Evaluating the Functional Pore Size of Chloroplast TOC and TIC Protein Translocons

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28   **ABSTRACT**

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30   The degree of residual structure retained by proteins while passing through

31   biological membranes is a fundamental mechanistic question of protein translocation.

32   Proteins are generally thought to be unfolded while transported through canonical

33   proteinaceous translocons, which has historically been the thought for the translocons of

34   the outer and inner chloroplast envelope membranes (TOC and TIC). Here, we readdressed

35   the issue and found that medium-sized tightly folded proteins such as the 22 kDa

36   dihydrofolate reductase (DHFR) can be tolerated by TOC and TIC. Chimeric DHFR fused

37   with RuBisCO small subunit transit peptide (tp22DHFR) was found to be imported into

38   chloroplasts in complex with its stabilizing ligand, methotrexate (MTX), in a folded

39   conformation. Following import, both mature tp22DHFR and MTX were found in the

40   chloroplast stroma. A subsaturating concentration of MTX was used to exclude the

41   possibility that MTX was stripped off tp22DHFR, independently imported into the

42   chloroplasts, and reassociated with imported tp22DHFR. Independent MTX import was

43   further excluded by use of fluorescein conjugated MTX (FMTX), which has very slow

44   membrane transport rates relative to unconjugated MTX. The TOC/TIC pore size was

45   determined by probing the translocons with particles of fixed diameter and found to be

46   greater than 25.6 Å, large enough to support folded DHFR import. The pore size is also

47   larger than those of the mitochondrial protein translocons that have a requirement for

48   protein unfolding.

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51 **SIGNIFICANCE**

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53   The chloroplast TOC and TIC translocons are responsible for the import of up to

54 95% of all chloroplast proteins and are therefore essential for plastid biogenesis and

55 photosynthesis. However, the mechanisms of protein import into chloroplasts are not well

56 understood. The TOC/TIC translocons have long been suggested to have a strong unfoldase

57 activity relative to other comparable protein translocons. Here, we present data suggesting

58 that this is not true, and that instead, they possess a relatively large pore size. This

59 identifies TOC and TIC as rather unique protein translocons capable of transporting folded

60 proteins across a double membrane barrier, which has important implications in the

61 mechanisms of TOC/TIC function and biogenesis of photosynthetic proteins.

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63 **INTRODUCTION**

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65   Protein import into chloroplasts is a vital prerequisite for photosynthesis and

66 therefore for plant growth and development. The protein translocons of the outer and

67 inner chloroplast membranes (TOC and TIC, respectively) are responsible for the import of

68 approximately 95% of all chloroplast proteins from the cytoplasm and are highly

69 conserved amongst all land plants (1). The TOC complex is composed of Toc75, Toc34, and

70 Toc159 at a 4:4:1 or 3:3:1 ratio (2). Toc75, a β-barrel BamA ortholog, is the major pore-

71 forming subunit at the outer membrane. The composition of the TIC complex has been

72 disputed, with Tic20, Tic21, and/or Tic110 proposed as major pore-forming subunits at the

73 inner membrane (2, 3). Structures are available for many of the soluble domains of

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74   TOC/TIC components, but little is known about the assembled complex structures. The

75   pore sizes of Toc75, Tic20, and Tic110 have been respectively estimated to be 14-26 Å, 7.8-

76   14.1 Å, and 15-34 Å by electrophysiological measurements in proteoliposomes (4-6).

77   However, these calculations rely on many assumptions and may not reflect the full

78   functional pore size range of the subunits within their native complex and membrane

79   environments. The driving force for protein import derives from ATPase activity of stromal

80   chaperones that exert a pulling force on the N-terminus of precursor proteins. Stromal

81   Hsp70 plays a major role, presumably analogous to the mechanism of mitochondrial

82   matrix-localized mtHsp70 (7, 8). Other chaperones proposed to be involved in parallel

83   and/or in series with Hsp70 are stromal Hsp93, stromal Hsp90, and an enigmatic

84   intermembrane space localized Hsp70 (9).

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86   Although most of the TOC/TIC components are known, the mechanisms of protein

87   translocation are not fully understood. A fundamental question in all protein translocation

88   systems is whether the translocon requires substrate proteins to be unfolded, or allows

89   folded proteins to cross the membrane by means of a larger pore. Proteins are generally

90   thought to traverse membranes in an unfolded conformation, as is the case for

91   mitochondrial membrane translocons (TOM and TIM), bacterial SecYEG, and Sec61 in the

92   ER (10-13). However, pathways that transport folded proteins also exist, such as in

93   peroxisomal import and in Tat transport in bacterial and thylakoid membranes (14, 15).

94   Unlike mitochondria, chloroplasts are known to tolerate small folded proteins, such as

95   internally cross-linked 6.5 kDa bovine pancreatic trypsin inhibitor, but have been generally

96   considered to unfold larger proteins (15, 16). The 22 kDa dihydrofolate reductase (DHFR)

97   is a model protein for folding/unfolding due to interactions with its stabilizing non-

98   covalently bound inhibitor, methotrexate (MTX). MTX binding is a good indicator for DHFR

99   folding because the kinetics of DHFR unfolding match those of DHFR/MTX complex

100   dissociation, implying unfolded DHFR has no affinity for MTX (17). MTX blocked DHFR

101   translocation through the TOM/TIM and SecYEG complexes by preventing DHFR unfolding,

102   but did not block DHFR transport through the Tat pathway (11, 13, 18). MTX was observed

103   not to block DHFR import into chloroplasts and both DHFR and MTX were found in the

104   stroma (19). In interpreting this experiment, the authors suggested that the TOC/TIC

105   translocons have a strong unfoldase activity capable of stripping MTX away from DHFR.

106   Here, we report that DHFR/MTX is in fact imported into chloroplasts as a folded complex.

107   Additionally, the TOC/TIC pore size was probed and determined to be significantly larger

108   than its functional cognate pore in mitochondrial TOM/TIM.

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110   **RESULTS**

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112   **The DHFR/MTX Complex Is Imported into Chloroplasts with Subsaturating [MTX].**

113   The mechanism of DHFR/MTX import into chloroplasts was previously described as

114   separate transport of protein and inhibitor across the envelope membranes, after which

115   they reassociate. The ability of MTX to independently cross the chloroplast envelope

116   membranes complicates the question of whether or not TOC/TIC unfolds the DHFR/MTX

117   complex. However, DHFR/MTX complex import can be differentiated from independent

118   DHFR and MTX import by monitoring the presence of MTX-bound DHFR in the stroma

119   when the import reaction is conducted with a subsaturating MTX concentration. This

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120   method was previously used to show DHFR/MTX complex transport through the cpTat

121   pathway (18). *In vitro*-translated DHFR fused to the RuBisCO small subunit transit peptide

122   ([^{3}H]-tp22DHFR) and bound to MTX can be detected by protease resistance conferred by

123   MTX binding (Fig. S1). The protease-resistant degradation product is the size of DHFR and

124   smaller than mature [^{3}H]-tp22DHFR due to a 22-residue linker between the cleavable

125   transit peptide and DHFR. [^{3}H]-tp22DHFR/MTX binding was saturated at MTX

126   concentrations above 4 nM and was negligible below 1 nM (Fig. 1A). [^{3}H]-tp22DHFR was

127   incubated with 133 nM MTX to saturate binding, then desalted and diluted into the import

128   reaction to achieve subsaturating MTX concentrations below 1 nM. Desalting reduced MTX

129   concentrations 660-fold, as determined by MTX absorbance at 304 nm (Fig. S2). MTX-

130   pretreated and -desalted [^{3}H]-tp22DHFR was more protease resistant after import relative

131   to the negative control without any MTX treatment (Fig. 1B). Since MTX was subsaturating

132   in the import reaction, this indicates that the originally bound MTX remained bound during

133   translocation. MTX-pretreated [^{3}H]-tp22DHFR was less protease resistant than the positive

134   controls where MTX was added to lysed chloroplast stroma samples after import. This

135   difference is most likely due to passive [^{3}H]-tp22DHFR/MTX complex dissociation under

136   the subsaturating conditions. [^{3}H]-tp22DHFR still utilized the TOC/TIC translocons in the

137   presence of MTX since it competed with a native precursor substrate (Fig. S3). An alternate

138   explanation to folded [^{3}H]-tp22DHFR/MTX complex import is that folate transporters in

139   the inner membrane with MTX affinity much higher than that of DHFR are able to actively

140   concentrate MTX in the stroma. This level of high affinity MTX transport has not been

141   described for characterized inner membrane folate transporters (20).

