding by 2.6 kcal/mol (A.M. and Alan R. Fersht, unpublished data); increased the extent of import 3-fold [Fig. 1D, (35)-176/88/96V barnase]. Destabilization probably enhanced spontaneous unfolding of the precursor, which reduced the requirement for the mitochondrial unfolding machinery when dealing with a short (35 aa) targeting sequence and, hence, accelerated import. These results suggest that properties of both the targeting sequence and the mature domain affect import efficiency and, therefore, contribute to the specificity of protein sorting to the mitochondrial matrix.

### The Structure of the Mature Domain Affects Mitochondrial Import.

The susceptibility of a precursor to mitochondrial unfolding depends on the mature domain's structure. To investigate the effect of protein structure on translocation, we compared the import of barnase with that of CheY and DHFR when attached to a series of targeting sequences ranging in length from 35 to 95 aa. Precursor proteins with shorter targeting sequences cannot fully engage the mitochondrial unfolding machinery, and, as shown above, their import is largely dependent on the precursor's spontaneous unfolding at the surface of the mitochondria (5). In contrast, precursors harboring targeting sequences of 75 to 95 aa can fully connect to the mitochondrial unfolding machinery and are imported significantly faster than their shorter counterparts (5) (Fig. 1E). In this case, import rates of the precursors were not determined by the equilibrium or kinetic stabilities of the mature domains against global spontaneous unfolding.

CheY has approximately the same thermodynamic stability as barnase [unfolding free energy = 8.5 kcal/mol for CheY (11) and 8.8 kcal/mol for barnase (20) at 25°C] and spontaneously





**Fig. 2.** Effect of ligand binding on protein import. The graphs show the inverse of the measured initial import rate vs. barstar concentration for (35)- and (95)-barnase precursors (A), or the inverse of the measured initial import rate vs. methotrexate concentration for (65)- and (95)-wild-type DHFR (B), (65)- and (95)-CP P25 DHFR (C), and (65)- and (95)-CP K38 DHFR precursors (D), standardized to the initial import rates of the unliganded precursors.

unfolds 10 times faster than barnase [unfolding rate constants =  $1.4 \times 10^{-3} \text{ s}^{-1}$  for CheY (11) and  $1.5 \times 10^{-4} \text{ s}^{-1}$  for barnase (21) at  $25^\circ\text{C}$ ]. However, CheY was imported 12 times more slowly than barnase in precursors with identical 75-aa-long targeting sequences (Fig. 1E). Similarly, DHFR is thermodynamically less stable than barnase [unfolding free energy = 6.1 kcal/mol (12) at  $15^\circ\text{C}$ ] and unfolds spontaneously more than 250 times faster than barnase [unfolding rate constant =  $4 \times 10^{-2} \text{ s}^{-1}$  at  $15^\circ\text{C}$  (22)] but is imported 6-fold slower with an identical 75-aa-long targeting sequence (Fig. 1E). Previous studies have shown that fusing these targeting sequences to the barnase or DHFR domains does not destabilize them against unfolding (5).

