Structural features of chloroplast trigger factor determined at 2.6 Å resolution

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The folding of newly synthesized polypeptides requires the coordinated action of molecular chaperones. Prokaryotic cells and the chloroplasts of plant cells possess the ribosome-associated chaperone trigger factor, which binds nascent polypeptides at their exit stage from the ribosomal tunnel. The structure of bacterial trigger factor has been well characterized and it has a dragon-shaped conformation, with flexible domains responsible for ribosome binding, peptidyl-prolyl cis–trans isomerization (PPIase) activity and substrate protein binding. Chloroplast trigger-factor sequences have diversified from those of their bacterial orthologs and their molecular mechanism in plant organelles has been little investigated to date. Here, the crystal structure of the plastidic trigger factor from the green alga Chlamydomonas reinhardtii is presented at 2.6 Å resolution. Due to the high intramolecular flexibility of the protein, diffraction to this resolution was only achieved using a protein that lacked the N-terminal ribosome-binding domain. The eukaryotic trigger factor from C. reinhardtii exhibits a comparable dragon-shaped conformation to its bacterial counterpart. However, the C-terminal chaperone domain displays distinct charge distributions, with altered positioning of the helical arms and a specifically altered charge distribution along the surface responsible for substrate binding. While the PPIase domain shows a highly conserved structure compared with other PPIases, its rather weak activity and an unusual orientation towards the C-terminal domain points to specific adaptations of eukaryotic trigger factor for function in chloroplasts.

1. Introduction

Molecular chaperones belong to a structurally diverse protein family which is dedicated to maintaining protein homeostasis in cells. Molecular chaperones assist their substrate or client proteins in de novo folding as well as in preventing substrates from misfolding or aggregation during stressful conditions (Balchin et al., 2016). Chaperone-assisted protein folding starts co-translationally when newly synthesized polypeptides emerge from the ribosomal exit tunnel (Frydman, 2001; Koubek et al., 2021; Pechmann et al., 2013). In bacteria, the highly abundant ATP-independent molecular chaperone that associates with nascent polypeptides is termed trigger factor and is a well studied protein (Hoffmann et al., 2010; Koubek et al., 2021). Bacterial trigger factor (hereafter referred to as TF) has been shown to assist a broad spectrum of cytosolic, periplasmic and outer membrane substrates and associates with nascent polypeptides once the first 60 to 70 amino acids are accessible (Oh et al., 2011). TF promotes the de novo folding of nascent polypeptides by shielding local partially folded...
conformations from premature distal interactions with other sections of the polypeptide (Mashaghi et al., 2013). Besides the bona fide chaperone function on nascent polypeptides, TF has also been postulated to bind full-length proteins, thereby contributing to complex assembly and ribosome biogenesis (Hoffmann et al., 2010; Liu et al., 2005; Martinez-Hackert & Hendrickson, 2009; Saio et al., 2014).

The Escherichia coli TF protein has a molecular weight of 48 kDa and has an elongated dragon-shaped conformation, bending over the ribosomal exit tunnel, which places the chaperone in an ideal position for embracing the emerging polypeptide (Deeng et al., 2016; Ferbitz et al., 2004). Ribosome interaction is modulated mainly via the N-terminal ribosome-binding domain (RBD; see Fig. 1a, bottom), which forms the so-called tail of the dragon molecule. Ribosome binding is mainly mediated via the conserved GFRxGxxP signature motif and two surrounding α-helices of TF contacting ribosomal proteins Rpl23 and Rpl29 as well as domain III of the 23S ribosomal RNA (Ferbitz et al., 2004; Kramer et al., 2002). The middle domain in the linear sequence forms the active center of a dome-shaped peptidyl-prolyl cis–trans isomerase (PPIase) with sequence homology to FK506-binding proteins (FKBPs). The PPIase constitutes the so-called head, opposite the RBD, in the three-dimensional structure of TF. The PPIase allows TF to actively catalyze the cis–trans conversion of peptidyl-prolyl bonds in vitro (Hesterkamp et al., 1996; Scholz et al., 1997; Stoller et al., 1995). However, the contribution of the PPIase to the chaperone function of TF is still under debate (Hoffmann et al., 2010; Kramer et al., 2004). PPIases can be classified into three subgroups: the FK506-binding proteins (FKBPs), cyclophilins (CYPs) and parvulins (He et al., 2004; Vallone, 2005). FKBPs are often found in tandem with other modules in multidomain proteins. Their functions are described as scaffolds, holdases and foldases, with regulatory roles in protein folding, cell signaling, transcription and apoptosis (Dunyak & Gestwicki, 2016; Tong & Jiang, 2015). The main chaperone module of TF, here termed the substrate-binding domain (SBD), is formed by the C-terminal sequence of TF. This domain is mainly α-helical and shapes the back and two arms of the dragon-shaped molecule (Ferbitz et al., 2004). The central cavity between the RBD and the two arms exhibits the peptide-binding capacity of the chaperone and is even capable of accommodating larger structural features of substrate proteins up to the size of the 14 kDa lysozyme (Ferbitz et al., 2004). In recent years, structural information on several different prokaryotic TFs has been published for isolated TF (Ludlam et al., 2004; Morgado et al., 2017; Saio et al., 2018; Ferbitz et al., 2004; Martinez-Hackert & Hendrickson, 2009), TF in association with ribosomal proteins (Deeng et al., 2016; Ferbitz et al., 2004; Merz et al., 2008; Baram et al., 2005; Schlünzen et al., 2005; Martinez-Hackert & Hendrickson, 2009) and with bound substrates (Saio et al., 2014; Kawagoe et al., 2018; Bhakta et al., 2019) or single domains (Vogtherr et al., 2002; Kawagoe et al., 2018; Martinez-Hackert & Hendrickson, 2007; Kristensen & Gajhede, 2003), demonstrating that the overall domain architecture remains relatively conserved among the bacterial kingdom. However, the relative orientations of the individual TF domains display a remarkable variance between the different functional states and between organisms.

