The NH$_2$-terminal Domain of the Chloroplast GrpE Homolog CGE1 Is Required for Dimerization and Cochaperone Function in Vivo*

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GrpE proteins function as nucleotide exchange factors for DnaK-type Hsp70s. We have previously identified a chloroplast homolog of GrpE in Chlamydomonas reinhardtii, termed CGE1. CGE1 exists as two isoforms, CGE1a and CGE1b, which are generated by temperature-dependent alternative splicing. CGE1b contains additional valine and glutamine residues in its extreme NH$_2$-terminal region. Here we show that CGE1a is predominant at lower temperatures but that CGE1b becomes as abundant as CGE1a at elevated temperatures. Comوضع measurementss revealed that CGE1, like GrpE, undergoes two thermal transitions, the first of which is in the physiologically relevant temperature range (midpoint ~45 °C). Truncating the COOH to the NH$_2$ termini: (i) the four-helix bundle, to which each GrpE monomer contributes two short $\alpha$-helices and which serves as a dimerization platform (15, 16); (ii) the extended, paired $\alpha$-helices, which require the four-

helix bundle for pairing (15, 16) and which appear to serve as a thermosensor (local melting of the paired helix at heat shock temperatures drastically lowers the efficiency of GrpE to catalyze nucleotide exchange in DnaK (15, 17, 18)); and (iv) the four-helix bundle, which to each GrpE monomer contributes two short $\alpha$-helices and which serves as a dimerization platform (15, 16); (iii) the extended, paired $\alpha$-helices, which require the four-helix bundle for pairing (15, 16) and which appear to serve as a thermosensor (local melting of the paired helix at heat shock temperatures drastically lowers the efficiency of GrpE to catalyze nucleotide exchange in DnaK (15, 17, 18)); and (iv) the unstructured NH$_2$ terminus, which may interact with the substrate-binding domain of DnaK (19, 20).

We have reported previously that by a temperature-dependent alternative splicing process, the gene encoding the chloroplast GrpE homolog CGE1 gives rise to two transcripts termed CGE1a and CGE1b (8). The CGE1b transcript contains six additional nucleotides coding for valine and glutamine, which are the fourth and fifth NH$_2$-terminal residues of mature CGE1b. In this study, we demonstrate that the CGE1 NH$_2$-terminal region strongly affects the biochemical properties of the protein; first, valine-glutamine in CGE1b increased its affinity for HSP70B, and second, dimerization and functionality of CGE1 in vivo required a coiled coil domain situated at the NH$_2$ terminus of the protein.

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Structure-Function Analysis of Chloroplast GrpE Homolog CGE1

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Chlamydomonas reinhardtii was grown mixotrophically in TAP medium (21) on a rotary shaker at 25 °C and ~30 microeinstein m⁻² s⁻¹. Wild type strain 137c (mt⁻) was used for the experiments of Fig. 1, and cw15 strain CF185 (22) was used for the isolation of HSP70B and the experiments of Figs. 3, C, and D.

Polyacrylamide Electrophoreses and Gel Blot Analyses—SDS-PAGE and gel blot analyses were performed as described earlier (23). Native PAGE was carried out according to Schägger et al. (24). Antiseras were used against HSP70B (22), CF1α (25), and CGE1 (8). Detections were done with ECL.

Cloning, Expression, and Purification of CGE1 Derivatives, DnaK, and HSP70B—The coding regions of CGE1β, Δ9, Δ9, Δ25, Δ45, and Δ71, were amplified by PCR from cDNA clone AV391963 (encoding CGE1b) with 5’-GGGCTCGTCTGGTGCTCAAGCCGGCTG-3’, 5’-GGGCTCTGGTGCTCAAGCCGGCTG-3’, 5’-GGGCTCTGGTGCTCAAGCCGGCTG-3’, 5’-GGGCTCTGGTGCTCAAGCCGGCTG-3’, 5’-CTTGGTGCTCTGGTGCTCAAGCCGGCTG-3’, 5’-GGACTAGTGCTCTTGAGACGGCC-3’, and 5’-GGGTCTCTGGTGCTCAAGCCGGCTG-3’, and 3’ primer T7. For ΔHB, primers 5’-GGGCTCTGGTGCTCTGGTGCTCAAGCCGGCTG-3’ and 5’-CCTGCCGGTCTTCCTGGTGCTCAAGCCGGCTG-3’ were used. For CGE1a, primers 5’-GGGCTCTGGTGCTCTGGTGCTCAAGCCGGCTG-3’ and T7 were used with pMS213 (cDNA clone encoding CGE1a) as a template (8). PCR products were digested with SapI and XhoI and cloned into SapI-XhoI-cloned pMS205 (8) (contains the NH2- and COOH-terminal hexahistidine tags in expression vector pQE-9 (Qiagen, Hilden, Germany)) with primers 5’-CAGAATTCATTAAGAGGAGAAATATACATATGTACGTTACGTCATC-3’ and 5’-CCCAAGCTTAGTGATGGTGATGGTAACC-3’. The 777-bp PCR product was digested with EcoRI and HindIII and ligated into EcoRI-HindIII-digested pQE-9, giving pMS397. Next, the coding regions of the CGE1 derivatives were PCR-amplified with primers 5’-GGATCCCCGTTTTATGCTACGAGGCTG-3’ and 5’-TTGGGTAACCCTCTCACAGAAGCTAGCCGCAGG-3’. These PCR products were then digested with SnaBI and BstXI and ligated into SnaBI-BstEII-digested pMS300, pMS301, pMS367, pMS369, and pMS295 as templates. PCR products were then digested with SnaBI and BstEI and ligated into SnaBI-BstXI-digested pMS397, generating pMS399 (1a), pMS400 (1b), pMS401 (Δ16), pMS402 (Δ25), and pMS398 (Δ45). To remove regions coding for COOH-terminal histidine tags, the latter constructs were digested with BstXI and BspI, and a ~1-kb BstXI-BspI fragment from BstXI-BspI-digested pMS301 was inserted, giving pMS404 (1a), pMS405 (1b), pMS406 (Δ16), pMS407 (Δ25), and pMS408 (Δ45). pMS408 (Δ9) was constructed by ligating a ~500-bp PCR product (using primers 5’-CAGAATTCTATTAAGAGGAGAAATATACTATGAGGCTG-3’ and 5’-CCCAAGCTTAGTGATGGTGATGGTAACC-3’) on pMS365 digested with SnaBI and BstXI into SnaBI-BstXI-digested pMS404. Correct cloning was verified by sequencing.