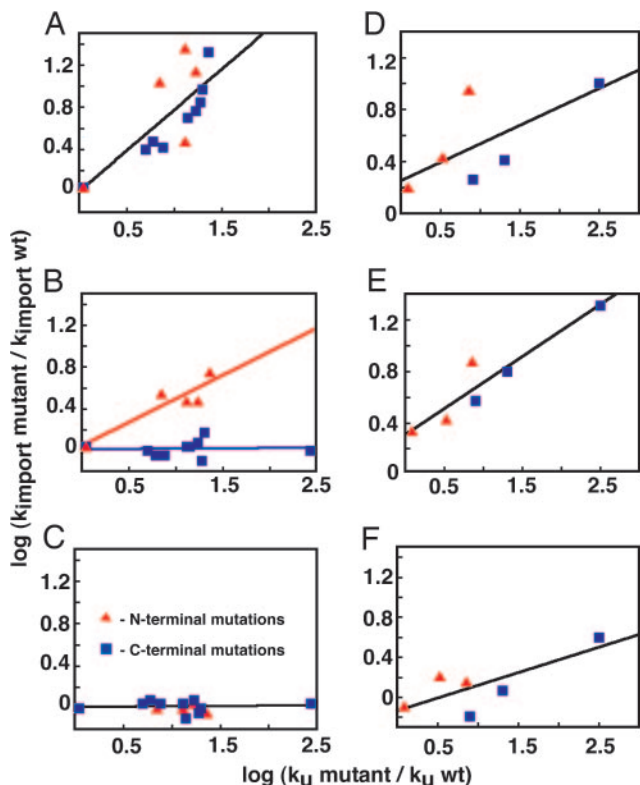
The differences in import efficiency between different precursors become even more pronounced when the proteins are stabilized against spontaneous unfolding by ligand binding. Barstar binds barnase tightly [ $K_D \approx 30 \text{ nM}$  (4)] and stabilizes the protein against spontaneous unfolding (23). Barstar inhibited the import of barnase precursors with shorter (35) targeting sequences, which is governed by spontaneous precursor unfolding (4) (Fig. 2A). However, import of barnase precursors with 95-aa-long targeting sequences, which can fully engage the mitochondrial unfolding machinery, remained largely unaffected by barstar (Fig. 2A). Similarly, DHFR can be stabilized against spontaneous unfolding by addition of its ligand methotrexate (24) ( $K_D \approx 0.7 \text{ nM}$ , determined as described in ref. 25). In this case, however, ligand-stabilized DHFR precursors were

not imported even when attached to long targeting sequences that engaged the import machinery fully (Fig. 2B). Together, these results suggest that a precursor's mitochondrial import efficiency depends on a property of the mature domain's structure that is not its thermodynamic or kinetic stability against spontaneous global unfolding.

**The Local Structure Adjacent to the Targeting Sequence Affects Mitochondrial Import.** What aspect of the structures of barnase and DHFR precursors determines their import efficiency? Mitochondria can unfold barnase precursors by unraveling them from their N-terminal targeting sequences and, thus, changing their unfolding pathway from that of spontaneous global unfolding (4). Therefore, it is possible that the local structure of a precursor's mature domain immediately adjacent to the targeting signal has a strong influence on the protein's susceptibility to unfolding by mitochondria. We investigated the effect of local structure of proteins on their susceptibility to unfolding by studying the import of CPs of DHFR. In these experiments, the original N and C termini of DHFR were connected by a short linker, new N termini were created at Pro-25 (CP P25) or Lys-38 (CP K38), and mitochondrial targeting sequences were fused to the proteins at the new N termini (26). The DHFR mutants have similar structures and stabilities and differ primarily in their topology (12). The amino acids immediately adjacent to the N terminus of CP P25 DHFR form an  $\alpha$ -helix at the surface of the protein, as does the N terminus of barnase. In contrast, the amino acids immediately following the N terminus of CP K38 DHFR form a  $\beta$ -strand buried in the core of the protein, as is the case for wild-type DHFR (Fig. 2A–D).

Ligand binding inhibited the import of both CP P25 DHFR and CP K38 DHFR precursors with 65-aa targeting sequences, indicating that import of the two proteins remained governed by spontaneous global unfolding of the mature domains (5). This result was expected because the length of these targeting sequences is just below the threshold required for fully engaging the mitochondrial unfolding machinery (Fig. 1E). Increasing the length of the targeting sequences to 95 aa led to methotrexate binding losing its effect on import of CP P25 DHFR, whereas methotrexate binding retained its effect on import of CP K38 DHFR (Fig. 2C and D). These results were surprising because CP P25 DHFR is more stable against global unfolding than CP K38 DHFR [unfolding-free energies 4.4 kcal/mol and 3.5 kcal/mol at  $15^\circ\text{C}$ , respectively (12)], and CP P25 DHFR precursors bind methotrexate more tightly than CP K38 DHFR precursors (Fig. 2C and D) ( $K_D \approx 35$  and  $K_D \approx 290 \text{ nM}$ , respectively). Together, these results suggest that the susceptibility to unfolding by mitochondria is determined by a precursor protein's local structure adjacent to the targeting signal. In the examples analyzed here, structures in which the N termini formed  $\alpha$ -helices at the surface of the folded domains (barnase and CP P25 DHFR) (Fig. 2A and C) were more susceptible to unfolding by mitochondria than structures in which the N termini formed  $\beta$ -strands buried inside the folded domains (wild-type DHFR and CP K38 DHFR) (Fig. 2B and D).