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143   **FMTX Import into Chloroplasts Is Dependent upon DHFR Import.** Unlike MTX,

144   fluorescein-conjugated methotrexate (FMTX) has extremely reduced passive membrane

145   diffusion rates (21). This property makes FMTX an ideal substrate to test whether it is

146   imported into chloroplasts independently or in complex with DHFR. FMTX also has

147   enhanced fluorescence when bound to DHFR, which allows for quantification of the

148   DHFR/FMTX complex (22, 23). Such FMTX fluorescence enhancement was seen upon

149   binding chimeric *E. coli* DHFR fused to RuBisCO small subunit transit peptide

150   (tp22EcDHFR) purified from *E. coli*, and was reversed by addition of excess MTX to

151   compete for tp22EcDHFR binding (Fig. 2).

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153   FMTX import into chloroplasts was analyzed by introducing FMTX to chloroplasts

154   during or after tp22EcDHFR import (Fig. 3A). To test for DHFR-dependent FMTX import,

155   both protein and inhibitor were added to the initial import reaction. The reaction was

156   stopped in cold IB and immediately washed to remove excess FMTX. To test for protein-

157   independent FMTX import, tp22EcDHFR was pre-imported so that independently imported

158   FMTX could potentially bind to DHFR in the stroma and accumulate therein. This was

159   achieved by importing tp22EcDHFR in an initial import reaction, followed by washing and

160   thermolysin treatment to remove unimported protein. FMTX was introduced to the

161   chloroplasts in a second mock import reaction with time, temperature, light, and ATP

162   conditions identical to the initial import reaction. The only difference between the DHFR-

163   dependent and -independent FMTX import was the initial location of DHFR (outside or

164   inside the chloroplast, respectively). Thermolysin treatment of chloroplasts should not

165   affect FMTX import since FMTX is small enough to freely pass through pores in the outer

166   membrane and therefore does not require any outer membrane transporters (24).

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168   All chloroplast samples were reisolated and imported FMTX was detected by

169   fluorescence. The initial fluorescence observed with DHFR-dependent FMTX import was

170   much higher than that of independent FMTX import, supporting the model that the

171   DHFR/FMTX complex was imported (Fig. 3B). Quantification of the imported

172   tp22EcDHFR/FMTX complex requires debinding of FMTX with excess MTX. However,

173   excess MTX did not efficiently replace bound FMTX in the stroma. MTX may not accumulate

174   in the stroma at high enough concentrations to compete for FMTX binding at a detectable

175   rate. Therefore, the chloroplasts were solubilized with 0.05% Triton after addition of

176   excess MTX to allow for complete FMTX debinding. Triton solubilization causes the

177   chloroplast solution to become more transmissive to light, increasing the fluorescence

178   signal at the four-minute time point. Solubilization also causes the effective solution

179   volume surrounding the tp22EcDHFR/FMTX complex to increase 1000-fold from the

180   stromal volume to the total bulk solution volume. This dilution shifts the binding

181   equilibrium towards the unbound state, causing the fluorescence signal to decline at the

182   five-minute time point even without prior addition of excess MTX. This dilution effect is

183   specific to DHFR/FMTX debinding, as it is not seen upon solubilizing chloroplasts with

184   imported precursor covalently conjugated to fluorescein (Fig. S4). In the presence of excess

185   MTX, the fluorescence decay after solubilization was faster due to the additive effects of

186   dilution and MTX/FMTX binding competition (Fig. 3B). The kinetics of FMTX replacement

187   with MTX was seen by subtracting traces with and without MTX addition (Fig. 3B, trace 2-1

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188   and trace 4-3; Fig. S5). This MTX effect is more pronounced for DHFR-dependent FMTX

189   import. To quantify the DHFR/FMTX debinding fluorescence decay, the background

190   chloroplast fluorescence (trace 5) was first subtracted from the other traces (Fig. 3C). The

191   fluorescence change was calibrated by measuring fluorescence of EcDHFR (without tp22

192   fusion) added stepwise to a solution of chloroplasts and saturating FMTX (Fig. 3C Inset).

193   The fluorescence change was taken as the difference in peak fluorescence without MTX

194   addition (at 5 minutes) and steady state fluorescence after complete FMTX debinding by

195   MTX (at 21 minutes). However, the five-minute peak does not represent the maximum

196   protein-bound FMTX since the dilution effect begins immediately after solubilization at

197   four minutes and competes with the fluorescence increase caused by chloroplast

198   solubilization between four and five minutes. The true peak was therefore determined by

199   linearizing the fluorescence decays and extrapolating back to the four-minute time point

200   when Triton was added (Fig. 3D). Quantitated DHFR-dependent FMTX import was much

201   higher than independent FMTX import, again indicating that folded tp22EcDHFR/FMTX

202   was translocated (Fig. 3F). Replicate samples were analyzed by Western blot to

203   independently quantify tp22EcDHFR import, which matched the extrapolated import of the

204   tp22EcDHFR/FMTX complex (Fig. 3E,F). This convergence of the data suggests a 1:1 ratio

205   of tp22EcDHFR and FMTX import. There was some inhibition of tp22EcDHFR import by

206   FMTX, which suggests that uncomplexed tp22EcDHFR may in fact be somewhat unfolded

207   and thereby imported more efficiently by chloroplasts.

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209   **The TOC/TIC Pore Size Is Greater Than 25.6 Å.** To further confirm that folded DHFR can

210   pass through the TOC/TIC translocons, the functional pore size was measured by probing

211   the translocons with particles of fixed diameter attached to precursor proteins. A rigid,

212   spherical, 20 Å monomaleimido Undeccagold particle was covalently conjugated to a single

213   C-terminal cysteine on RuBisCO small subunit containing FLAG and HIS tags for detection

214   and purification (RSSUFHC). The Undeccagold-labeled RSSUFHC, detected by a gel shift,

215   was imported and localized to the chloroplast stroma (Figs. 4A and 6A). The labeled mature

216   protein was protease-protected and detected in the soluble stromal fraction (Fig. 4A). To

217   control for the effect of protein modification on import, a smaller 12-14 Å particle,

218   fluorescein maleimide, was conjugated to RSSUFHC and also imported into the chloroplast

219   stroma. Another RuBisCO small subunit construct was made with a single internal cysteine

220   at residue S58 (RSSU58CFH). Since the diameter of a linear protein chain is 4-6 Å,

221   Undeccagold conjugated to the internal cysteine produces an effective probe diameter of at

222   least 24 Å (25). The internally labeled Undeccagold probe was also imported into the

223   chloroplast stroma, indicating the TOC/TIC pore size is greater than 24 Å (Fig. 4B).