Glutaraldehyde Cross-links—Proteins were incubated in KMH buffer (20 mM Hepes-KOH, pH 7.2, 80 mM KCl, and 2.5 mM MgCl₂) for 10–30 min. Then glutaraldehyde (0.05–0.1%) was added, and the incubation was continued for another 10–20 min. Cross-linking was stopped by the addition of one volume of 2× Laemmli buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.005% bromphenol blue) containing 400 mM glycine.

Immunoprecipitations and Native Dimer Assays—For affinity assays, 20-μl reaction mixtures containing 50 ng of HSP70B, 15 μg of bovine serum albumin, and 0.75 units of apyrase in KMH buffer were incubated at 23 °C for 15 min. Mixtures were then diluted to 100 μl with KMH buffer (20 mM Hepes-KOH, pH 7.2, 80 mM KCl, and 2.5 mM MgCl₂). Lysates were loaded onto a sucrose density gradient and centrifuged in a TI50 rotor for 30 min at 155,000 g. 2 ml of 10% bovine serum albumin, and 0.75 units of apyrase were added. After 1 h on an overhead shaker, beads were washed four times with KMH and twice with 10 mM Tris-HCl, pH 7, and proteins were eluted by boiling for 45 s after the addition of one volume of 2× Laemmli buffer. For immunoprecipitations from cell extracts, Chlamydomonas cells from a 300-ml culture with a density of ~2 × 10⁶ cells/ml had been grown overnight at 37 °C or subjected to heat shock at 41 °C for 4 h. Cells were harvested and lysed by sonication on ice in 4 ml of lysis buffer (20 mM Hepes, pH 7.2, 1 mM MgCl₂, 20 mM KCl, 150 mM NaCl, 0.25× protease inhibitor mixture (Roche Applied Science), 10 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone, and 5 units of apyrase). Lysates were loaded onto a sucrose cushion (20 mM Hepes-KOH, pH 7.2, 0.6 M sucrose) and centrifuged in a T150 rotor for 30 min at 152,000 × g and 4 °C. 2 ml of the supernatant were incubated with 100 μl of Protein A-Sepharose beads coupled with 20 μl of polyclonal anti-HSP70B serum. After mixing for 1 h on an overhead shaker,
beads were washed four times with lysis buffer and twice with 10 mM Tris-HCl, pH 7, and proteins were eluted by boiling after the addition of one volume of 2× Laemmli buffer. Quantification of CGE1a/b bands was done from scanned ECL films using the QuantityOne-4.5.1 program (Bio-Rad).

For dimerization assays, 1 μM CGE1a, 1 μM CGE1b, or a 0.5 μM concentration of both was incubated in 10 mM Hepes-KOH, pH 8, 50 mM KCl, 10 mM β-mercaptoethanol, 0.1 mM EDTA, 2.5 mM MgCl2, 20% glycerol for 30 min at 23 °C. The reaction mixture was then diluted 10-fold in 50 mM BisTris-HCl, pH 7.0, 0.5 mM ε-aminocaproic acid, 15% glycerol, 1 mM MgCl2, 10 mM KCl, 0.004% Ponceau-S, and 10% of the mix was loaded onto a native gel.

Homology Modeling of CGE1b—As a template for homology modeling, the structure of the E. coli GrpE dimer bound to its HSP70 chaperone partner DnaK was used (13) (Protein Data Bank entry 1DKG, chains A and B). Pairwise alignment of CGE1b and GrpE was done based on a multiple alignment of CGE1 with GrpE homologs of bacterial and mitochondrial origins (8). Secondary structure prediction of the NH2-terminal region of CGE1, which is not conserved between CGE1 and GrpE, was done with the programs JPRED, PHD, PROF, PSIPred, PSSP, and SSpro accessed via the Columbia metamask (available on the World Wide Web at cubic.bioc.columbia.edu/predictprotein/submit_meta.html). Tropomyosin (29) (Protein Data Bank entry 1C1G, amino acids 70–122 of chain A and 355–407 of chain B) as an appropriate template for the modeling of the CGE1 NH2-terminal region was found by using the hydrophobic pattern identified in this region as a query for BLAST-P. Pairwise alignment of CGE1b and Tropomyosin was done on the basis of similar hydrophobicity. 20 series of 100 models each with plausible alternative alignments (including an α-helical restraint for Ala1–Ala12 and Ala222–Ala233, respectively, based on secondary structure prediction) was generated with the MODELLER 7v06 software within InsightII (Accelrys, San Diego, CA). The five models with highest ranking (based on probability density function and energy values) and best root mean square values as determined by ProFit (A. C. R. Martin; available on the World Wide Web at www.bioinf.org.uk/software/profit/) were tested with the Procheck version 3.5.4 program (30) and checked manually. The model chosen was then energy-minimized three times using the CHARMM force field in InsightII. During the first minimization (600 steps and 0.001 final convergence) the backbone and the region between Arg24–Glu221 and Ala295–Glu442 were fixed. During the second minimization (again 600 steps and 0.001 final convergence), only the region Arg24–Glu221 and Ala295–Glu442 was fixed. The entire model was fixed during the final minimization (30 steps).