In eukaryotic cells, trigger factors are only found in the chloroplasts of plants (Ries et al., 2017). Chloroplast trigger factors (hereafter referred to as TIG1s) display a remarkable sequence variance compared with prokaryotic TFs, while the typical organization of the three domains remains conserved. Recent low-resolution small-angle X-ray scattering (SAXS) analysis and ab initio modeling of TIG1 from Chlamydomonas reinhardtii (Chlamydomonas) and from Arabidopsis thaliana (Arabidopsis) demonstrated that the trigger-factor proteins have maintained the dragon-shaped conformation; however, their domain orientation seems to vary when compared with the conformation of TF from E. coli (EctF; Ries et al., 2017). In addition, chloroplast TIG1 is not able to functionally replace the bacterial TF in E. coli, suggesting that the chaperone has evolved for its specific task in chloroplasts. However, ribosome association and aggregation preventing chaperone function is also preserved in the chloroplast ortholog (Rohr et al., 2019).

The ribosome-binding signature motif of plastidic TIG1 is highly conserved within the land plants lineage (GFRP-GxxXP), whereas algal TIG1s show greater sequence variance in this region, with only the first glycine and the last proline being conserved in the motif. Interestingly, the algal trigger-factor binding site on the ribosomal Rpl23 protein shows equal sequence variance at this site, which points to co-evolution of the trigger-factor ribosome-binding interface in these phyla (Ries et al., 2020; Rohr et al., 2019). Disruption of bacterial and chloroplast trigger-factor genes results in no obvious growth defects under ambient conditions, but tig1 mutants display retarded growth under heterotrophic conditions (Rohr et al., 2019). However, the molecular mechanism of action of eukaryotic trigger factor and its substrate proteins during the biogenesis of chloroplast protein maturation are not known to date. A better understanding of this ortholog requires a deeper knowledge of the structural conformation of this protein, especially of the chaperone domain. We thus set out to solve the structure of Chlamydomonas TIG1 (CrTIG1) at the atomic level. We resolved the X-ray structure of truncated CrTIG1 with the PPIase domain and the C-terminal SBD. The structure shows an uncommon tilted PPIase domain which interacts intermolecularly with the SBD over a coiled-coil motif. The SBD does not contain the common ‘open arms’ features of the bacterial TF structure, but rather a conformation with aligned arms. The inner part of the cave built by the arms together with the PPIase domain shows a very negatively charged surface. The PPIase domain exhibits a low but measurable PPIase activity.

2. Methods
2.1. Protein purification

CrTIG1 and EctF were expressed and purified as published previously (Ries et al., 2017). Selenomethionine-substituted
CrTIG1 (hereafter referred to as CrTIG1-SeMet) was produced in *E. coli* using a modified two-step protocol as described by Guerrero *et al.* (2001). A preculture was diluted in LB medium supplemented with ampicillin (100 µg ml⁻¹), grown for 16 h, harvested and washed with sterile deionized water. The cells were then diluted in 2 l SelenoMethionine medium (Molecular Dimensions) supplemented with the same antibiotic and 40 µg ml⁻¹ L-selenomethionine (Molecular Dimensions) to an OD₆₀₀ of 1.0. After incubation for 30 min at 303 K, CrTIG1-SeMet was expressed by the addition of

Figure 1
Crystal structure of CrTIG1ΔRBD. (a) Domain assignment of full-length CrTIG1. The missing N-terminal ribosome-binding domain (RBD) is colored gray, the long linker spanning the backbone is colored green, the PPlase domain is colored red and the long linker helix spanning from the PPlase domain to the C-terminal arms is colored blue. Arm 1 of the C-terminal chaperone activity domain (SBD) is colored light blue and arm 2 is colored magenta. (b) Ribbon presentation of the secondary-structure elements presented from the front view and from a side view rotated 90° counterclockwise (coloring is according to (a)). (c) Scheme of the domain arrangement (coloring is according to (a)). (d) Electrostatic surface potential from the front view (red indicates negative charge and blue indicates positive charge). The electrostatic surface potential was calculated with the *APBS* plugin from *PyMOL* (Baker *et al.*, 2001).
0.4 mM isopropyl β-d-1-thiogalactopyranoside for 17 h at 290 K. CrTIG1-SeMet was purified as described for the native construct, except that 1 mM DTT was maintained in all buffers subsequent to elution from the chitin matrix.