**The Mechanism of Mitochondrial Unfolding Is Determined by the Mature Domain's Local Structure Adjacent to the Targeting Sequence.** Wild-type and CP K38 DHFR may be more difficult to unfold by mitochondria than barnase because the import machinery is somehow unable to induce similarly extensive changes to their unfolding pathways. The unfolding kinetics of DHFR *in vitro* are complex (27), and it is not feasible to determine the unfolding pathway of DHFR precursors during import. Therefore, we compared the unfolding pathways of barnase and CheY precursors by analyzing the effects of point mutations throughout their mature domains on import. CheY resembles DHFR in that both

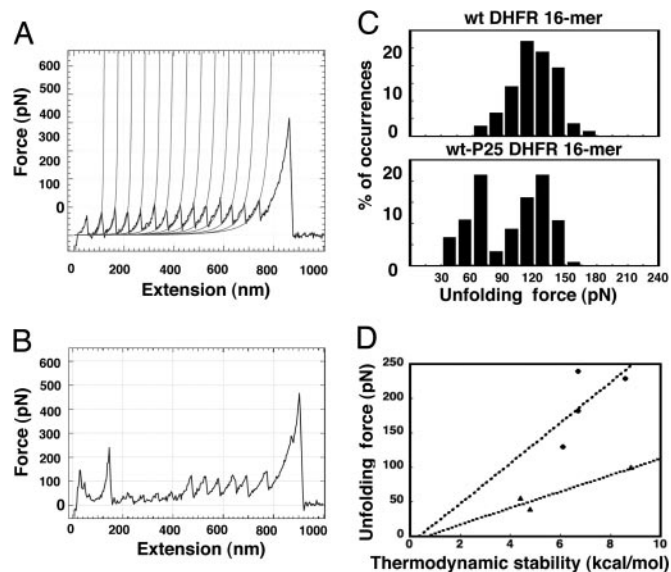


**Fig. 3.** Effect of mutations on mitochondrial import of barnase and CheY precursors. For each mutant analyzed, the initial import rate is plotted against the spontaneous unfolding rate ( $k_u$ ), both standardized to the wild-type import or spontaneous unfolding rates. The graphs show dependence of the mutational effects on import on their effect on spontaneous unfolding for (35)-barnase (A), (65)-barnase (B), (95)-barnase (C), (35)-CheY (D), (65)-CheY (E), and (95)-CheY (F).

proteins have  $\alpha/\beta$  folds and N termini that lead into buried  $\beta$ -strands.

Mutations throughout both barnase (4) and CheY precursors with short (35 aa) targeting sequences accelerated import in proportion to the mutations' known effects on both the thermodynamics and kinetics of spontaneous global unfolding *in vitro* (Fig. 3 A and D). When the lengths of the targeting sequences were increased to 65 aa in length, mutations throughout barnase lost their effect on import and only mutations that destabilized the N terminus of the protein continued to accelerate translocation (4) (Fig. 3B). This observation indicates that mitochondria are able to change the unfolding pathway of barnase as the targeting sequence begins to engage the unfolding machinery (4). When the targeting sequence length was further increased to 95 aa to allow the unfolding machinery to engage the precursors fully, all mutations throughout barnase lost their effect on import, and unfolding no longer determined import rates (4, 5) (Fig. 3C). In CheY precursors, no such change was observed, and all mutations retained their full effect on import as the targeting sequence was increased to 65 aa (Fig. 3E). Moreover, all mutations retained a significant if reduced effect on CheY precursor import when the targeting sequence length was increased to 95 aa. Thus, the mitochondrial unfolding machinery is unable to induce the large changes in the unfolding pathway of CheY that it induces in barnase even when the mitochondrial import machinery fully engages the precursors.