Circular Dichroism Measurements—Circular dichroism was measured with a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) using a thermostated cuvette with a 1-mm path length. Temperature was controlled with a programmable water bath. At fixed temperatures (25 °C), three spectra between 300 and 190 nm (bandwidth 1 nm) were recorded every 0.1 nm at a scan speed of 200 nm min −1 and averaged. For these experiments, proteins had been diazylated against 20 mM sodium phosphate, pH 7.5, and proteins were used at a concentration of 10 μM. Time courses of temperature-induced conformational changes were followed by continuously monitoring the ellipticity at 222 nm (bandwidth 1 nm). The cuvette was heated by 0.5 °C min −1, and measurements were taken once every 2 min. For these experiments, proteins had been diazylated against KH buffer, and proteins were used at 20 μM concentration.

RESULTS

Temperature-dependent Alternative Splicing of CGE1 Transcripts Leads to the Differential Accumulation of CGE1a and CGE1b Isoforms—The recently available Chlamydomonas genome sequence (available on the World Wide Web at genome.jgi-psf.org/Chlr3/Chlr3.home.html) allowed us to elucidate that the CGE1 gene consists of eight exons and seven introns and that it is the first CGE1 intron that is alternatively spliced (Fig. 1A). Alternative splicing was shown to be temperature-dependent (i.e. CGE1a represented 60–80% of the CGE1 message at 25 °C but declined to ~30% in favor of CGE1b after a 40-min heat shock) (8). To test whether temperature-dependent changes in CGE1 message composition also were reflected at the protein level, we sub-
jected *Chlamydomonas* cells to different temperature treatments and analyzed the cellular accumulation of CGE1a and CGE1b protein. As shown in Fig. 1B, CGE1a was much more abundant than CGE1b at 15 and 25 °C. However, CGE1b levels increased slightly at 30 °C, and CGE1b became equally abundant as CGE1a at 37 and 41 °C.

We were surprised to see that we could separate CGE1a and CGE1b by SDS-PAGE, since the two proteins differ only by 227.3 Da, the mass of valine and glutamine. To verify that the CGE1 double band observed in Fig. 1B originated from the CGE1a/b isoforms, we compared the migration pattern of CGE1 from heat-shocked *Chlamydomonas* cells with that of purified CGE1a/b proteins that had been heterologously expressed in *E. coli*. The masses of heterologously expressed CGE1a and CGE1b determined by mass spectrometry were 23812.0 and 24040.0, respectively, which matched the masses calculated from the amino acid sequences (23812.19 and 24039.46, respectively). As presented in Fig. 1C, also purified CGE1a and CGE1b could be separated in SDS-polyacrylamide gels, and they co-migrated exactly with the two isoforms from *Chlamydomonas* cells. We conclude that the temperature-dependent alternative splicing of the *CGE1* transcript leads to a temperature-dependent accumulation of CGE1a and CGE1b proteins.

**HSP70B Purified from *E. coli* Does Not Interact with CGE1, but HSP70B Purified from *Chlamydomonas* Does**—According to current knowledge, GrpE-type proteins do not have any enzyme activities by themselves, but they act as cochaperones for Hsp70s (14). Hence, if the differential accumulation of the CGE1a/b isoforms was of any biological significance, it was expected to be by interaction with HSP70B, the chloroplast Hsp70 partner of CGE1 (8). Since we intended to study possible effects of CGE1a/b on HSP70B, we first purified HSP70B. This we did from *E. coli* cells that heterologously expressed HSP70B (Bec) and from *Chlamydomonas* cell extracts (Bcr; Fig. 2A) (23, 26). Next, we used glutaraldehyde cross-linking to test whether our HSP70B preparations were capable of interacting with CGE1 in vitro. As shown in Fig. 2B, most of purified CGE1a existed as dimers, as expected from previous observations with native gels (8). In the absence of ATP, CGE1a readily formed a complex with Bcr, which was disrupted by ATP. In contrast, CGE1a did not interact with Bec. Interestingly, Bec and Bcr, cross-linked in the absence of CGE1 already displayed different migration properties; whereas Bec migrated as oligomers, dimers, and as a compact species of ~50 kDa, Bcr appeared not to form oligomers or dimers but mainly occurred as the compact ~50-kDa species and as a less compact ~70-kDa species. Since the latter vanished entirely upon the addition of CGE1a in the absence of ATP but reappeared upon the addition of ATP, it appears to be this less compact ~70-kDa species of HSP70B that is able to interact with CGE1. In summary, recombinant CGE1a efficiently formed dimers and formed ATP-sensitive complexes with HSP70B isolated from *Chlamydomonas* but not with recombinant HSP70B from *E. coli*. Apparently, recombinant HSP70B stably assumed non-functional conformations.

