

Renaturation of *Bacillus thermoglucosidasius* HrcA Repressor by DNA and Thermostability of the HrcA-DNA Complex In Vitro

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HrcA, a negative control repressor for chaperone expression from the obligate thermophile *Bacillus thermoglucosidasius* KP1006, was purified in a His-tagged form in the presence of 6 M urea but hardly renatured to an intact state due to extreme insolubility. Renaturation trials revealed that the addition of DNA to purified *B. thermoglucosidasius* HrcA can result in solubilization of HrcA free from the denaturing agent urea. Results from band shift and light scattering assays provided three new findings: (i) any species of DNA can serve to solubilize *B. thermoglucosidasius* HrcA, but DNA containing the CIRCE (controlling inverted repeat of chaperone expression) element is far more effective than other nonspecific DNA; (ii) *B. thermoglucosidasius* HrcA renatured with nonspecific DNA bound the CIRCE element in the molecular ratio of 2.6:1; and (iii) *B. thermoglucosidasius* HrcA binding to the CIRCE element was stable at below 50°C whereas the complex was rapidly denatured at 70°C, suggesting that the breakdown of HrcA is induced by heat stress and HrcA may act as a thermosensor to affect the expression of heat shock regulatory genes. These results will help to determine the nature of HrcA protein molecules.

In the heat shock response of gram-positive bacteria and α -proteobacteria, the CIRCE (controlling inverted repeat of chaperone expression)-HrcA systems play an important role in the mechanism for regulation of expression of heat shock genes (14, 15, 29). HrcA, a repressor protein encoded by the *hrcA* gene, is responsible for negative control of heat shock genes by binding to the CIRCE element located in the region controlling transcription. The CIRCE element in *Bacillus* species is composed of a pair of conserved 9-bp inverted repeats and intervening nonconservative 9-bp spacers. The *groE* and *dnaK* operons of *Bacillus* species have been demonstrated to be under this control system (28, 29). Indeed, analyses of the entire genome sequence of *Bacillus subtilis* revealed that the strictly conserved inverted repeat is restricted to these two chaperone operons (15). Further studies on *B. subtilis* HrcA and *Bacillus stearothermophilus* HrcA indicated that the GroE chaperonin machine modulates the activity of the HrcA repressor (12). In *Clostridium acetobutylicum* DSM1731, however, the HrcA protein can be refolded by the chaperones DnaK, DnaJ, and GrpE (17). Furthermore, in *Bradyrhizobium japonicum* 110spc4, elevated levels of GroESL in an *hrcA* mutant had no influence on free-living growth and symbiotic nitrogen fixation (11). The many examples of cooperation between HrcA and these chaperones for proper function of HrcA in diverse bacteria (1) are consistent with the common and stable characteristics that HrcA is hardly soluble and easily forms aggregates when expressed and purified from *Escherichia coli*. This unfavorable characteristic was reported for *B. subtilis* HrcA (19), *B. stearothermophilus* HrcA (12), *Staphylococcus aureus* HrcA (16), and *C. acetobutylicum* HrcA (17). Although in vitro experiments performed with *B. stearother-*

mophilus HrcA revealed the participation of GroEL in modulating HrcA function, the purification process and band shift assays still required the presence of 8 and 0.5 M urea, respectively (12). The insolubility of HrcA has thus far impeded in vitro analyses aimed at elucidating the nature of HrcA. On the other hand, many DNA-binding proteins tend to form inclusion bodies upon overproduction, which is a serious obstacle to purification of the proteins after gene cloning (4, 6).

Based on our interest in protein thermostability, we characterized the *dnaK* operon genes from the obligate thermophile *Bacillus thermoglucosidasius* KP1006 and showed that there are significant differences in the proline content of the *dnaK* operon proteins (DnaK, DnaJ, and GrpE) which are closely correlated with the thermostability of enzyme proteins (25, 26). *B. thermoglucosidasius* KP1006 is an obligate thermophile that can grow at temperatures between 42 and 69°C, with optimum growth between 61 and 63°C (21). *B. thermoglucosidasius* strains show considerable phenotypic resemblance to *B. stearothermophilus*. The stress-inducible proteins from *B. thermoglucosidasius* and *B. stearothermophilus* are proposed to share a high degree of identity (26). We are now shifting our focus to *B. thermoglucosidasius* HrcA with the aim of investigating its thermostability and function. To study the native features of *B. thermoglucosidasius* HrcA, it is necessary to solubilize the purified protein in the absence of the denaturing agent urea. In many trials, we devised a convenient method to renature *B. thermoglucosidasius* HrcA in a soluble form, excluding urea even in the absence of the chaperonin GroEL. Here we show that the presence of DNA is crucial to the solubilization and thermostability of HrcA. Later, we found that the method we devised is essentially the same as that previously reported by Egan and Schleif (5). On the basis of results of our in vitro studies, we discuss the possibility that HrcA binding to the CIRCE element may act as a thermosensor for regulation of the two chaperone operons in *B. thermoglucosidasius* KP1006.