2.2. Crystallization and crystal harvesting

For crystallization, native and selenomethionine-labeled CrTIG1 were concentrated to 23 mg ml⁻¹. Initial crystals were obtained in 96-well plates by the sitting-drop vapor-diffusion method at 291 K using the automated crystallization facility at the Department of Structural Biology, Saarland University (Müller & Lancaster, 2013). Equal amounts of protein solution (at protein concentrations of 23 and 11.5 mg ml⁻¹, respectively) and reservoir solution were mixed and equilibrated against the reservoir solution. Crystals appeared between 10 and 90 days and were further optimized in 24-well plates using the hanging-drop vapor-diffusion method. Crystals were obtained at 277, 283 and 291 K in various crystallization conditions. Only crystals that grew at 283 and 291 K could be used for diffraction experiments; they diffracted to between 2.6 and 6 Å resolution. The crystals obtained at 277 K were very fragile and did not tolerate any handling or cryoprotectant. Crystals growing at 283 or 291 K tolerated glycerol over PEG 3350 as the best cryoprotectant. High-resolution structural information was obtained for native and selenomethionine-labeled proteins, which were crystallized under conditions containing magnesium sulfate (0.1–0.3 M) or ammonium sulfate (0.76–1.76 M) and MES buffer (pH 6.0–6.5), both with PEG 3350 as a precipitant.

2.3. Data collection, structure determination and refinement

The protein crystals were transferred to buffers consisting of the reservoir solution supplemented with 30% PEG 3350 or 30% glycerol for cryoprotection and were flash-cooled in liquid nitrogen. After initial diffraction experiments at the home source at the Department of Structural Biology (an Oxford Diffraction Nova system), X-ray diffraction data were collected at 100 K on beamline ID23-1 (Nurizzo et al., 2006) at the European Synchrotron Radiation Facility (ESRF), Grénoble, France. Data were processed with either XDS (Kabsch, 2010) or iMosflm (Battye et al., 2011) and were scaled with XSSCALE, SCALA or AIMLESS (Evans, 2006) from the CCP4 software package (Potterson et al., 2003) or processed with the automatic pipelines at the ESRF (Monaco et al., 2013). The structure of CrTIG1 was solved by phasing with selenomethionine using SHELX (Sheldrick, 2015). Coot (Emsley et al., 2010) was used for manual rebuilding and completion of the model, and refinement was performed using REFMAC5 (Murshudov et al., 2011). All data-collection and refinement statistics are summarized in Table 1. The coordinates and associated structure factors have been deposited at the Protein Data Bank as PDB entry 7zgi. Graphical representations of the structural model were created using PyMOL (DeLano, 2006). Further screening for crystals containing the full-length protein was performed on various beamlines at the ESRF and the Swiss Light Source (SLS).

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| 2.4. Chymotrypsin-coupled PP1ase activity assay |

Peptidyl-prolyl cis–trans isomerization (PP1ase) activity was measured with a protease-coupled assay (Fischer et al., 1992) with modifications at 273 K. 50 μM succinyl-Ala-Ala-Phe-Pro-Phe-nitroanilide as the substrate peptide (Bachem Biochemica GmbH) was diluted in 35 mM HEPES buffer pH 7.6, 150 mM KCl and incubated with 0.1 mg ml⁻¹ bovine α-chymotrypsin for 30 s, in which the pre-existing trans isomser of the substrate peptide was cleaved by chymotrypsin. To start the reaction, CrTIG1 was added in the concentration range 1–25 μM. Absorption at 395 nm was followed in a UV–Vis photometer (Ultraspec 2100 pro). EcTF was used as a positive control and the spontaneous isomerization reaction without trigger factors was used as a negative control. Reaction rates were derived by nonlinear curve fitting to a first-order rate equation using OriginPro and were corrected for spontaneous isomerization.

| 2.5. Small-angle X-ray scattering experiments and data analysis |

Small-angle X-ray scattering (SAX) data were collected on the BM29 beamline (Pernot et al., 2013) at the ESRF with a
PI-LATUS 1M detector (16.9 × 17.9 cm) at a wavelength of 0.9919 Å (12.5 keV) and a sample-to-detector distance of 2.867 m, corresponding to a q-range of 0.025–5 nm⁻¹. Static measurements with three different protein concentrations of CrTIG1 supplemented with 20-meric peptides from Chlamydomonas chloroplast-encoded AtpB (the β subunit of the plastidic ATP synthase) and RbcL (the large subunit of Rubisco) were measured with 1 s exposure times per frame and ten frames per concentration at 293 K. The peptides were first dissolved in DMSO and added to the protein in a tenfold molar excess. Scattering by the corresponding buffer (20 mM Tris pH 7.5, 150 mM KCl, 1 mM DTT, 2% DMSO) was measured before and after one run, averaged and subtracted from the protein scattering. As a control, CrTIG1 alone in buffer with the corresponding DMSO concentration was also measured. BSA standards were used to calibrate the I(0) values and the scattering of pure water was used to calibrate the intensity to absolute units (Orthaber values and the scattering of pure water was used to calibrate Tris pH 7.5, 150 mM KCl, 1 mM DTT, 2% DMSO). The frames collected from the static measurements were checked for radiation damage before averaging and buffer subtraction and those derived from different concentrations were merged.