**CGE1b in Vitro and in Vivo Has a Higher Affinity for HSP70B than CGE1a**—We suggested previously that the CGE1a/b isoforms may differ in their affinity for HSP70B (8). To test this idea, we used HSP70B isolated from *Chlamydomonas*, mixed it with equal amounts of CGE1a and CGE1b in the absence of ATP, immunoprecipitated HSP70B from the mixture, and assayed how much CGE1a/b coprecipitated with HSP70B. Although crude, the advantage of this assay was that it required only small amounts of the precious Bcr. The assay was performed at 23 °C and at 37 °C to monitor possible temperature effects. As demonstrated in Fig. 3A, little but reproducibly more CGE1b than CGE1a coprecipitated with HSP70B at 23 °C. At 37 °C, the affinity of both CGE1a and CGE1b for HSP70B was dramatically reduced compared with 23 °C, but also at 37 °C.
CGE1b had a higher affinity for HSP70B than CGE1a. To estimate to what extent CGE1b has a higher affinity for HSP70B than CGE1a, we increased the ratio of CGE1a to CGE1b in the CGE1 mixture that was incubated with HSP70B. Only when the amount of CGE1a exceeded that of CGE1b by about 25%, both CGE1 isoforms coprecipitated at equal amounts with HSP70B (Fig. 3B). To test whether also in vivo CGE1b had a higher affinity for HSP70B than CGE1a, we immunoprecipitated HSP70B from Chlamydomonas cell extracts and compared the ratio of coprecipitating CGE1a/b with the ratio of CGE1a/b present in the extracts prior to immunoprecipitation. To have roughly equal ratios of CGE1b to CGE1a prior to immunoprecipitation, we grew cells at 37 °C overnight or subjected them to heat stress at 41 °C for 4 h. Quantification of the CGE1a/CGE1b ratios in cell extracts and in HSP70B coprecipitates in three independent experiments revealed that CGE1b was 5–10% more abundant in coprecipitates than in cell extracts (Fig. 3C). Hence, in vivo, the affinity of CGE1b for HSP70B seemed to be less than half of that observed in vitro. An explanation that may account for this discrepancy is that CGE1a and CGE1b possibly exist as homodimers and heterodimers in vivo but only as homodimers in vitro. If this was true, the higher affinity of CGE1b for HSP70B would be "neutralized" by more than half (i.e. from 25 to 11.8%) in heterodimers. According to the crystal structure of GrpE in complex with the ATPase domain of DnaK, only the GrpE molecule of the dimer that is situated proximal to Hsp70 interacts with the chaperone (13). Hence, if CGE1b in a CGE1ab heterodimer was distal to HSP70B, its higher affinity for HSP70B would be useless; if it was proximal, its higher affinity for HSP70B would also lead to coprecipitation of CGE1a.

To analyze the composition of CGE1 dimers in vivo, we isolated soluble cell extracts from Chlamydomonas cells that were grown under nonstress conditions or from cells that were subjected to heat stress for 2 h, separated native protein complexes by CN-PAGE (8, 24), and immunodetected CGE1. Purified CGE1a and CGE1b alone or in mixture were treated likewise. As shown in Fig. 3D, CGE1a/b in Chlamydomonas cell extracts did form heterodimers, but CGE1a/b mixed in vitro did not. As expected from proteins present at about equimolar amounts, CGE1aa/CGE1ab/CGE1bb dimers in cell extracts after heat stress were present at a 1:2:1 stoichiometry. In contrast, CGE1b homodimers were hardly detectable under nonstress conditions. This is not surprising, considering that CGE1b was expected to form homodimers in vivo.

The Extreme NH2-terminal Segment of CGE1 Appears to Contain a Coiled Coil Motif and Is Essential for Dimer Formation and Cochaperone Function in Vivo—Our finding that the additional valine-glutamine amino acids in the extreme NH2-terminal region of CGE1 mediate a higher affinity of the protein for HSP70B suggested that this region might have a significant impact on the protein’s biochemical properties. We therefore decided to analyze the NH2-terminal region of CGE1 in more detail. Overall, Chlamydomonas CGE1a and E. coli GrpE share 32% identical and 49% similar residues. When we compared both proteins, we noticed that their amino acid sequences aligned without gaps but that mature CGE1a has a
Structure-Function Analysis of Chloroplast GrpE Homolog CGE1

A

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\begin{align*}
&\text{CGE1a} \\
&\text{GrpE} \\
&\text{CGE1a} \\
&\text{GrpE} \\
&\text{CGE1a} \\
&\text{GrpE} \\
\end{align*}
\]