224

225   The TOC/TIC translocons were additionally probed with cylindrical monomeric

226   streptavidin (mSA) with a 25.6 Å diameter and a 2.8 nM K_d for biotin (26). His-tagged

227   RuBisCO small subunit was biotinylated at a single C-terminal cysteine (RSSUHC-Bt), to

228   which mSA was non-covalently bound. Although mSA is not a rigid particle like

229   Undeccagold, it can still be used as a size probe since it must remain folded to retain affinity

230   for RSSUHC-Bt during import. Without RSSUHC-Bt present, mSA did not bind to

231   chloroplasts (Fig. 5). When imported with RSSUHC-Bt, mSA was localized to the chloroplast

232   stroma, indicating the maximum TOC/TIC pore size is greater than 25.6 Å. Imported mSA

233   was found in the soluble stromal fraction and was protease-protected from thermolysin

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234   and trypsin, but not in the presence of Triton. mSA import into chloroplasts was saturated

235   above a 35-fold molar excess of mSA over RSSUHC-Bt in the import reaction (Fig. S6).

236   Under saturating conditions, mSA imported into chloroplasts at a 1:1 ratio with RSSUHC-Bt,

237   ruling out the idea that the RSSUHC-Bt/mSA complex was dissociated by a strong unfoldase

238   activity applied on mSA at the chloroplast membrane surface (Fig. 6B).

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240   **The TOC/TIC Pore Size Is Larger Than That of TOM/TIM**. The import efficiencies of the

241   fixed-diameter probes were determined as the ratio of probe-labeled to -unlabeled

242   precursor imported after eight-minute reactions. The eight-minute time point falls at the

243   end of the linear portion of the reaction, and therefore correlates with the initial import

244   rate (Fig. S6). As is to be expected, the probe import efficiency decreased with increasing

245   probe diameters (Fig. 6C). Protein dimensions are given as minor axis diameters since the

246   longest major axis can, in principle, be imported perpendicular to the TOC/TIC pore

247   diameter. Thus, these minor axis values represent minimum pore size dimensions. The

248   import efficiency of mSA supports the model of folded DHFR import since they have

249   roughly similar minor axis diameters. The probes were imported more efficiently into

250   chloroplasts than identical (20 Å Undeccagold) or similarly sized probes (26 Å Nanogold)

251   were imported into mitoplasts through the TIM complex as determined by Schwartz and

252   Matouschek (Fig. 6C)(27). In our hands, the Nanogold-precursor conjugate was unstable in

253   the presence of chloroplasts and could not be used as a probe.

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257 **DISSCUSION**

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259   In this study, we readdressed the tolerance of the TOC/TIC translocons for folded

260 proteins and measured their functional pore size. We show that the DHFR/(F)MTX complex

261 can be imported in a folded conformation by three independent methods: 1) By using

262 subsaturating MTX concentrations, the possibility of independently imported MTX

263 quantitatively reassociating with imported DHFR was ruled out. 2) FMTX import was

264 significantly greater during concurrent DHFR import, indicating that DHFR/FMTX was

265 imported as a complex. 3) The TOC/TIC pore size was greater than 25.6 Å, which is larger

266 than the TOM/TIM pores and large enough to accommodate folded DHFR. Tetrameric

267 avidin has previously been shown to block chloroplast import, suggesting the pore size has

268 an upper limit of 50 Å (28). However, based on the reduced import efficiencies of mSA and

269 DHFR/FMTX (Fig. 6C), the maximum pore size appears likely to be no more than 30-35 Å.

270 In mitochondria, the TIM complex pore size is only slightly larger than 20 Å (27). The TOM

271 pore is larger than the TIM pore, but less than 26 Å. Here, the TOC pore size was not

272 determined independently of TIC, but it is tempting to speculate that the TOC pore may be

273 larger since the inner membrane is solute selective, while the outer membrane is not (29).

274 Since FMTX does have some inhibitory effect on DHFR import efficiency, DHFR is likely

275 imported in a somewhat unfolded state in the absence of FMTX. The foldedness of proteins

276 with diameters roughly 20-30 Å during import is probably determined by both the stability

277 and size of the protein. Proteins below 20 Å are likely to remain folded during their import,

278 while those above 30-35 Å are probably at least partially unfolded. However, given the

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279   pore size that we measured here, even large proteins may retain significant amounts of

280   residual structure based on their particular mechanical unfolding pathways.

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282   Prior evidence for protein foldedness during chloroplast import has mostly been

283   limited to measuring protease sensitivity of tightly folded precursors bound to the

284   chloroplast surface. These studies with different precursors yielded conflicting results.

285   Purified ferredoxin reductase was found to be protease-resistant when bound to intact

286   chloroplasts, but a purified chimeric OE33-RicinA protein was observed to be protease-

287   sensitive (30, 31). An *in vitro*-translated ferredoxin-DHFR fusion protein complexed with

288   MTX was found to be protease-sensitive when bound to purified envelope membranes

289   (32). *In vitro*-translated plastocyanin-DHFR fusion protein complexed with MTX was found

290   to be protease-sensitive when bound to intact chloroplasts (19). In this case, the protease

291   may have cleaved the transit peptide from DHFR, leaving the resistant DHFR/MTX complex

292   in the supernatant, which was not analyzed. The same study reported a lack of [^{3}H]-MTX

293   associated with chloroplast-bound DHFR, however, the [^{3}H]-MTX was likely diluted away

294   due to equilibrium debinding during chloroplast reisolation and washing, just as FMTX was

295   debound by dilution in Fig. 3B. It seems unlikely that these tightly folded proteins could be

296   unfolded by a passive mechanism at the chloroplast surface, yet an energy dependent

297   unfoldase at the outer membrane has not been found. The quantitative import of mSA also

298   indicates that there is no global unfoldase activity at the chloroplast surface (Fig. 6B).

299

300   The length of loosely structured N-terminal extensions (including transit peptides)

301   on tightly folded proteins is critical for import efficiency into both mitochondria and

302   chloroplasts as the transit peptide/presequence must reach internal chaperones that

303   provide the energy for unfolding and/or translocation. Mitochondria require an

304   approximately 80 residue N-terminal extension to span two membranes and reach

305   mtHsp70 in the matrix (33). Here also, the chloroplast data is conflicting, with ferredoxin

306   reductase (FNR) requiring roughly 80 residues for efficient import and titin requiring only

307   60 (34, 35). It was suggested that the unfoldase activity for titin resided in the

308   intermembrane space (IMS) such that the transit peptide only had to cross one membrane

309   to reach the chaperone. However, the average minor axis diameters of FNR and titin are 40

310   Å and 22 Å, respectively, which suggests that titin does not necessarily need to be unfolded

311   prior to chloroplast import, unlike FNR. The force required for mechanical unfolding of titin

312   by atomic force microscopy is roughly twice the DHFR/MTX unfolding force, which

313   strongly suggests titin would also remain folded during chloroplast import since it is both

314   smaller and more stable than DHFR/MTX (36, 37). IMS ATPase activity has been implicated

315   in precursor translocation through TOC when uncoupled from TIC, but no IMS chaperone

316   has been directly implicated in the coupled TOC/TIC import process to date (9, 38, 39).

317   While it remains possible for IMS ATPase activity to play a role in precursor unfolding, the

318   simpler explanation for the FNR/titin discrepancy would be related to the respective sizes

319   of the proteins. Accordingly, folded proteins too large to pass through the TOC/TIC pore

320   likely require the 80 residue N-terminal extension for efficient import, as in mitochondria.

321

322   Titin provides an ideal translocon-mediated protein-unfolding model because its

323   mechanical unfolding pathway has been elucidated by atomic force microscopy. With

324   mechanical force applied to the N and C termini of titin, the N-terminal β-strand A unravels

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325   first at approximately 100 pN, followed by the next β-strand A' at 200 pN, after which the

326   rest of the protein is easily unfolded at lower force (36). Stabilizing mutations in the A

327   strand inhibit import efficiency of titin into mitochondria, while destabilizing mutations

328   enhance import (40). The localized N-terminal stability of proteins is important for

329   mitochondrial import because transient N-terminal unfolding is rate limiting. In

330   chloroplasts, destabilizing strand A' mutations, but not strand A mutations, affected import

331   efficiency, and it was concluded that chloroplasts have a fundamentally different unfoldase

332   mechanism, possibly involving an IMS chaperone (41). An alternate explanation is that

333   strand A mutations did not affect chloroplast import because titin is small enough to be

334   imported as a folded protein and unfolding of the A strand does not significantly reduce the

335   minor axis diameter of the protein. Unlike strand A, destabilizing mutations in strand A'

336   and the core of titin likely allow the entire protein to unfold, thereby increasing chloroplast

337   import efficiency. Strand A' mutations probably did not affect mitochondrial import

338   because mtHsp70 turnover and not protein unfolding was rate limiting (40). Due to the

339   complication of folded titin import into chloroplasts, the mechanism of TOC/TIC-mediated

340   protein unfolding remains unclear.