**Mitochondrial Import Efficiency Correlates with the Mature Domain's Resistance to Mechanical Unfolding.** The vectorial nature of protein translocation and the directed unraveling of precursors from



**Fig. 4.** Changing the location of the N terminus of DHFR alters its mechanical stability. (A) Force extension trace of wild-type DHFR 16-mer. Wild-type DHFR unfolds at a pulling force of  $131 \pm 20$  pN. Data were recorded at a loading rate of 90,000 pN/s. (B) Force extension trace of wild-type-CP P25 DHFR 16-mer. Wild-type DHFR unfolds at a pulling force of  $130 \pm 10$  pN. CP P25 DHFR unfolds at a pulling force of  $56 \pm 12$  pN. Data were recorded at a loading rate of 135,000 pN/s. (C) Graph showing the fraction of recorded unfolding events at each force for experiments as those described in A and B. (D) The correlation between thermodynamic stability and unfolding forces of unrelated proteins is  $R^2 = 0.58$  ( $\blacktriangle$  and  $\bullet$ ). The correlation is stronger when similar structures are compared, i.e., for structures with at least one  $\alpha$ -helical terminus  $R^2 = 0.94$  ( $\blacktriangle$ ) and for structures with  $\beta$ -strand termini  $R^2 = 0.63$  ( $\bullet$ ). Unfolding forces were extrapolated to a loading rate of 135,000 pN/s.

their N termini by mitochondria suggest a scenario in which mitochondria unfold precursors by pulling at their N termini. Accordingly, the resistance of proteins against unfolding by direct mechanical pulling from their N termini as determined by AFM may be a better predictor of their susceptibility to mitochondrial unfolding than their global stability against unfolding measured by solvent or heat denaturation experiments. To test this hypothesis, we compared the amounts of force required to mechanically unfold several mature domains of the precursor proteins by AFM. We created recombinant DHFR polyproteins by fusing 16 wild-type DHFR domains to each other in frame. Plots of the unfolding force exerted on the proteins against the extension of the protein measured in AFM pulling experiments produced the expected sawtooth pattern (28). The collected force peaks for wild-type DHFR denaturation showed that individual domains unfolded at an average force of  $131 \pm 20$  pN ( $n = 50$ , loading rate = 90,000 pN/s) (Fig. 4A and C). To analyze the mechanical unfolding of CP P25 DHFR, we created a chimeric eight-domain protein composed of alternating wild-type and CP P25 DHFR entities. Both the N and C termini of wild-type DHFR lead into  $\beta$ -strands buried within a  $\beta$ -sheet, whereas the N and C termini of CP P25 DHFR lead into  $\alpha$ -helices and unstructured loops, respectively. The force traces collected for wild-type CP P25 DHFR fusion proteins displayed two different types of force peaks ( $n = 84$ , loading rate = 135,000 pN/s) (Fig. 4B and C), one at  $130 \pm 10$  pN, which corresponded to the unfolding of wild-type DHFR, and another at  $56 \pm 12$  pN, which we identified as corresponding to the unfolding of single CP P25 DHFR domains. Wild-type DHFR, therefore, resisted mechanical unfolding at least 2.3-fold more strongly than CP P25 DHFR, whereas its free energy of spontaneous global unfolding is only 1.7 kcal/mol or 40% larger. Previous mechanical unfold-





There are also some clear differences between protein unfolding by pulling in AFM and mitochondrial import. In AFM, force is applied to both ends of the protein, whereas in mitochondria, force is applied from one end and the substrate is pulled against the narrow opening of the import channel. In the first case, the tension is concentrated on the first mechanical resistance points at the N- and C-terminal structures in the protein, whereas in the second, it is only applied to the point of resistance in the N-terminal structure adjacent to the target sequence. Nonetheless, the correlation between the susceptibility to mechanical unfolding of proteins by AFM and by the mitochondrial import machinery points to a basic similarity between these processes.