(B) GrpE

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\begin{align*}
&\text{CGE1b} \\
&\text{GrpE} \\
&\text{CGE1b} \\
&\text{GrpE} \\
&\text{CGE1b} \\
\end{align*}
\]
14-amino acid longer NH$_2$-terminal region than GrpE (Fig. 4A). Moreover, whereas the extended, paired α-helix of GrpE ends at Pro$^{39}$ (13), that of CGE1a by all secondary structure prediction programs is used to end at Pro$^{18}$. Thus, the paired α-helix of CGE1 appears to be 34 amino acids longer than that of GrpE. Whereas the prediction for the region between Pro$^{18}$ and Pro$^{39}$ was ambivalent, the region between Ala$^3$ and Pro$^{18}$ is likely to form an α-helix (in both CGE1a and CGE1b). Most strikingly, however, the CGE1 α-helix from Leu$^{20}$ to Ala$^{72}$ contains a canonical i + 3, i + 4 heptad repeat of hydrophobic residues typical for coiled coils (31) (Fig. 4A). This repeat is only interrupted by Glu$^{30}$ and Lys$^{34}$, which, however, might stabilize a coiled coil by forming salt bridges with Lys$^{34}$ and Glu$^{30}$ of the opposite CGE1 molecule. To illustrate how CGE1 with an NH$_2$-terminal coiled coil structure might look like compared with GrpE lacking a coiled coil, we modeled CGE1b using as templates the crystal structures of the GrpE dimer bound to DnaK (13) and of tropomyosin (29) (Fig. 4B). Tropomyosin is a classical example for a coiled coil protein and was chosen because it shares a highly similar hydrophobic pattern with the NH$_2$-terminal segment of CGE1. Tropomyosin could easily be superimposed on the ends of the two GrpE α-helices, which are slightly shifted with respect to each other and point somewhat outwards.

Since coiled coils may form very stable structures, we wondered whether the putative coiled coil in the CGE1 NH$_2$-terminal region may affect dimer stability. To test this, we compared CGE1 derivatives from which the NH$_2$-terminal 9, 16, 25, and 45 amino acids had been deleted with CGE1a and CGE1b (Fig. 4). Dimerization properties of these CGE1 derivatives were assayed by glutaraldehyde cross-linking at different temperatures (Fig. 5A). CGE1a, CGE1b, Δ9, and Δ16 were capable of dimer formation at all temperatures tested. Δ25 formed dimers at 22 and 30 °C but only to a strongly reduced extent at 42 °C and not at all at 55 °C. Δ45 was even more severely affected; it formed dimers only at 22 °C and not at 30 °C or higher temperatures.

To test whether the six CGE1 derivatives were all capable of functioning as cochaperones in vivo, we expressed them in E. coli strain OD212 and assayed for growth at 23, 42, and 45 °C (Fig. 5B). OD212 carries both a deletion of the grpE gene and the compensatory dnaK332 allele and is incapable of growth at temperatures above 30 °C (32). As judged from their ability to grow normally at 23 °C (over)expression of the six CGE1 derivatives appeared not to have negative effects on cell viability (Fig. 5, B and C). OD212 containing the empty expression vector also grew normally at 23 °C but as expected did not grow at 42 or 45 °C. In contrast, OD212 expressing CGE1a, CGE1b, Δ9, and Δ16 did grow at 42 and 45 °C, although colonies formed by cells expressing Δ16 were smaller at elevated temperatures. Expression of Δ25 only partially complemented temperature-sensitive growth, and no complementation of temperature sensitivity was observed in OD212 expressing Δ45. The most straightforward explanation for this observation would be that Δ25 and Δ45 also in vivo were unable to form dimers at elevated temperatures and therefore could not interact with DnaK to function as cochaperones.

To address this idea, we incubated purified DnaK with CGE1a, Δ9, Δ16, Δ25, and Δ45 in the absence of ATP and cross-linked the proteins with glutaraldehyde at 30 and 44 °C (Fig. 5D). As before, dimer formation of Δ45 at 30 °C and of Δ25 and Δ45 at 44 °C was abolished, whereas CGE1a, Δ9, and Δ16 formed dimers at both temperatures. The slightly faster migration of the proteins cross-linked at 44 °C may originate from more efficient intramolecular cross-linking at higher temperatures that may have increased their compactness. At 30 °C, all five CGE1 derivatives formed complexes with DnaK at ~130 kDa, thus consistent with CGE1 dimers interacting with DnaK monomers. In some experiments, DnaK formed complexes even more efficiently with Δ45 than with the other CGE1 derivatives. At 44 °C, however, DnaK-CGE1$_2$ complexes were absent for Δ25 and Δ45 but still detectable for CGE1a, Δ9, and Δ16 (Fig. 5D). In summary, the NH$_2$-terminal region of CGE1 is longer than that of GrpE and appears to contain a coiled coil motif. Perturbing this coiled coil by deletions strongly impairs the ability of CGE1 to form dimers and to function as a cochaperone at elevated temperatures in vivo. The latter effect appears to be due to the inability of CGE1 derivatives affected in their coiled coil to interact with DnaK at elevated temperatures.

**CGE1 Dimer Formation Is Mediated by the NH$_2$-terminal Coiled Coil and Not by the Four-helix Bundle**—Dimerization of E. coli GrpE was shown to be mediated exclusively by its four-helix bundle, which is situated immediately COOH-terminally of the extended α-helix (Fig. 4) (13, 15, 16). In contrast, our data suggest that CGE1 dimerization is mediated mainly by its NH$_2$-terminal coiled coil. To test whether the four-helix bundle also predicted to be present in CGE1 (Fig. 4B) contributes to dimer formation, we generated two CGE1 derivatives lacking either the entire coiled coil domain (Δ71) or the four-helix bundle and the succeeding β-sheet domain (ΔHB). Again, we assayed dimerization properties of these derivatives by glutaraldehyde cross-linking at different temperatures (Fig. 6). Whereas CGE1a and ΔHB formed dimers equally well at 22, 30, and 42 °C, dimer formation was virtually absent for Δ71 at all temperatures and, as before, was seen only at 22 °C for Δ45. Thus, we conclude that CGE1 appears to require its NH$_2$-terminal coiled coil for dimer formation, whereas the four-helix bundle is dispensable.