2.6. Modeling of the full-length structure of CrTIG1 into the SAXS shape

The protein structure of CrTIG1ΔRBD and an ab initio model of the RBD were fitted manually into the SAXS shape derived from previous experiments (Ries et al., 2017) using UCSF Chimera (Yang et al., 2012). The ab initio model of the RBD was calculated with I-TASSER (Zhang, 2008) based on the EcTF structure in PDB entry 2mlz (Saio et al., 2014). The fit between the SAXS data and the structure was evaluated with CRYSOL (Svergun et al., 1995).

3. Results

3.1. Crystallization trials of CrTIG1

For crystallization of the chloroplast trigger-factor ortholog from C. reinhardtii (CrTIG1), the mature protein, lacking the N-terminal 67 amino acids of the chloroplast transit peptide, was heterologously expressed in E. coli and purified, yielding protein without remaining affinity tags (Ries et al., 2017). Data sets were collected from selenomethionine-labeled CrTIG1 to 2.72 Å resolution with one molecule in the asymmetric unit and to 2.6 Å resolution with two molecules in the asymmetric unit in space group C2121. The statistics for the data set at 2.6 Å resolution are summarized in Table 1. Although the structural domain arrangement was expected to be similar to that of the bacterial counterparts, molecular replacement with bacterial TF structures failed. Phasing with the anomalous scatterer selenium was successful and 11 of the 17 available atom positions were found and could be assigned to the sequence of the PPIase domain and the C-terminal substrate-binding domain (SBD). No suitable electron density was observed for the N-terminal ribosome-binding domain (RBD), which suggests that the RBD was absent in these high-resolution protein crystals. The model was further built by density modification and manual refinement. This initial structure was then used as a model to solve structures of CrTIG1 from further data sets which were derived from several different native and selenomethionine-labeled CrTIG1 protein preparations and diverse crystallization conditions. However, crystals containing the full-length protein exclusively diffracted anisotropically to diffraction limits lower than 6 Å in space groups P1, P2₁, P2₂, P2₁, 1422 and H32. The diffraction limits of the crystals of the full-length protein could not be increased through variation of the incubation temperature, the use of different crystallization methods or seeding experiments. Small-angle X-ray scattering experiments on full-length CrTIG1 and ArTIG1 proteins point to highly flexible conformations (Ries et al., 2017). Thus, chemical cross-linking was examined, as well as cocrystallization experiments with 20-meric peptides from the putative substrate proteins AtpB and RbcL, which had previously been identified by in vitro chaperone assays and peptide-spot assays, respectively (Rohr et al., 2019). Due to the very fragile crystals of the full-length protein, the crystal-harvesting system (Zander et al., 2016) and in situ crystallography at the ID30-B beamline (McCarthy et al., 2018) at the ESRF were also tested. Through a combination of all these methods, the diffraction limits of full-length CrTIG1 could be improved to 4 Å, which was still too low to solve the structure. Similar problems with anisotropy and lower diffraction limits of crystals of the full-length protein has also been described for Thermotoga maritima TF and is mainly caused by the high flexibility of the three domains (Martinez-Hackert & Hendrickson, 2009).

Dissolved crystals obtained from different crystallization conditions were investigated via SDS–PAGE and ESI mass spectrometry, which confirmed that the well diffracting crystals lack the N-terminal domain, whereas the crystals with low diffraction limits comprise the full-length protein. Of note, SDS–PAGE analyses indicated no degradation of the CrTIG1 protein preparations when samples were stored over three months at 291 K (data not shown) and thus did not confirm the truncation of the RBD during crystallization. No putative protease cleavage site by E. coli proteases was predicted between the RBD and the PPIase domain using the ExPASy PeptideCutter tool and MEROPS (Gasteiger et al., 2005; Rawlings et al., 2018).

3.2. Unique domain arrangement of chloroplast trigger factor

The resulting atomic structure of CrTIG1 lacks the first 137 amino acids of the mature protein (without the chloroplast
signal peptide) and will be referred to here as CrTIG1ΔRBD. Numbering of the amino-acid sequence starts with Ala1 and the secondary-structure elements are numbered according to the full-length EcTF structure (Ferbitz et al., 2004). Visible electron density starts with amino acid Val138 at the beginning of the linker region between the N-terminal RBD and the middle PPIase domain, with β5 being the first secondary-structure element. The protein resembles the dragon-like structure described for bacterial trigger factors, but without the tail (the RBD; Figs. 1a–1c). The C-terminal SBD is completely visible except for 15 amino acids at the C-terminus. The middle PPIase domain extends from Phe181 to Leu277. The C-terminal domain contains seven helices and one β-strand and can be divided into a long linker helix (Pro278–Asp332), arm 1 built from three helices (Met333–Leu406) and a second arm (Val407 to the end) with two helices. A long linker (Val138–Gly180) containing a β-strand followed by an α-helix connects the missing N-terminal domain and the PPIase domain and spans the complete backbone of the protein. The linker is stabilized via a parallel β-sheet built between the β-strand in the linker (β5) and the only β-strand at the C-terminus (β11) (Figs. 1b and 1c).

Calculation of the surface potential reveals an overall mixed pattern of acidic and basic patches in CrTIG1ΔRBD, while the cradle formed by the PPIase domain and the C-terminal arms is mainly negatively charged (Fig. 1d). Prominent hydrophobic patches are only found in the cavity of the PPIase domain and in arm 2 of the SBD (Supplementary Fig. S1).