Protein unfolding during mitochondrial import is remarkably similar to protein unfolding during AAA<sup>+</sup>-protease degradation. In both processes, substrates are recognized by N- or C-terminal targeting sequences. Substrate unfolding requires ATP hydrolysis, and unfolding is coupled with the movement of the extended polypeptide through a channel. Thus, not surprisingly, the efficiency with which a precursor is imported into mitochondria correlates with the efficiency with which a similar substrate is degraded by AAA<sup>+</sup>-proteases (37, 38).

In summary, we show that the efficiency of protein targeting to the mitochondrial matrix is determined by the ability of the mitochondrial targeting sequence to engage the import machinery and the susceptibility of the mature domain to unfolding by mitochondria. This susceptibility to mitochondrial unfolding depends on the protein's local structure adjacent to the targeting sequence, just as is the case for mechanical unfolding. Accordingly, differences in local stability between protein substrates determine the extent to which mitochondria can change these substrates' unfolding pathway and therefore accelerate unfolding.

We thank R. Morimoto, A. Rosenzweig, E. Sontheimer, and members of the Matouschek laboratory for valuable discussion and advice. This work was supported by National Institutes of Health Grant GM063004 (to A.M.) and the Howard Hughes Medical Institute (C.B.). In addition, we gratefully acknowledge support from the Leukemia and Lymphoma Society (Scholar Award 1252-03 to A.M.) and the National Institutes of Health (Cellular and Molecular Biology of Disease Training Grant to A.J.W.), as well as use of the Keck Biophysics Facility at Northwestern University.