341

342   It is unknown which TOC/TIC components, if any, are specifically involved in

343   precursor unfolding. One of the proposed stromal chaperones, Hsp93, has been ruled out

344   by Kovacheva *et al.* (42). They show that DHFR import efficiency into Arabidopsis *hsp93-*

345   *V/III-1* double mutant chloroplasts was not affected by MTX, while DHFR import into WT

346   chloroplasts was affected. In light of folded DHFR/MTX import, their interpretation might

347   still hold if DHFR is unfolded in the absence of MTX, which would provide the differential

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348   import efficiency readout based on protein stability required for interpreting their

349   experiment.

350

351   The import of folded proteins has structural implications for the TOC and TIC

352   complexes. The transport pathways of substrates utilizing β-barrel translocases such as

353   mitochondrial Tom40 and bacterial FhaC pass through the central channel of the β-barrels,

354   as determined by extensive cross-linking studies (43, 44). By analogy, the transport

355   pathway of Toc75 substrates should also be through the central channel, but this has not

356   been tested. Although the central channel is too small to accommodate folded DHFR, it has

357   been speculated that the weakly interacting first and last β-strands of Toc75 could open

358   (2). These Toc75 β-strands are structurally similar to those of its bacterial ortholog, BamA,

359   and dissimilar from those of other β-barrel translocases, which have more tightly

360   interacting terminal β-strands. The emerging model of BamA function in OMP biogenesis

361   involves the terminal β-strands opening and pairing with newly inserted β-strands of

362   substrate OMPs (45). In this model, once the substrate OMP is fully inserted into the

363   membrane, its terminal β-strands would be paired with the terminal β-strands of BamA

364   forming one large β-barrel. The substrate OMP would subsequently bud off from BamA and

365   close its β-barrel structure. This mechanism requires BamA to be somewhat flexible and

366   lends credibility to the prospect of the Toc75 β-barrel opening and potentially forming a

367   large pore in cooperation with other TOC components. Pore flexibility is certainly not

368   uncommon and is important even for SecYEG (46), where restricting the opening of the

369   lateral gate caused transport inhibition of soluble proteins (25). The Toc159 membrane

370   domain has been cross-linked to precursor proteins and may therefore cooperate with

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371   Toc75 to form part of the functional pore (47). Multiple Toc75 subunits within the TOC

372   complex may also cooperate to form the pore. It is much harder to speculate on the nature

373   of the TIC pore since there is very little structural information and continued controversy

374   over the identity of the complex components. If multiple TIC complexes exist, they may also

375   have different maximum pore sizes. Both TOC and TIC pores are likely to be somewhat

376   flexible and expandable to accommodate folded proteins.

377

378   The import of folded proteins through the TOC/TIC translocon raises many

379   questions for future investigation. Is the pore size of TOC larger than TIC? What is the

380   mechanism of chaperone action that allows for folded protein translocation? Finally, the

381   biological relevance for the large TOC/TIC pore size needs to be understood. It will be of

382   interest to find native chloroplast proteins that are imported in a folded conformation,

383   perhaps even when tightly bound to a cofactor.

384

385   **MATERIALS AND METHODS**

386

387   **Plasmid Constructs.** *P. sativum* RSSU cDNA was cloned into pET23a with 5' NdeI and 3'

388   XhoI restriction sites. The three native cysteines were mutated (C-1S, C41V, and C112V)

389   and a single C-terminal cysteine was inserted after the 6xHis tag by QuickChange PCR,

390   yielding the plasmid pET23a-RSSUHC. A C-terminal FLAG tag was added 5' of the His tag to

391   yield pET23a-RSSUFHC. The RuBisCO construct with a single internal cysteine (pET23a-

392   RSSU58CFH) was created by making a S58C mutation on pET23a-RSSUFHC and religating

393   into pET23a with NdeI and XhoI sites to remove the C-terminal cysteine.

394

395   The pET23a-tp22DHFR plasmid was cloned by fusing the first 79 residues of *P. sativum*

396   RSSU from the plasmid pET23a-tp22GFP (48) with mouse cytosolic DHFR DNA from the

397   plasmid AtPC-DHFR (49) flanked by 5' PstI and 3' XhoI sites. The pET23a-tp22EcDHFR

398   plasmid was made by replacing RSSU residues 80-180 in RSSUHC-pET23a with *E. coli*

399   DHFR residues 2-159 flanked by two FLAG tags and 5' BamHI/3' XhoI sites. tp22EcDHFR

400   (from *E. coli*) was made because full-length tp22DHFR (from mouse) could not be

401   expressed in *E. coli.* EcDHFR without the tp22 fusion was cloned into pET23a with 5' NdeI

402   and 3' XhoI sites.

403

404   pRSET-mSA was a gift from Sheldon Park (Addgene plasmid # 39860)(26). It encodes mSA

405   with 6xHis and FLAG tags for purification and detection.

406

407   **Protein Production and Labeling.** Proteins were expressed in *E. coli* BL21 cells and

408   purified under native (EcDHFR) or denaturing (other purified proteins) conditions with Ni-

409   NTA Agarose according to the manufacturer (Qiagen). mSA was refolded by rapid dilution

410   into a stirred PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.2)

411   solution overnight at 4 °C. Soluble mSA was concentrated and buffer exchanged into 50 mM

412   NaCl, 50 mM HEPES pH 7.2 with Ultra-15 10K Centrifugal Filters (Amicon). EcDHFR and all

413   other purified proteins were respectively buffer exchanged from elution buffer into 50 mM

414   HEPES pH 7.2 or Labeling Buffer (8 M urea, 50 mM HEPES pH 7.2). Proteins were

415   quantified on Coomassie-stained SDS-PAGE gels against BSA standards. [^{3}H]-tp22DHFR

18

416   was *in vitro*-transcribed and translated with T7 RNA polymerase and wheat germ extract,

417   respectively, according to the manufacturer (Promega).

418

419   Maleimide reactions were carried out in Labeling Buffer containing 7 mM TCEP at room

420   temperature for two hours. Fluorescein maleimide (Vector Labs), monomaleimido

421   Undeccagold (Nanoprobes), and biotin maleimide (Sigma) stocks (10, 1, and 100 mM,

422   respectively) were made in DMSO. RSSUFHC or RSSU58CFH (1mg/mL) was reacted with

423   0.2 mM fluorescein maleimide or 0.05 mM Undeccagold and added directly to import

424   reactions. RSSUHC was labeled with 5 mM biotin maleimide, precipitated with 15% TCA,

425   washed with cold acetone, and resuspended in labeling buffer.

426

427   **Chloroplast Import Assays.** Chloroplasts were isolated from 9-12 day old peas as

428   described previously (50). Import reactions were conducted under 100 $\mu E/m^2 s$ light with 3

429   mM ATP and isolated chloroplasts (0.33 mg/mL Chl) in Import Buffer (IB, 330 mM sorbitol,

430   3 mM $MgCl_2$, 50 mM Tricine–KOH pH 8.0). Reactions with purified precursor protein

431   contained 33 $\mu g/mL$ protein unless otherwise specified. All protease treatments included 5

432   mM $CaCl_2$. Those that included 1% Triton X-100 were subsequently precipitated in 15%

433   TCA and washed with cold acetone. All samples were finally solubilized in 2X Laemmli

434   Sample Buffer and analyzed by SDS-PAGE followed by fluorography or Western blotting.

435   Input standards in Fig. 6B were diluted with cold Sample Buffer containing solubilized

436   chloroplasts. Fluorography and fluorograph quantification were performed as previously

437   described (48).