A global structural alignment of CrTIG1ΔRBD with bacterial trigger factors was difficult due to the high flexibility of the domain arrangement, which explains the failure of our approach to solve the structure via molecular replacement based on all available prokaryotic TF structures. The closest structural homologue is the E. coli trigger factor, with a root-mean-square deviation (r.m.s.d.) of between 4.6 and 6.9 Å as determined with DALI (Holm, 2020; Fig. 2). Structural alignment of seven available full-length EcTF structures shows that the PPIase domain can undergo major movement dependent on the binding or the interaction mode (substrate, ribosome or unbound; Supplementary Fig. S2). The most similar domain arrangement to that of the PPIase domain of CrTIG1ΔRBD is found in the dimeric structures of EcTF [PDB entries 5owi (Morgado et al., 2017) and 6d6s (Saio et al., 2018)], yet with clear differences. For example, the tips/loops of the CrTIG1ΔRBD PPIase domain are tilted by 7.5 Å and an angle of 9° when compared with the dimeric EcTF structure (PDB entry 6d6s; Saio et al., 2018). In great contrast, the tilt is 35 Å and 48° between the positions of the PPIase domains in CrTIG1ΔRBD and the structure of monomeric EcTF (PDB entry 1w26; Ferbitz et al., 2004; Fig. 2). The well characterized structures of TF from Vibrio cholerae (PDB entry 1t11; Ludlam et al., 2004) and T. maritima (PDB entry 3gty; Martinez-Hackert & Hendrickson, 2009) are even less comparable, with the chloroplast ortholog showing an r.m.s.d. of 9.8 and 7.1 Å, respectively. Structural comparison of the different domains was also performed individually using the DALI server. Their characteristics and putative functions will be discussed separately.

3.3. The chloroplast trigger factor contains a conserved PPlase domain

The PPIase domain of CrTIG1ΔRBD shows the typical conformation of FK506-binding proteins (FKBPs), consisting of a classical four-stranded antiparallel β-sheet forming a half β-barrel with two inserted 310-helices. This sheet can be extended through a fifth β-strand (Van Duyne et al., 1993). CrTIG1ΔRBD also possesses a short fifth β-strand (β6 in our annotation) at the N-terminus. However, in contrast to the classical FKBP fold, this N-terminal β-strand does not extend the half β-barrel; instead, it builds a separate antiparallel β-sheet together with the C-terminal β-strand 10, which is very unusually elongated (Fig. 3a). Prominent deviations from other FKBp proteins are further present in the 40s loop between β-strands 7 and 8 and in the 80s loop or so-called ‘flap’ (Fig. 3c). The 40s loop often divides a β-strand into two parts, but in CrTIG1ΔRBD only the second part of β-strand 8 is present. Instead, a long loop completely differently oriented to EcTF is observed. In the 80s loop, an unusual 310-helix is inserted at the tip of the loop. A second 310-helix is inserted in the 50s loop. β-Strand 7 is extended and is surrounded by an extra-long loop between β-strands 6 and 7, causing a loop crossing. This motif is structurally rare since it is energetically unfavorable (Finkelstein et al., 1993), and it needs a robust hydrogen-bond pattern in CrTIG1 for stabilization.
comparable conformation is stabilized by disulfide bridges in human FKBP12 (Schultz et al., 1994). In CrTIG1ΔRBD, this huge loop is stabilized via hydrogen bonds to the 50s loop and the loop preceding β-strand 9. The half-barreled β-sheet builds a hydrophobic cavity lined with mostly aromatic amino acids. The surface of the PPIase represents an extreme bipolar charge distribution, with the back of the half-barrel being positively charged and the opposite site being extremely negatively charged, especially within the 80s and 50s loops (Fig. 3b).

The PPIase domain, which forms the head of the dragon-like structure, is prominently tilted downwards in CrTIG1ΔRBD towards the arms. This conformation is stabilized via several hydrogen bonds. Two hydrogen bonds are directed towards arm 1 of the C-terminal domain (Phe257 O to Arg383 N; Gln209 N to Lys369 O): one formed from the unusual 3₁₀ helix in the flap of the PPIase domain and the second one from the neighboring loop after β-strand 7 (Fig. 3d). Additional stabilizing hydrogen bonds are observed from Lys177 of the linker helix α5 to the prominent loop between β-strands 6 and 7 (to Glu224 and Asp222) and to Gly193 at the end of β-strand 8. Asp222 is also involved in a hydrogen bond to Gln174 in the linker helix (Fig. 3e).

Within the PPIase domain of EcTF, Glu178, Ile195, Phe198, Tyr221 and Phe233 are conserved and identified as key residues for PPIase activity (Liu et al., 2010). The equivalent residues in the PPIase domain of CrTIG1 are Pro212, Leu228, Ile230, Tyr253 and Val265 (Table 2, Supplementary Fig. S3). The best conserved region between the trigger factors is found in the region surrounding the position of Tyr253 in CrTIG1.