- Eilers, M. & Schatz, G. (1986) *Nature* **322**, 228–232.
- Wiedemann, N., Frazier, A. E. & Pfanner, N. (2004) *J. Biol. Chem.* **279**, 14473–14476.
- Schwartz, M. P., Huang, S. & Matouschek, A. (1999) *J. Biol. Chem.* **274**, 12759–12764.
- Huang, S., Ratliff, K. S., Schwartz, M. P., Spenner, J. M. & Matouschek, A. (1999) *Nat. Struct. Biol.* **6**, 1132–1138.
- Matouschek, A., Azem, A., Ratliff, K., Glick, B. S., Schmid, K. & Schatz, G. (1997) *EMBO J.* **16**, 6727–6736.
- Hurt, E. C. & van Loon, A. P. G. M. (1986) *Trends Biochem. Sci.* **11**, 204–207.
- Van Steeg, H., Oudshoorn, P., Van Hell, B., Polman, J. E. & Grivell, L. A. (1986) *EMBO J.* **5**, 3643–3650.
- Verner, K. & Lemire, B. D. (1989) *EMBO J.* **8**, 1491–1495.
- Allison, D. S. & Schatz, G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9011–9015.
- Lemire, B. D., Fankhauser, C., Baker, A. & Schatz, G. (1989) *J. Biol. Chem.* **264**, 20206–20215.
- Lopez-Hernandez, E. & Serrano, L. (1996) *Fold. Des.* **1**, 43–55.
- Iwakura, M., Nakamura, T., Yamane, C. & Maki, K. (2000) *Nat. Struct. Biol.* **7**, 580–585.
- Arretz, M., Schneider, H., Guiard, B., Brunner, M. & Neupert, W. (1994) *J. Biol. Chem.* **269**, 4959–4967.
- Beasley, E. M., Müller, S. & Schatz, G. (1993) *EMBO J.* **12**, 2303–2311.
- Hase, T., Muller, U., Riezman, H. & Schatz, G. (1984) *EMBO J.* **3**, 3157–3164.
- Glick, B. S. & Pon, L. A. (1995) *Methods Enzymol.* **260**, 213–223.
- Yang, G., Cecconi, C., Baase, W. A., Vetter, I. R., Breyer, W. A., Haack, J. A., Matthews, B. W., Dahlquist, F. W. & Bustamante, C. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 139–144.
- Florin, E. L., Rief, M., Lehmann, H., Ludwig, M., Dornmair, C., Moy, V. T. & Gaub, H. E. (1995) *Biosens. Bioelectron.* **10**, 895–901.
- Huang, S., Ratliff, K. S. & Matouschek, A. (2002) *Nat. Struct. Biol.* **9**, 301–307.
- Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A. & Fersht, A. R. (1992) *J. Mol. Biol.* **224**, 783–804.
- Serrano, L., Matouschek, A. & Fersht, A. R. (1992) *J. Mol. Biol.* **224**, 805–818.
- Touchette, N. A., Perry, K. M. & Matthews, C. R. (1986) *Biochemistry* **25**, 5445–5452.
- Martinez, J. C., Filimonov, V. V., Mateo, P. L., Schreiber, G. & Fersht, A. R. (1995) *Biochemistry* **34**, 5224–5233.
- Kraut, J. & Matthews, D. A. (1987) in *Biological Macromolecules and Assemblies*, eds. Jurnak, F. A. & McPherson, A. (Wiley, New York), Vol. 3, pp. 1–72.
- Huang, S., Murphy, S. & Matouschek, A. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 12991–12996.
- Nakamura, T. & Iwakura, M. (1999) *J. Biol. Chem.* **274**, 19041–19047.
- Wallace, L. A. & Robert Matthews, C. (2002) *J. Mol. Biol.* **315**, 193–211.
- Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J. M. & Gaub, H. E. (1997) *Science* **276**, 1109–1112.
- Carrion-Vazquez, M., Li, H., Lu, H., Marszalek, P. E., Oberhauser, A. F. & Fernandez, J. M. (2003) *Nat. Struct. Biol.* **10**, 738–743.
- Li, H., Carrion-Vazquez, M., Oberhauser, A. F., Marszalek, P. E. & Fernandez, J. M. (2000) *Nat. Struct. Biol.* **7**, 1117–1120.
- Brockwell, D. J., Paci, E., Zinober, R. C., Beddard, G. S., Olmsted, P. D., Smith, D. A., Perham, R. N. & Radford, S. E. (2003) *Nat. Struct. Biol.* **10**, 731–737.
- Branden, C. & Tooze, J. (1998) *Introduction to Protein Structure* (Garland, New York).
- Purdue, P. E., Takada, Y. & Danpure, C. J. (1990) *J. Cell Biol.* **111**, 2341–2351.
- Motley, A., Lumb, M. J., Oatey, P. B., Jennings, P. R., De Zoysa, P. A., Wanders, R. J., Tabak, H. F. & Danpure, C. J. (1995) *J. Cell Biol.* **131**, 95–109.
- Purdue, P. E. & Lazarow, P. B. (2001) *Annu. Rev. Cell Dev. Biol.* **17**, 701–752.
- Carrion-Vazquez, M., Oberhauser, A. F., Fowler, S. B., Marszalek, P. E., Broedel, S. E., Clarke, J. & Fernandez, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3694–3699.
- Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M. & Matouschek, A. (2001) *Mol. Cell* **7**, 627–637.
- Kenniston, J. A., Baker, T. A., Fernandez, J. M. & Sauer, R. T. (2003) *Cell* **114**, 511–520.
- Rief, M., Pascual, J., Saraste, M. & Gaub, H. E. (1999) *J. Mol. Biol.* **286**, 553–561.
- Kusunoki, H., Minasov, G., Macdonald, R. I. & Mondragon, A. (2004) *J. Mol. Biol.* **344**, 495–511.
- Best, R. B., Li, B., Steward, A., Daggett, V. & Clarke, J. (2001) *Biophys. J.* **81**, 2344–2356.
- Oberhauser, A. F., Marszalek, P. E., Erickson, H. P. & Fernandez, J. M. (1998) *Nature* **393**, 181–185.
- Hamill, S. J., Meekhof, A. E. & Clarke, J. (1998) *Biochemistry* **37**, 8071–8079.
- Khorasanizadeh, S., Peters, I. D., Butt, T. R. & Roder, H. (1993) *Biochemistry* **32**, 7054–7063.