438

19

439   **Western Blotting.** SDS-PAGE resolved proteins were transferred to PVDF membranes. α-

440   FLAG blots were blocked with 5% fat-free milk in TBST (150 mM NaCl, 0.1% Tween 20, 50

441   mM Tris pH 7.5) and probed with monoclonal α-FLAG M2 (Sigma) diluted 1:1000 in

442   blocking buffer. Goat α-mouse IgG-HRP secondary antibody (SCBT) was diluted 1:10000 in

443   blocking buffer. α-Biotin blots were blocked with 1% gelatin in TBST and probed with

444   Avidin-HRP (Sigma) diluted 1:10000 in blocking buffer. Blots were developed with ECL

445   substrate (GE Amersham) on film or on a ChemiDoc imager (Bio-Rad).

446

447   **Fluorescence Spectroscopy.** Fluorescence measurements were conducted in a Fluorolog

448   3-22 spectrofluorometer (Horiba) set to λ_{ex}=494 nm, λ_{em}=518 nm, and 5 nm slit widths.

449   Kinetics were measured in stirred 3 mL cuvettes.

450

451   **ACKNOWLEDGMENTS**

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594

Fig. 1. Imported tp22DHFR/MTX complex is detected under subsaturating MTX conditions. (A) Protease protection saturation curve of [^{3}H]-tp22DHFR/MTX binding. *In vitro*-translated [^{3}H]-tp22DHFR was incubated with methotrexate (MTX) at indicated concentrations on ice for 10 min followed by addition of 200 μg/ml trypsin and 5 min incubation at 37 °C. Reactions were quenched with 2 mg/ml soy bean trypsin inhibitor (SBTI) for 10 min on ice, diluted in Sample Buffer, and analyzed by SDS-PAGE and fluorography. Blue arrow indicates expected MTX concentration of desalted [^{3}H]-tp22DHFR/MTX solution used in panel B. **(B)** Import of desalted [^{3}H]-tp22DHFR/MTX complex into chloroplasts. Before import, [^{3}H]-tp22DHFR was incubated with Import Buffer (IB, Lanes 1-6) or MTX (133 nM, lanes 7-12) on ice for 10 min. The treated precursor samples were then filtered through Zeba Spin Desalting Columns (7K MWCO). Chloroplasts were incubated with above precursor mixtures supplied with 3 mM ATP and 1 mM DTT for 20 min under light. After import, chloroplasts were treated with 200 μg/ml thermolysin on ice for 15 min, washed with IB containing 50 mM EDTA and reisolated by centrifugation. The pellets were resuspended in 15 μl lysis buffer (10 mM MES, 5 mM MgCl$_2$, pH 6.5) and sonicated in icy water bath for 30 s. The released stroma was collected by centrifugation at 16000 x g for 15 min at 4 °C. MTX (1 μM) or IB was added into stromal samples and the resulting mixtures were then subjected to trypsin (Tryp, 800 μg/ml, lanes 4, 6, 10, and 12) or mock (with IB, lanes 3, 5, 9, and 11)) treatments at 37 °C for 10 min. Reactions were quenched by with SBTI (8 mg/mL). Three stromal samples derived from those treated with MTX before import (Lanes 9-12) were combined and TCA precipitated, while lanes 3-6

24

616   each derive from a single import reaction. Stromal samples were subjected to SDS-PAGE

617   and fluorography. pr, precursor; m, mature; DP, protease-protected degradation product.

618

619   **Fig. 2. Fluorescence enhancement of FMTX upon binding tp22EcDHFR.** tp22EcDHFR

620   (40 nM) was added to 80 nM FMTX followed by addition of excess MTX (800 nM) at

621   indicated time points.

622

623   **Fig. 3. FMTX Import into chloroplasts is dependent upon concurrent DHFR import.**

624   **(A)** Schematic of FMTX import experiment. tp22EcDHFR (470 nM) was imported into

625   chloroplasts in 300 µl reactions with or without 17 µM FMTX as shown (Initial Import).

626   Reactions were stopped with cold IB. Chloroplasts were washed and resuspended in 400

627   µg/mL thermolysin for 1 hour on ice and quenched with 12.5 mM EDTA. Chloroplasts were

628   resuspended in IB with ATP and with or without 17 µM FMTX as shown, and were again

629   exposed to light for 20 min (Mock import). Chloroplasts were reisolated on 40% Percoll

630   cushions, washed with IB, and assayed for fluorescence. **(B)** Fluorescence kinetics of DHFR-

631   dependent FMTX import (traces 1 and 2) or independent FMTX import (traces 3 and 4).

632   Trace 5 represents background chloroplast fluorescence derived from chloroplasts treated

633   as shown in A, except without any addition of protein or FMTX. Excess MTX (17 µM, traces

634   2 and 4) or DMSO (traces 1, 3, and 5) was added at t=2 min and 0.05% Triton-X 100 (TX)

635   was added to all traces at t=4 min. All kinetics represent averages of three independent

636   experiments. **(C)** Trace 5 was subtracted from traces 1 through 4 to remove background

637   fluorescence. Replicates were offset adjusted to the same initial fluorescence.

638   tp22EcDHFR/FMTX complex import was determined as the change in fluorescence due to

639   debinding of FMTX from DHFR (base fluorescence at t=21 min in MTX added trace

640   subtracted from peak fluorescence at t=5 min in the DMSO added trace). (C, Inset)

641   Fluorescence change (ΔF) was calibrated by adding EcDHFR in 14 pmol increments to a

642   saturating 170 nM FMTX solution in IB containing chloroplasts (17 μg/mL Chl). **(D)** The

643   true tp22EcDHFR/FMTX peak at t=4 min was determined by extrapolating linearized

644   DMSO added traces (1 and 3) back to t=4 min. Decay curves were linearized by taking the

645   natural log of normalized data. Sample extrapolation is shown. **(E)** tp22EcDHFR import

646   was determined by α-FLAG blotting, shown along with a Coomassie stained loading control

647   (CBB). **(F)** tp22EcDHFR protein import and tp22EcDHFR/FMTX complex import (non-

648   extrapolated and extrapolated) were quantified from Western blots and fluorescence data,

649   respectively. Error bars indicate SEM (n=3).

650

651   **Fig. 4. The TOC/TIC pore size is greater than 24 Å.** Undeccagold (UG) and fluorescein (F)

652   labeled RSSUFHC **(A)** or RSSU58CFH **(B)** were localized to the chloroplast stroma. Import

653   reactions were conducted for 20 minutes and stopped in cold IB (Imp). For protease

654   treatments, chloroplasts were further resuspended in 400 μg/mL thermolysin (Therm) or

655   240 μg/mL trypsin (Tryp) 30 minutes on ice and quenched with 12.5 mM EDTA or

656   resuspended in 1 mg/mL SBTI. All samples were reisolated on 40% Percoll cushions and

657   washed with IB. After reisolation, some non-protease treated samples were separated into

658   membrane (M) and soluble (S) fractions by lysis in 2 mM EDTA for 10 minutes on ice and

659   pelleting membranes at 16000 x *g*. Soluble fractions were precipitated in 15% TCA and

660   washed with cold acetone. Samples were analyzed by SDS-PAGE and α-FLAG blotting.

661

662 **Fig. 5. The TOC/TIC pore size is greater than 26.5 Å.** Biotinylated RSSUHC (RSSUHC-Bt)

663 was incubated with 4-fold molar excess mSA in IB prior to import reaction. Stromal

664 localization of mSA was determined as in Fig. 4, except that the membrane/stroma

665 fractionation was done on thermolysin treated chloroplasts and trypsin treatments were

666 conducted at 25 °C. Additionally, protease treatments were conducted in the presence of

667 1% Triton X-100 (TX). RSSUHC-Bt and mSA were detected on α-biotin and α-FLAG blots,

668 respectively.