The next structurally related neighbor of the CrTIG1 PPIase domain is the respective domain in the conformation of monomeric E. coli TF (EcTF; PDB entry 1w26; Ferbitz et al., 2004), with an r.m.s.d. of 1.7 Å and a sequence identity of 22%, followed by other EcTF structures. However, the PPIase domain of CrTIG1 is also structurally related to peptidyl-prolyl cis-trans isomerases such as human FKBP13 (PDB entry 4nr; Schultz et al., 1994), FK506-binding protein 2 (PDB entry 2pbc; Structural Genomics Consortium, unpublished work) and SlyD (Quistgaard et al., 2016), with r.m.s.d. values of between 2.0 and 2.4 Å and sequence identities of 12–17%. The next structural homologues in plants are AtFKBP42 (PDB entry 2f4e; Weiergräber et al., 2006) and the plastidic...
ArFKBP13 (PDB entry 1u79; Gopalan et al., 2004), with low sequence identities of 12% and 11%, respectively, and an r.m.s.d. of 2.4 Å (Supplementary Fig. S4). In contrast to CrTIG1, these Arabidopsis PPIases differ in the number of β-strands and in the inlets in the 80s loop. Also, ArFKBP13 and ArFKBP42 contain disulfide bridges (Gopalan et al., 2004). No such cysteines are present in the PPIase domain of CrTIG1, again suggesting a different task of this domain in CrTIG1 when compared with other plastidic PPIases.

3.4. CrTIG1 exhibits a weak PPIase activity

The PPIase activity was measured with a protease-coupled enzyme assay in which trans isomer-specific proteolytic cleavage of the substrate succinyl-Ala-Phe-Pro-Phe-paranitroanilide (suc-AFPF-pNA) was detected. The performance of the enzyme assay is based on the tetrapeptide assay described previously for EcTF and is also used for determining the activities of chloroplast PPIases (Stoller et al., 1995; Kramer et al., 2004). EcTF was used as a positive control and the spontaneous isomerization reaction without trigger factors was used as a negative control (Fig. 4). The catalytic activity \( k_{\text{cat}}/K_m \) was determined to be 0.0057 \( \mu \text{M}^{-1} \text{s}^{-1} \) for CrTIG1, which is 100-fold lower compared with the measured activity of EcTF (0.64 \( \mu \text{M}^{-1} \text{s}^{-1} \)). Previously determined \( k_{\text{cat}}/K_m \) values for EcTF with the suc-AFPF-pNA peptide as a substrate were 0.52 \( \text{mM}^{-1} \text{s}^{-1} \) (Kramer et al., 1995) and 0.74 \( \text{mM}^{-1} \text{s}^{-1} \) (Stoller et al., 1995). Interestingly, CrTIG1 only shows PPIase activity in an environment with a physiological salt concentration and therefore 150 mM KCl was used in the reaction buffer. This is consistent with a melting-point determination, in which CrTIG1 and ArTIG1 showed higher stability in buffers supplemented with increased salt conditions (Ries et al., 2017).

3.5. Unique properties of the C-terminal domain of CrTIG1

The C-terminal domain of CrTIG1 stands out by its unique features and differently arranged arms, which are not found in bacterial trigger factors. The arms are oriented towards each other and do not show the prominent ‘open arms’ conformation of the bacterial trigger factors, which build a cavity for substrate protein binding (Saio et al., 2014; Figs. 5a and 5b). The thus built lap in CrTIG1 mostly contains negative charged amino acids and is mainly surrounded by hydrophobic amino acids (Figs. 5c and 5d). The conformation of the SBD structure most closely resembles the structures of the E. coli trigger factor [PDB entries 1w26 (Ferbitz et al., 2004) and 2mlz (Saio et al., 2014)] and the periplasmic chaperone SurA (Xu et al., 2007) (Figs. 5a and 5b). In CrTIG1 the location of arm 1 is comparable to the respective arm of EcTF, but arm 2 is arranged differently, with a distance of 24 Å and an angle of 27° with monomeric EcTF (PDB entry 1w26; Ferbitz et al., 2004) and a distance of 39 Å and an angle of 54° with the dimeric EcTF NMR structure (PDB entry 6d6s; Saio et al., 2018) (Fig. 2). Comparison of both chains in the asymmetric unit of the crystal structure shows differences in the position of arm 1 but smaller differences in arm 2. Also, the backbone helix has a kink in chain B compared with chain A (Fig. 5e). Helix α10 in arm 1 is unusually kinked and contains a coiled-coil motif at amino acids 360–392 as predicted by the SMART server (Letunic et al., 2021; Fig. 5f).