**FIGURE 4. Alignment and models of CGE1 and GrpE.** A. E. coli GrpE (accession P09372) and Chlamydomonas CGE1a (accession AAK96223) were aligned pairwise. Conserved residues are shaded in black. Deletion end points of CGE1 derivatives used in this study are indicated with arrows, as is the position of Val-Gln (VQ) in CGE1b. The canonical heptad repeat of hydrophobic residues (i + 3, i + 4) in CGE1 starting with Leu$^{20}$ and ending with Ala$^{72}$ is given. The asterisks indicate Glu$^{30}$ and Lys$^{34}$, which disturb the hydrophobic pattern but might form salt bridges with Lys$^{34}$ and Glu$^{30}$ of the opposite CGE1 molecule. Domain functions and borders of GrpE were taken from Ref. 15. B, ribbon presentation of the structures of GrpE (13) (left) and modeled CGE1b (right). Since the NH$_2$-terminal 33 amino acids of GrpE were unstructured and had to be removed for crystallization, they are not shown; because of their low resolution in the crystal structure, loops connecting helices of the four-helix bundle in GrpE are also not shown (13). Hydrophobic residues interacting in the paired α-helices are drawn with space-filling symbols; blue spheres represent nitrogen, red spheres represent oxygen, and gray spheres represent carbon atoms. Residues representing the deletion end points of CGE1 derivatives in the backbone are colored in magenta and indicated by the arrows. Due to ambiguous predictions of the secondary structure formed by the residues between Pro$^3$ and Pro$^{18}$, this region was drawn as a random coil. Also, the positions of the NH$_2$-terminal α-helices predicted between Ala$^3$ and Ala$^{12}$ are drawn arbitrarily.
Thermal Unfolding of CGE1a/b Reveals Two Reversible Transitions—To analyze how CGE1 secondary structure was affected in CGE1 deletion derivatives, we measured circular dichroism properties of CGE1a and CGE1b and of /H900425, /H900445, /H900471, and /H9004HB. The far UV circular dichroism spectrum of CGE1b displays strong peaks of negative ellipticity at 208 and 222 nm indicative of substantial /H9251-alpha-helical content (Fig. 7, top left panel). The spectrum for CGE1a was identical (data not shown). Thus, the CGE1a/b spectra strongly resemble those of GrpE homologs from /E. coli/ (15, 17, 33), Thermus thermophilus (34), human mitochondria (35), and yeast mitochondria (36). Compared with CGE1b, ellipticity is slightly reduced in the /H900425, /H900445, and /H9004HB deletions. This is consistent with a loss of alpha-helical content due to removal of part of the NH2-terminal coiled coil in /H900425 and /H900445 and of the four-helix bundle in /H9004HB (Fig. 7, left panels). Strikingly, ellipticity is completely lost in the /H900471 deletion, suggesting that removal of the CGE1 NH2-terminal coiled coil abolishes its ability to fold correctly. This observation is consistent with the results from glutaraldehyde cross-linking, which indicated that CGE1 /H900471 could not form dimers even at 22 °C (Fig. 6).

Two transitions were resolved in circular dichroism thermal unfolding curves of CGE1a/b (Fig. 7, top right panel; CGE1a curves were identical to those of CGE1b and therefore are not shown). The first transition started at ~37 °C and had its midpoint at ~45 °C. The second transition started at ~51 °C and had its midpoint at ~59 °C. Only one thermal transition was resolved in the /H900425 deletion, which, similar to the first transition in CGE1a/b, started at ~37 °C and had its midpoint at ~45 °C (Fig. 7, right panels, second from top). Moreover, unfolding of the /H900425 deletion was completed at ~55 °C, whereas that of CGE1a/b was completed at ~65 °C. This finding correlates with the observation that the /H900425 deletion could not form dimers at temperatures above 42 °C and showed only partial complementation of temperature sensitivity of the OD212 strain (Fig. 5, A and B). Also, in the /H900445 deletion, only
one thermal transition was resolved, which started at \(-19^\circ C\), had its midpoint at \(-29^\circ C\), and ended at \(-37^\circ C\), most likely because of complete unfolding of the protein (Fig. 7, right panels, third from top). Also, this behavior correlates with the inability of the \(\Delta 45\) deletion to form dimers at \(30^\circ C\) and temperatures above and to complement the temperature sensitivity of the OD212 strain (Fig. 5, A and B). The unfolding curve of the \(\Delta HB\) deletion also exhibited only one thermal transition, which, starting at \(-46^\circ C\), had its midpoint at \(-57^\circ C\) (Fig. 7, right panels, fourth from top). Similar to CGE1a/b, unfolding was completed around 65°C, suggesting that CGE1a/b and the \(\Delta HB\) deletion shared similarly stable secondary structures, which again was supported by similarly stable dimer formation observed for CGE1a/b and \(\Delta HB\) in glutaraldehyde cross-links (Fig. 6). For the \(\Delta 71\) deletion, no change in circular dichroism at 222 nm was observed over the temperature range between 15 and 80°C (Fig. 7, bottom right panel). This suggests that the \(\Delta 71\) deletion is constitutively unfolded, thus explaining the inability of this deletion to form dimers at \(22^\circ C\) (Fig. 6). Unfolding of the CGE1a/b, \(\Delta 25\), and \(\Delta HB\) derivatives after heating to 80°C was almost completely reversible, whereas that of the \(\Delta 45\) deletion was only partly reversible, suggesting some extent of aggregation (Fig. 7, right panels). We noticed that reversibility of thermal unfolding (and resolution of the two thermal transitions in CGE1a/b) was strongly dependent on the solvent; proteins dissolved in 20 mM sodium phosphate buffer, pH 7.5, gave smoother spectra, but reversibility of unfolding was impaired, whereas proteins dissolved in 20 mM HEPES-KOH, pH 7.2, 80 mM KCl gave more noisy spectra, but refolding was facilitated (Fig. 7; data not shown). Finally, we wondered whether thermo-
stability of homodimers of CGE1a and CGE1b differs from CGE1a/b heterodimers. To test this, both proteins were mixed at equimolar concentrations, and circular dichroism curves for unfolding, refolding, and repeated unfolding were recorded. We reasoned that CGE1a and CGE1b homodimers would monomerize during the first unfolding and that during refolding half of the reconstituted dimers would be heterodimers. Since both unfolding curves and the refolding curve for the mixtures of CGE1a and CGE1b were indistinguishable from the curves recorded for the pure proteins (data not shown), it appears that the CGE1 homo- and heterodimers are equally thermostable. In summary, circular dichroism revealed that upon thermal unfolding, CGE1a/b homo- and heterodimers exhibit two almost fully reversible transitions, the first of them being in the physiologically relevant temperature range. This first transition was absent in CGE1 lacking the four-helix-bundle and the β-domain. Progressive deletion of the NH₂-terminal coiled coil domain progressively shifted the second transition to lower temperatures.