669

670 **Fig. 6. Import efficiencies of fixed-diameter probes.** Import reactions of fluorescein (F)

671 and Undeccagold (UG) labeled RSSUFHC **(A)** and mSA labeled RSSUHC-Bt **(B)** are

672 quantified **(C).** RSSUHC-Bt (8.3 µg/mL in import reaction) was incubated with a 70-fold

673 molar excess of mSA in IB prior to import. All reactions were stopped after 8 min with cold

674 400 µg/mL thermolysin and incubated 1 hour on ice. Samples were then treated as in Figs.

675 4 and 5. Quantifications were plotted as the labeled:unlabeled mature protein ratio. The

676 internal control unlabeled protein was used for fluorescein and Undeccagold

677 quantifications. Error bars (SD) for fluorescein, Undeccagold, and mSA import efficiency

678 represent multiple replicates from 1, 2, and 2 independent experiments, respectively.

679 tp22EcDHFR/FMTX import efficiency was plotted as the ratio of tp22EcDHFR import in the

680 presence and absence of FMTX from Fig. 3F. Protein dimensions are given as minor axis

681 diameters with error bars representing standard deviation away from a perfectly circular

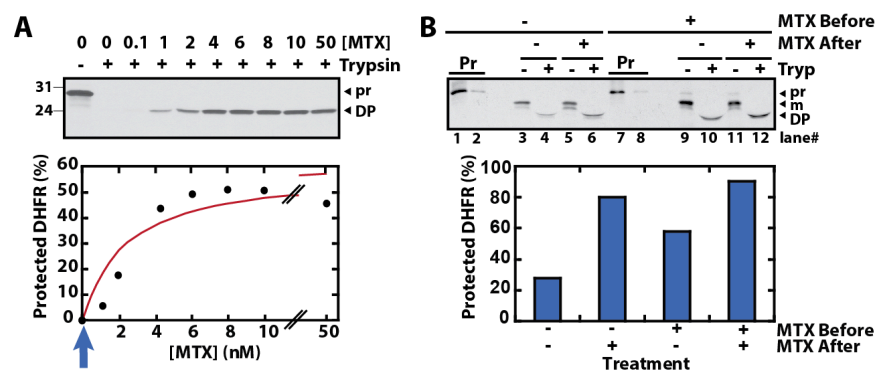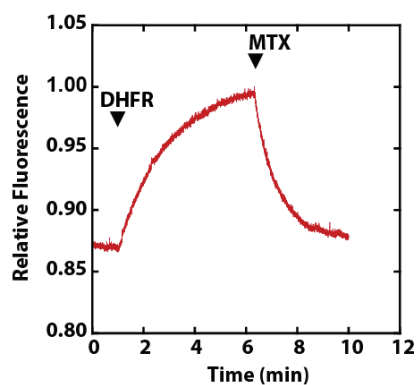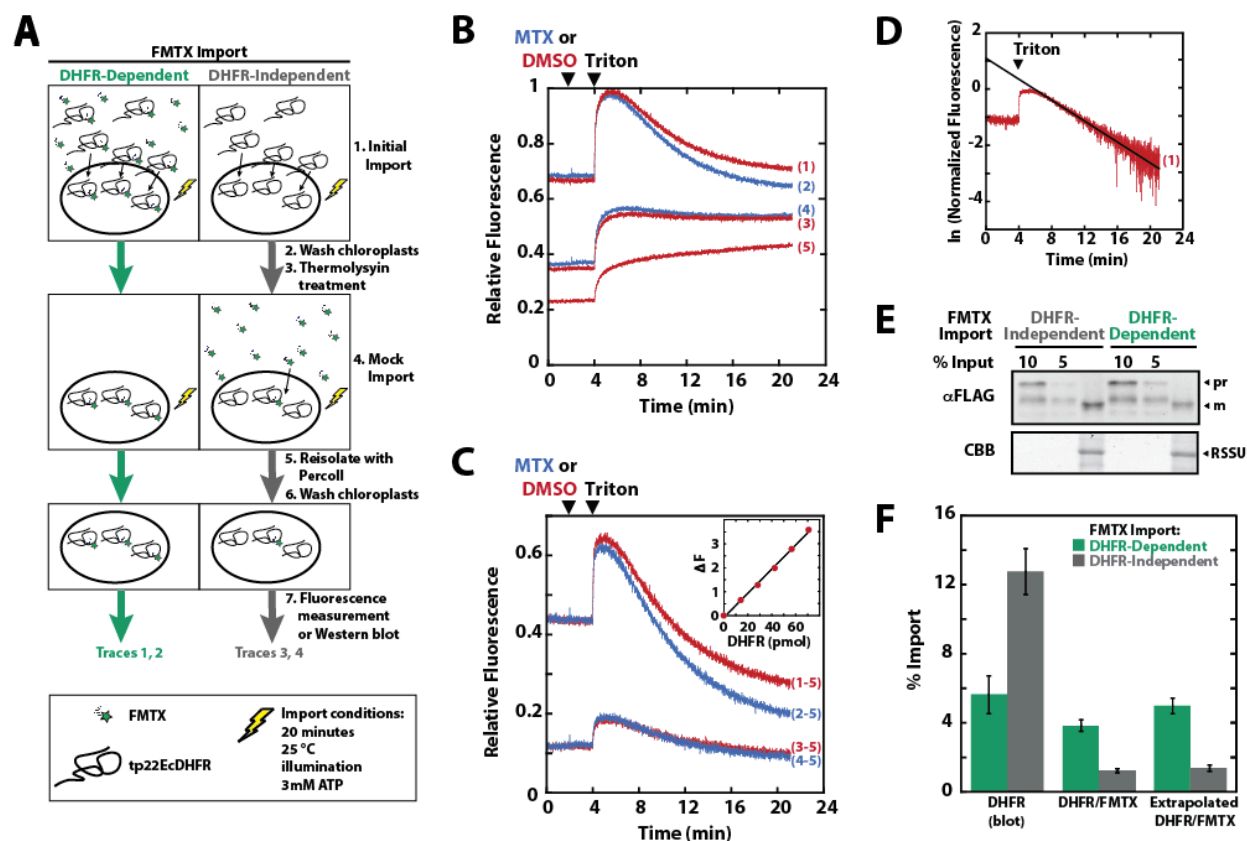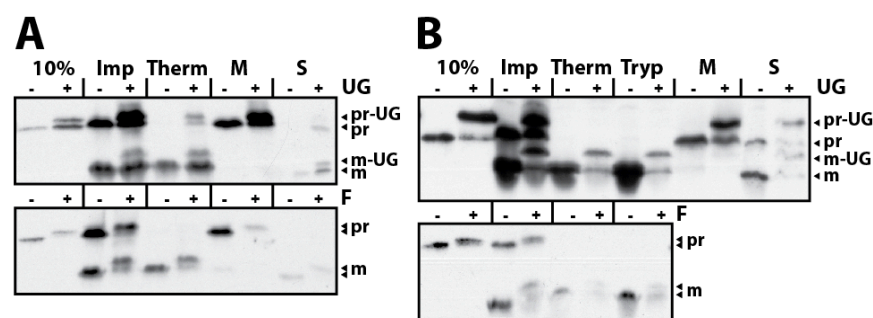682 cross section in the minor axis plane. *nonspecific chloroplast protein.