3.6. CrTIG1 shows a more compact form with a tilted PPIase domain and closed arms

To determine whether CrTIG1ΔRBD might have artificial arrangements caused by truncation of the N-terminal domain, we fitted the X-ray structure together with the rigid-body model of the N-terminal RBD into the SAXS bead model of full-length CrTIG1, which we had published previously (Ries et al., 2017). We fitted the X-ray structure together with the rigid-body model of the N-terminal RBD into the SAXS bead model of full-length CrTIG1, which we had published previously (Ries et al., 2017).
Figure 5
Structural features of the C-terminal substrate-binding domain (SBD). (a, b) Comparison of the C-terminal arms of CrTIG1 with other chaperones in a front view (a) and a side view (b). Left, CrTIG1; middle, EcTF with bound substrate colored green (PDB entry 2mlz; Saio et al., 2014); right, SurA complexed with peptide (PDB entry 2pv3; Xu et al., 2007). (c) Electrostatic surface potential of the CrTIG1 arms alone; view from the upper side. (d) Hydrophobicity plot of the CrTIG1 arms alone; view from the upper side. (e) Alignment of chain A (red) and chain B (blue) found in the asymmetric unit of the CrTIG1ΔRBD crystal. (f) Coiled-coil motif (colored in orange) in arm 1 of the C-terminal SBD.
The final model was evaluated with CRYSTOL ($\chi^2 = 1.88$), which indicates a good alignment. While the N-terminal domain might have more latitude to move in the SAXS envelope, the PPIase domain is likewise tilted down to the SBD and the arms are still in the closed conformation. There seems to be no free space for an open conformation of the PPIase domain and the arms of the SBD. This indicates that full-length CrTIG1 in solution has a comparable closed conformation with interaction between the PPIase domain and the arms as in the crystal structure.

We also investigated the conformation of CrTIG1 with peptides from the putative interaction partners AtpB (the $\beta$ subunit of the plastidic ATP synthase) and RbcL (the large subunit of Rubisco) as extracted from peptide-spotting arrays (Rohr et al., 2019). The structural parameters point to slight conformational changes but no relocation of domains, as shown for EcTF structures (Supplementary Fig. S5). Taken together, the structure of CrTIG1ARBD resembles the bacterial counterparts but displays unique features such as an unusual orientation of the head and arms and also a special surface-charge pattern.

4. Discussion

Over the past 15 years, numerous structures of bacterial trigger factors have been obtained by NMR, X-ray crystallography and cryo-EM, with EcTF being by far the best investigated trigger factor and possibly one of the best-understood molecular chaperones in the literature. In contrast, no structural information on chloroplast trigger factors had been obtained until now despite their unique presence in eukaryotic cells and detectable functional deviations from their prokaryotic origin (Rohr et al., 2019). Comparison of our chloroplast trigger-factor structure with all full-length structures of bacterial trigger factors from E. coli, T. maritima and V. cholerae revealed the very flexible arrangement of the three domains within the molecule. This agrees with a previous SAXS analysis of CrTIG1 and ArtTIG1, showing that both of these proteins are likewise intrinsically flexible (Ries et al., 2017).

4.1. The PPIase domain of chloroplast trigger factor

In the crystal structure of CrTIG1ARBD and in the SAXS model of full-length CrTIG1 in solution, the PPIase domain is very tilted down and
connects to arm 1 of the SBD. Similar behavior is found in the structures of dimeric EcTF in solution (Morgado et al., 2017; Saio et al., 2018) and in the X-ray structure of a trigger-factor dimer from V. cholerae (Ludlam et al., 2004). Dimerization appears to be a molecular strategy to control the activity of the trigger factor at the ribosome and in its free state. The dimeric TF associates faster with proteins and exhibits stronger anti-aggregation and holdase activity than the monomeric TF (Patzelt et al., 2002; Morgado et al., 2017; Saio et al., 2018). In the asymmetric unit of the crystal packing of CrTIG1ΔRBD two molecules were found, but analysis with the PISA server (Krissinel & Henrick, 2007) indicated no formation of a dimeric complex. This goes in hand with previous small-angle X-ray scattering results and size-exclusion chromatography experiments, which showed that CrTIG1 is mainly monomeric in solution (Ries et al., 2017). However, it cannot be excluded that the interfaces might be crystal-packing contacts.

The conformation of the PPIase domain of chloroplast CrTIG1 shows remarkable overall conservation compared with other PPIase domains across kingdoms. Also, the binding pocket in the inner cavity of the half barrel contains nonpolar and aromatic amino acids which are conserved among FKBPs. However, we observed several structural features that seem to be unique to the PPIase domain of CrTIG1 when compared with the respective domains of other trigger factors or FKBPs proteins. The similarities and differences of key residues in the active site of the CrTIG1 PPIase domain, which are related to PPIase activity, are summarized in Table 2. The structural elements and sequences in the 40s and 80s loops are particularly important for substrate recognition by FKBPs and protein–protein interactions, as demonstrated for the interplay between hFKBP12 and FRAP (FKBP-rapamycin-associated protein; Choi et al., 1996; Lücke & Weiwad, 2011). Interestingly, the 40s loop of CrTIG1 does not show clear homology to either human FKBP13 or to the EcTF PPIase domain as the next structural homologues. An intramolecular hydrogen bond between Tyr72 and Asp38 inside the binding pocket is described in human FKBP12 and other active PPIases, but this bond is missing in EcTF and CrTIG1. In CrTIG1 the corresponding part preceding the 40s loop is disordered and only contains nonpolar residues. It is stabilized through a hydrogen-bond network in the peptide backbone to the neighboring β-strand 7. Likewise, the 80s loop or ‘flap’ of CrTIG1 shows structural particularities, being of equal length but differently oriented. The 50s loop seems to be responsible for substrate specificity (Weiergräber et al., 2006). The backbone confirmation of this loop appears to play the most significant role for PPIase activity in substrate binding and is stabilized via a structural water molecule (Szep et al., 2009). In CrTIG1, Asp226 stabilizes this loop instead of a water molecule. Also, the amino-acid composition in this stretch is important for activity (Gollan et al., 2011). In CrTIG1, the sequence P221DTEAD226V227L228 indicates PPIase activity. Furthermore, the complete PPIase domain of CrTIG1 shows an entirely reverted distribution of its surface charge. While hFKBP12 and EcTF show a mixed pattern of negative and positive patches in this segment, the CrTIG1 PPIase domain has a negative bottom and a positive backbone. In the altered charge distribution, the ‘flap’ stands out with its very negative polarity, a feature that is not observed for EcTF or hFKBP12.