**DISCUSSION**

Here we show that the CGE1b isoform has an about 25% higher affinity for its chloroplast chaperone partner HSP70B than CGE1a (Fig. 3). From data available from the homologous DnaK-GrpE chaperones of E. coli, we can envision three explanations that may account for this finding. (i) The NH₂-terminal 33 amino acids of bacterial GrpE have been demonstrated to interact with the substrate-binding domain of DnaK, although with low affinity (20). Since the NH₂-terminal 33 amino acids of GrpE compete with DnaK substrates for binding to DnaK, it was suggested that the GrpE NH₂-terminus may occupy the substrate-binding pocket of DnaK (19). Assuming that also the CGE1 NH₂-terminus may occupy the substrate-binding pocket of HSP70B, the CGE1b NH₂-terminal segment containing the valine-glutamine dipeptide might be a better substrate than that of CGE1a. In fact, an algorithm estimating the suitability of a given peptide as a substrate for DnaK (37) indeed predicted that the CGE1b NH₂-terminal region due to its higher hydrophobicity would be a slightly better substrate for DnaK than that of CGE1a. (ii) Valine and glutamine are located at positions 4 and 5 in mature CGE1b in a region predicted to be α-helical (Fig. 4). Thus, they might introduce a half-helical turn leading to the positioning of Glu³ such that it can form a salt bridge with a lysine or arginine residue in the HSP70B substrate-binding domain. Alternatively, Glu³ in CGE1a might be unfavorably positioned toward a negative charge in HSP70B; introduction of a half-helical turn in CGE1b might turn away the unfavorable position negative charge and thus increase the affinity of CGE1b for HSP70B. (iii) The interaction of DnaK and GrpE is mediated largely by an exposed conserved loop in the ATPase domain of DnaK (6, 38). Thus, contacts of the NH₂-terminal segment of CGE1 with the substrate-binding domain of HSP70B might trigger intramolecular conformational changes affecting the ATPase domain of the chaperone, where residues of the exposed loop required for the interaction with CGE1 are withdrawn (CGE1a) or more favorably presented (CGE1b). What might be the in vivo effect of an improved affinity of CGE1b for HSP70B as compared with CGE1a? It was shown recently that GrpE stabilized the affinity of DnaK for its substrate α2 more than 2-fold (19). If a similar effect exists for the HSP70B-CGE1 complex, CGE1b having a higher affinity for HSP70B might enhance this effect. Consequently, under stress conditions when ATP concentrations are low, CGE1b might stabilize HSP70B-substrate interactions to avoid release of aggregation-prone substrates that cannot be folded. Increased levels of CGE1b relative to CGE1a under increasingly stressful conditions would be consistent with this interpretation.

The extended α-helix of the NH₂-terminal region of CGE1 is predicted to be 34 amino acids longer than that of GrpE (Fig. 4). The distal part of this extended α-helix contains a canonical i + 3, i + 4 heptad repeat of hydrophobic amino acids characteristic for coiled coils (31). This is surprising, since the extended α-helix of E. coli GrpE does not form a coiled coil due to a stuffer in the heptad repeat, which abolishes superhelicity and most likely reduces the stability of the interaction between the helices relative to a coiled coil (13, 15). Consequently, GrpE dimerization of CGE1 depended exclusively on its NH₂-terminal coiled coil and was independent of the four-helix-bundle (Figs. 5–7).