683

A

| 0 | 0 | 0.1 | 1 | 2 | 4 | 6 | 8 | 10 | 50 | [MTX] |
| - | + | + | + | + | + | + | + | + | + | Trypsin |

31 — ◄ pr
24 — ◄ DP

B

				-					+				MTX Before
					-		+			-		+	MTX After
Pr		-	+	-	+	Pr		-	+	-	+	Tryp

◄ pr
◄ m
◄ DP

1 2   3 4 5 6   7 8   9 10 11 12 lane#

Fig. 1 plot: Protected DHFR (%) vs [MTX] (nM)

Fig. 1 bar chart: Protected DHFR (%) vs Treatment

	MTX Before	MTX After
-	-	
+	-	
-	+	
+	+	

684
685   **Fig. 1.**
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Fig. 2 plot: Relative Fluorescence vs Time (min), with DHFR and MTX markers.

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695   **Fig. 2.**
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698   **Fig. 3.**
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707   **Fig. 4.**
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714   **Fig. 5.**

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726   **Fig. 6.**
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734 **SUPPLEMENTARY INFORMATION**
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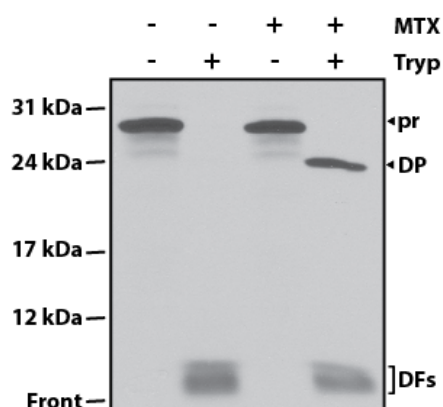
737
738 **Fig. S1. Stabilization of [³H]-tp22DHFR by MTX against trypsin digestion**. *In vitro*-
739 translated [³H]-tp22DHFR was incubated with 100 nM methotrexate (+, MTX) on ice for 10
740 min. Trypsin (+, Tryp) was then added to the above mixture reaching a final concentration
741 of 200 μg/ml. Protease treatment was conducted at 37 °C for 5 min. Reactions were
742 quenched with 2 mg/ml trypsin inhibitor (soy bean) on ice for 10 min, diluted in Sample
743 Buffer and analyzed by SDS-PAGE and fluorography. pr, [³H]-tp22DHFR precursor; DP,
744 DHFR degradation product; DFs, degradation fragments; Front, gel front.
745
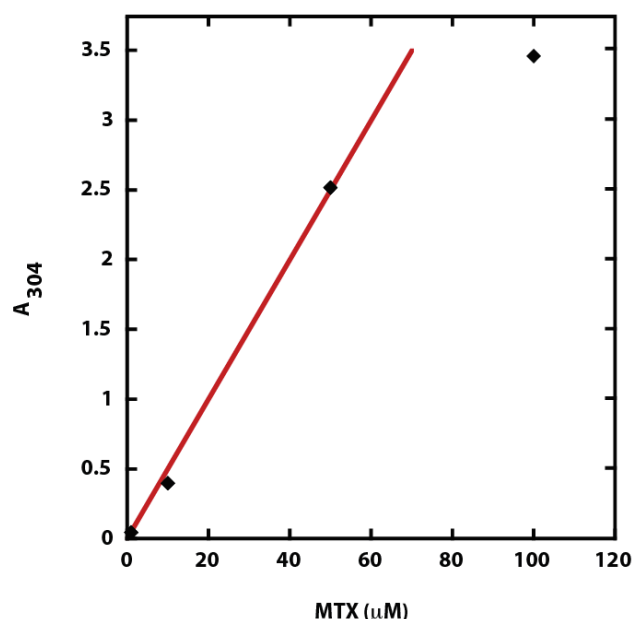
746
747 **Fig. S2**. The A_{304} extinction coefficient of MTX in Import Buffer was determined to be 0.05
748 μM^{-1}cm^{-1} within the linear range of 0 to 50 μM MTX. Within this linear range, the A_{304} of
749 MTX decreased 33-fold after passing through a Zeba Spin Desalting Column (7K MWCO),
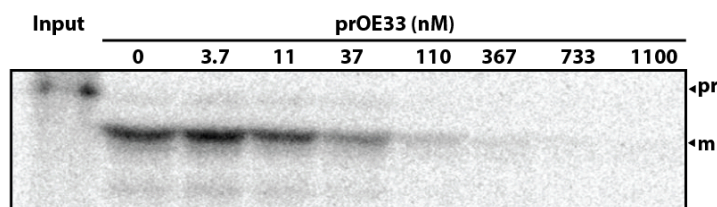750 which indicates a 660-fold decrease in MTX concentration.

31

751
752 **Fig. S3. Import of [^{3}H]-tp22DHFR competes with the precursor to the oxygen evolving**
753 **complex 33 kDa subunit (prOE33) in the presence of MTX.** His-tagged prOE33 was
754 over-expressed in *E. coli*, purified under native conditions with Ni-NTA Agarose, and added
755 at indicated concentrations to import reactions, while the concentrations of [^{3}H]-tp22DHFR
756 and MTX were held constant. Higher concentrations of prOE33 ultimately led to [^{3}H]-
757 tp22DHFR/MTX import quenching, indicating the native protein and the chimeric [^{3}H]-
758 tp22DHFR/MTX complex utilize the same TOC/TIC translocation machinery. pr, [^{3}H]-
759 tp22DHFR precursor; m, mature [^{3}H]-tp22DHFR.
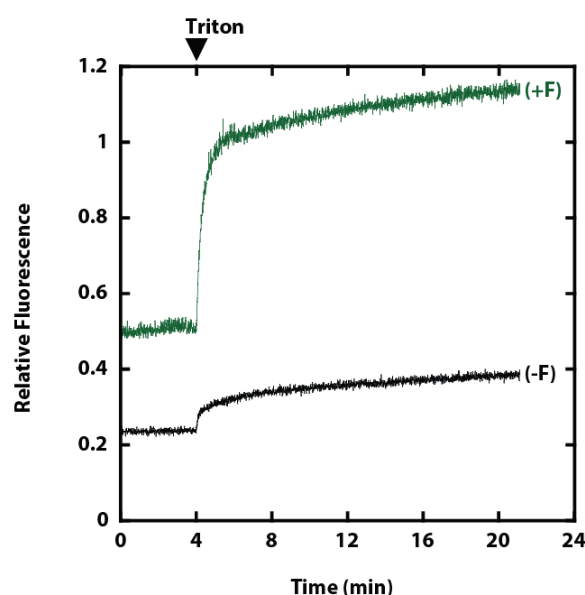760
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763
764 **Fig. S4. Fluorescence decay after chloroplast solubilization is specific to FMTX**
765 **debinding and not fluorescein covalently conjugated to precursor protein.** RSSUFHC
766 labeled with fluorescein maleimide was imported into 0.33 mg/mL chlorophyll
767 chloroplasts in a 240 µl reaction. Chloroplasts were thermolysin treated, reisolated, and
768 assayed for fluorescence as in Fig. 3B. Triton X-100 was added as indicated. (+F), RSSUFHC-
769 fluorescein imported chloroplasts; (-F), mock imported chloroplasts.
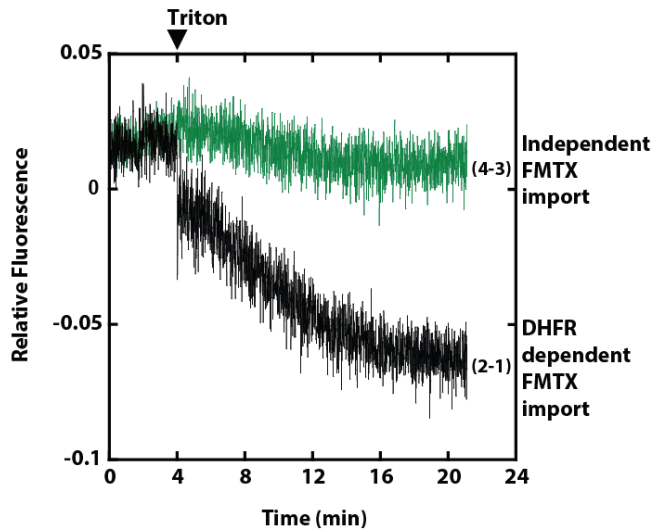770