However, neither the substitution of the conserved key active-site residues within the hydrophobic pocket of the PPIase domain of CrTIG1 nor the structural differences in the loops necessarily point to loss of its PPIase activity (Lücke & Weiwad, 2011). Also, comparisons of the active-site residues in CrTIG1 with EcTF and related PPIase domains as well as the amino-acid sequence in the 50s loop indicate an active domain. Indeed, we measured a PPIase activity of CrTIG1 of 0.0057 μM⁻¹ s⁻¹, which is 100-fold lower than that of EcTF with the substrate, but still measurable and within the range of other plant FKBPs proteins (Singh et al., 2020). An FK506-binding protein with comparable low-level PPIase activity (kcat/Km = 0.021 μM⁻¹ s⁻¹) is AtFKBP20-2, which is involved in the accumulation of the PSII supercomplex in Arabidopsis (Lim et al., 2006). Thus, the chloroplast trigger factor possesses a conserved, albeit adapted, PPIase domain with measurable activity. However, the contribution of this domain to the function of the chaperone remains elusive, and it cannot be excluded at this point that the PPIase domain might have evolved towards rather atypical substrates.

4.2. The substrate-binding interfaces of chloroplast trigger factor

EcTF exhibits a broad substrate spectrum and seems to bind several hundred nascent polypeptides during their synthesis (Oh et al., 2011). In contrast, fewer than 100 different proteins are expressed from the chloroplast genome and in vitro studies indicate less general substrate specificity of CrTIG1 (Rohr et al., 2019). EcTF preferably binds peptides with a positive net charge and recognizes aromatic and basic amino-acid residues in peptide substrates (Patzelt et al., 2001). In CrTIG1 the distribution of the electrostatic and hydrophobic potential shows a mixed pattern on the backbone of the protein, but the inner core between the PPIase domain, the arms and the linker is particularly negatively charged. The amino acids in the peptides identified to bind to both of the eukaryotic trigger factors CrTIG1 and AtTIG1 described in Rohr et al. (2019) have no enrichment in basic or acidic peptides (11%/12%), with an average content of 34% nonpolar residues. The content of aromatic residues in these peptides is 12%, while nonbinding peptides mostly lack aromatic amino acids. Regarding peptides exclusively bound by CrTIG1, the content of nonpolar residues increases to 40% and the ratio of basic/acidic peptides increases to 14%/9%.

The substrate-binding domain of CrTIG1 shows structural homology to SurA. The chaperone activity of SurA in the periplasm of E. coli is essential for outer membrane protein biogenesis (Sklar et al., 2007). Ribosome profiling for EcTF showed that β-barrel outer-membrane proteins were the most prominent substrates (Oh et al., 2011). CrTIG1 is mainly localized in the stroma and it still remains to be shown whether it contributes to nascent polypeptide maturation of...
thylakoid membrane proteins, which would explain the similar structural features to SurA.

Arm 1 of the SBD forms an unusual coiled-coil motif and also appears to be more flexible than arm 2. Coiled-coil motifs in chaperones have been discussed to mediate the binding of unfolded proteins (Martin et al., 2004). Archaeal prefoldin (PFD), for example, interacts with unfolded proteins via a coiled-coil network (Siegert et al., 2000). The role of the putative coiled-coil motif in CrTIG1 remains unclear, but it might be the anchor point or interaction surface for unfolded peptide chains in the active state of the trigger factor. In the inactive state, the coiled-coil motif may stabilize the PPIase domain. It remains to be shown whether formation of the coiled coil may be absent in the ribosome-associated chloroplast TIG1 and thus promote nascent polypeptide binding.

Also, for EcTF, MD simulation shows a more compact structure in solution and a framework of intermolecular interactions between the head and arm 1 (Singhal et al., 2013).

5. Conclusion
The crystal structure of a eukaryotic trigger factor is presented. The structure of the trigger factor of the green alga C. reinhardtii resembles that of prokaryotic trigger factors, but with different domain orientations as well as an unusual conformation of the arms in the SBD and also uncommon intramolecular domain interactions. While bacterial trigger factors exhibit promiscuous substrate binding and high plasticity, the more distinct negative and hydrophobic patches of CrtTIG1ARBD in the cradle of the arms and the PPIase domain are compatible with a more specialized function in the chloroplast. Further studies specifically aimed at substrate identification of chloroplast TIG1, such as selective ribosome profiling, are required to precisely understand its role during protein biogenesis. However, the present structure already indicates specific structural adaptation for the binding of proteins in plant organelles. Further, structural availability of the RBD would allow the nature of the ribosome interaction in chloroplasts to be modelled.

6. Related literature
The following references are cited in the supporting information for this article: Gouet et al. (1999) and Madeira et al. (2019).

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