When we monitored loss of α-helical content at increasing temperatures by circular dichroism spectroscopy, we observed two almost fully reversible transitions for CGE1a/b (Fig. 7), the first transition started at ~37 °C and had its midpoint at ~45 °C. As judged from glutaraldehyde cross-linking analysis (Fig. 5A), the first transition is not accompanied by monomerization and is more likely due to local unfolding. The second transition started at ~51 °C and had its midpoint at ~59 °C. Progressive truncation of the NH₂-terminal coiled coil of CGE1 increasingly shifted the second transition to lower temperatures, whereas it remained essentially unaltered in CGE1 lacking the four-helix bundle and the β-domain (Fig. 7). Therefore, we attribute the second transition to unfolding of the NH₂-terminal coiled coil, which most likely is accompanied by monomerization (Figs. 5 and 6). The first transition is lost in CGE1 lacking the four-helix bundle and the β-domain (Fig. 7). Since β-sheets are invisible in these measurements (39), the first transition might be attributed to the unfolding of the four-helix bundle. Alternatively, the first transition observed for CGE1a/b might be due to local unfolding of the paired α-helices proximal to the coiled coil. Since folding and stabilization of the paired α-helices of E. coli GrpE require the four-helix bundle and the β-domain (15, 16), deletion of the latter in CGE1 might cause constitutive unfolding of the paired α-helices and therefore the loss of the first transition. Thermal unfolding of E. coli GrpE also revealed two transitions; the first (fully reversible) transition started at ~35 °C, had a midpoint at ~48 °C, and was attributed to the unfolding of the paired α-helices (15, 17, 18). The second transition had a midpoint at ~75–80 °C and was attributed to unfolding of the four-helix bundle and dissociation of the dimer (15, 17). Two transitions upon thermal unfolding were also observed for T. thermophilus GrpE (39); the first transition had a midpoint at 90 °C and was attributed to unfolding of the β-domain. The midpoint of the second transi-
tion was at 100–105 °C and was attributed to dimer dissociation, with the paired α-helices as dimerization platform. Only one (fully reversible) transition with a midpoint at 40 °C was observed for Mge1p, the GrpE homolog from yeast mitochondria. The transition was attributed to protein unfolding and dimer dissociation (36). Taken together, dimerization of GrpE homologs from different organisms appears to be realized by different domains; GrpE from E. coli employs the four-helix bundle, GrpE from T. thermophilus employs the paired α-helices, and CGE1 from Chlamydomonas reinhardtii chloroplasts employs an NH2-terminal coiled coil. How dimerization is realized by Mge1p is not yet clear.

Monomerization of Mge1p already took place at temperatures that inflict heat stress on yeast (i.e. temperatures above 37 °C) (36). In contrast, monomerization of the bacterial GrpEs and of chloroplast CGE1 occurs at temperatures above the respective organisms’ physiological temperature ranges. These lie between 10 and 45 °C for E. coli, 40 and 80 °C for T. thermophilus, and 15 and 43 °C for C. reinhardtii (39). However, in temperature ranges corresponding to heat shock conditions for the respective organism, all three proteins exhibit local unfolding of a distinct domain: the paired α-helices in E. coli GrpE, the β-domain in T. thermophilus GrpE, and the paired α-helices or the four-helix bundle in Chlamydomonas CGE1. Hence, complete or at least local unfolding at heat shock temperatures appears to be a widespread trait of GrpE homologs from different organisms.

The GrpE-mediated conversion from the ADP-bound to the ATP-bound state of E. coli DnaK, yeast mitochondrial Hsp70 (Ssc1p) and T. thermophilus DnaK, was shown not to exhibit Arrhenius temperature dependence; this means that nucleotide exchange was slowed down at temperatures higher than ~40, ~37, and ~80 °C, respectively (17, 36, 39). This effect is believed to preferably shift DnaK into the ADP-bound state under stress conditions, thus enhancing sequestration of substrates to DnaK, which at elevated temperatures would otherwise aggregate upon release (40). The loss of GrpE-mediated nucleotide exchange activity at elevated temperatures was demonstrated to be due to local unfolding of the paired α-helices and the β-domain of E. coli and T. thermophilus GrpE, respectively (15, 18, 39), and due to complete unfolding of yeast Mge1p (36). Whereas local unfolding of E. coli GrpE appeared to reduce its interaction with DnaK (41), complete unfolding of Mge1p abolished its interaction with Ssc1p (36). Hence, the local unfolding of the paired α-helices or the four-helix bundle in CGE1 (Fig. 7) and the reduced affinity of CGE1 for HSP70B at heat shock temperatures (Fig. 3A) suggest that under heat shock also the ability of CGE1 to catalyze nucleotide exchange in HSP70B might be impaired.

Clearly, more experiments are needed to test whether CGE1 like its bacterial GrpE homologs has a reduced nucleotide exchange activity on HSP70B at elevated temperatures. However, such experiments are hampered by our finding that HSP70B expressed in E. coli is nonfunctional (Fig. 2). Interestingly, yeast mitochondrial Hsp70 (Ssc1p) was also found to be nonfunctional when expressed heterologously in E. coli (32) unless it was co-expressed with the Hsp70-escort protein Hep1 (42). We have identified a chloroplast homolog of Hep1 (termed HEP2) that interacts with HSP70B. We hope that co-expression of HSP70B and HEP2 might enable us to generate the quantities of functional HSP70B required for more detailed studies of the chloroplast HSP70B-CGE1 chaperone pair.

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Structure-Function Analysis of Chloroplast GrpE Homolog CGE1