771
Fig. S5. Kinetics of the MTX/FMTX binding replacement reaction. Derived from Fig. 3B, DMSO added traces were subtracted from MTX added traces (Traces 2-1 and 4-3)
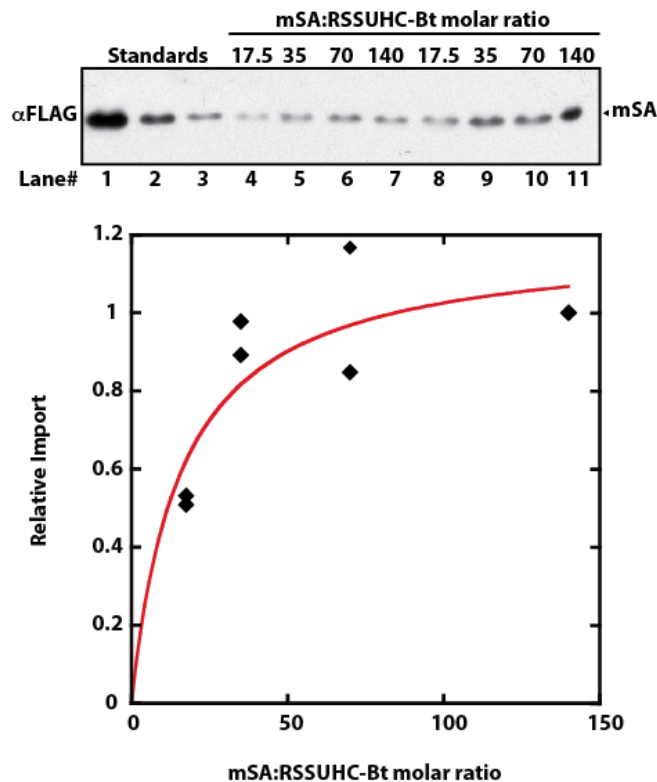

776
Fig. S6. Saturation curve of mSA import. Import reactions were conducted as in Fig. 6B, except that chloroplast samples in lanes 8-11 were not reisolated on 40% Percoll cushions. RSSUHC-Bt was preincubated with excess mSA at indicated ratios. mSA import in lanes 4-7 and lanes 8-11 were each normalized to the 140-fold molar excess data points (lanes 7 and 11).
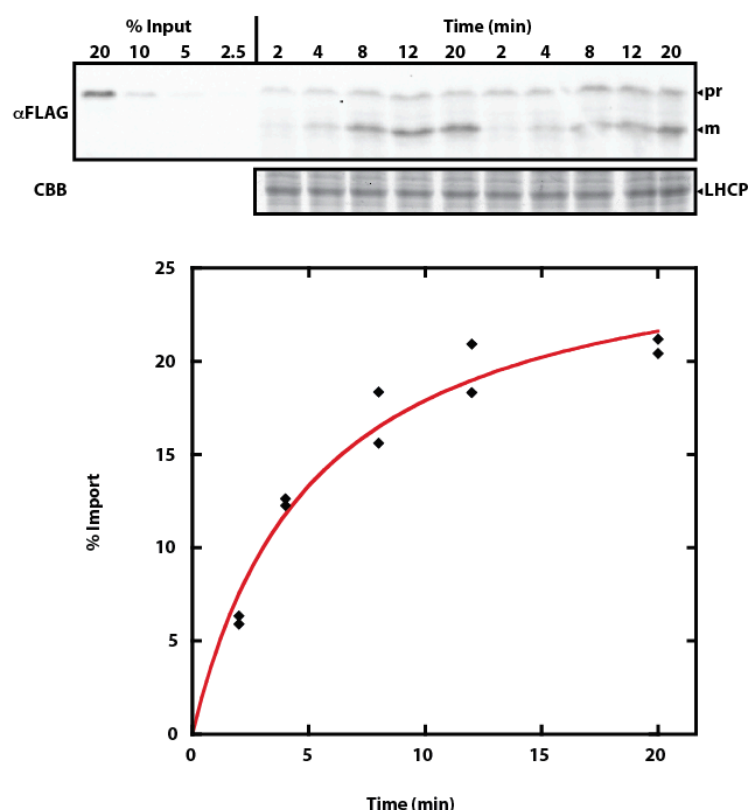
782

783
784 **Fig. S7. Time course import of RSSUFHC.** Import reactions were stopped in cold 400
785 µg/mL thermolysin at indicated time points. Quantification of mature RSSUFHC is shown.
786 CBB, Coomassie Brilliant Blue.
787
788
789 **Primer List:**

790 Amplification of mouse DHFR with 5' PstI and 3' XhoI sites:
791 F: CTGCAGATGGTTCGACCATTGAACTGCATCGTC
792 R: CTCGAGTTAGTCTTTCTTCTCGTAGACTTCAAACTTATACTTGATG
793
794 Amplification of *P. sativum* RSSU with 5'NdeI and 3'XhoI sites:
795 F: ATCGCATATGGCTTCTATGATATCCTCTTCA
796 R: GAATCTCGAGGTAGGATTCTGGTGTGTGGG
797
798 RSSU C41V QuickChange PCR:
799 F: GGAAGGGATGGGTTCCTGTCTTGGAATTTGAGTTGGAGAAAGG
800 R: CCTTTCTCCAACTCAAATTCCAAGACAGGAACCCATCCCTTCC
801
802 RSSU C112V QuickChange PCR:
803 F: GACAACGTTCGTCAAGTTCAAGTCATCAGTTTCATTGCCCAC
804 R: GTGGGCAATGAAACTGATGACTTGAACTTGACGAACGTTGTC

805
806   RSSU C-1S QuickChange PCR:
807   F: GGTGGAAGAGTAAAGAGCATGCAGGTGTGGCCTC
808   R: GAGGCCACACCTGCATGCTCTTTACTCTTCCACC
809
810   RSSUH-pET23a addition of C-terminal cysteine QuickChange PCR:
811   F: GCACCACCACCACCACCACTGTTGAGATCCGGCTGCTAACAAAG
812   R: CTTTGTTAGCAGCCGGATCTCAACAGTGGTGGTGGTGGTGGTGC
813
814   Amplification of RSSU with 5'Nde and 3'XhoI and FLAG tag with 2xGly linker:
815   F: ATCGCATATGGCTTCTATGATATCCTCTTCA
816   R: GAATCTCGAGCTTGTCGTCATCGTCTTTGTAGTCTCCACCGTAGGATTCTGGTGTGTGGG
817
818   RSSU S58C QuickChange PCR:
819   F: CGTGAGCACAACAAGTGCCCAGGATACTATGATG
820   R: CATCATAGTATCCTGGGCACTTGTTGTGCTCACG
821
822   Insertion of BamHI and FLAG tag between residues 79 and 80 of RSSUFHC:
823   F: AGTTGGATCCAGAGATCAGTTGTTGAAAGAAGTTGAA
824   R: ACTCGGATCCCTTGTCGTCATCGTCTTTGTAGTCTGGTGGCAAATAGGAAAGAGTC
825
826   Amplification of *E. coli* DHFR with 5' BamHI and 3'Xho and FLAG tag with 2xGly linker:
827   F: ATCTGGATCCATCAGTCTGATTGCGGCG
828   R: GAATCTCGAGCTTGTCGTCATCGTCTTTGTAGTCTCCACCCCGCCGCTCCAGAAT
829
830   Amplification of *E. coli* DHFR with 5' NdeI and 3'Xho sites:
831   F: ACTGCATATGATCAGTCTGATTGCGGC
832   R: CTTACTCGAGCCGCCGCTCCAGAAT