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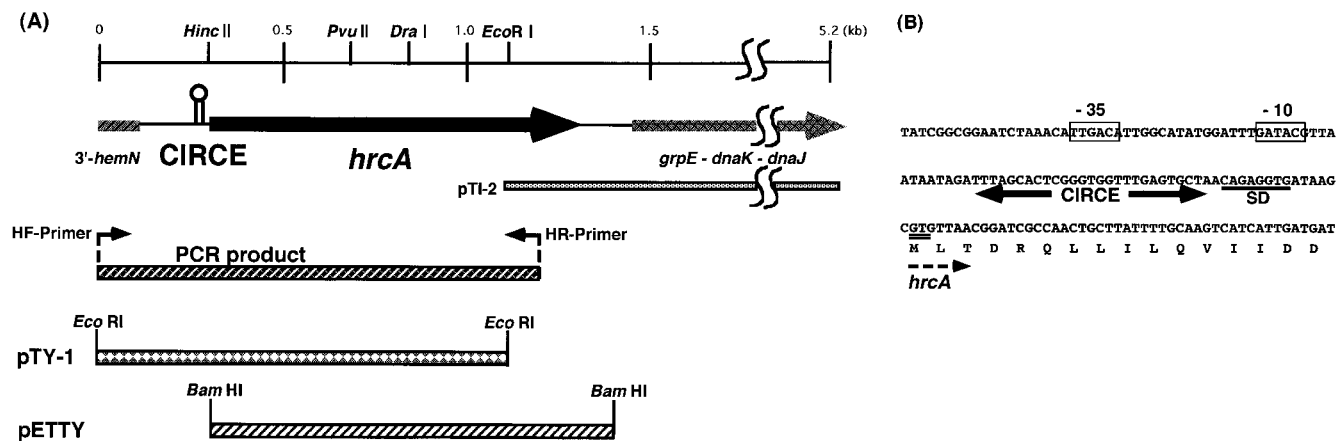


FIG. 1. (A) Schematic representation and physical map of the *hrcA-dnaK* operon. The CIRCE element and the *hrcA* gene are indicated with a hairpin and thick arrow, respectively. A 1.1-kbp PCR product obtained from primers HF and HR, the *EcoRI* fragment cloned in pTY-1, and the *BamHI* fragment in pETTY are shown as bars, as are pTI-2 (26) containing the C terminus of the *hrcA* gene with the *grpE*, *dnaK*, and *dnaJ* genes. (B) DNA sequence of the N terminus of the *hrcA* gene and its 5'-flanking region. The vegetative promoter (-35 and -10 consensus sequence) is boxed. The CIRCE element is indicated with thick arrows. A Shine-Dalgarno sequence (SD) for ribosome binding is underlined.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains used in this study were *B. thermoglucosidarius* KP1006 (DSM2542) (21), *E. coli* MV1184 {*ara* Δ (*lac-proAB*) *rpsL thi* (ϕ 80 *lacZ* Δ M15) Δ (*srl-recA*)306::Tn10(Tet^r)F' [*traD*306 *proAB*⁺ *lacI*^q *lacZ* Δ M15]} (23), and *E. coli* BL21(DE3) [*F*⁻ *ompT hsdS*_B(*r*_B⁻ *m*_B⁻) *gal dem*(DE3)] (20). Plasmids pUC119 (23) and pSTV29 (Takara Shuzo, Kyoto, Japan) were used as cloning vectors, and pET15-b (Novagen, Madison, Wis.) was used as a His-tagging fusion vector. *B. thermoglucosidarius* KP1006 was grown as previously described (22). The *E. coli* cells were aerobically grown at 37°C in L broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose [pH 7.2]) supplemented with ampicillin (50 μ g/ml).

DNA manipulations. Extraction of genomic DNA from *B. thermoglucosidarius* KP1006 was carried out as described before (24). Two primers, HF (CGGAATCGAAGAAATGTTTTTGGG) and HR (AGGCTGCAGTTTTCCATT), were used to amplify the DNA fragment containing the *B. thermoglucosidarius hrcA* gene and its flanking region by PCR with *B. thermoglucosidarius* genomic DNA as the template. The latter corresponds to the nucleotide sequence of a part of *B. thermoglucosidarius HrcA* (amino acid residues 276 to 294) (26), and the former was designed from the nucleotide sequences for the 5'-flanking regions of *B. subtilis hrcA* (9) and *B. stearothermophilus hrcA* (13). PCR was performed with LA-Taq DNA polymerase (Takara Shuzo) for 30 cycles according to the manufacturer's directions. Denaturation, annealing, and extension temperatures were 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, respectively. Other DNA manipulations followed protocols described before (2).

Purification and renaturation of *B. thermoglucosidarius HrcA*. To purify *B. thermoglucosidarius HrcA* overexpressed in *E. coli*, the *hrcA* gene was tagged with a stretch of six His residues at its N terminus. A DNA fragment containing the gene was amplified by PCR using primer 1 (CGGGATCCCATGTTAACGGA TCGCCAAC, corresponding to the region for the initiation codon of *hrcA*) and primer 2 (CGGGATCCAAATAGTCAAGGA, corresponding to the sequence between *hrcA* and *grpE*), both with *BamHI* sites at their termini, with *B. thermoglucosidarius* genomic DNA as the template. The amplified DNA fragment was digested with *BamHI* and cloned into a *BamHI* site of the His-tagging vector pET15-b. The resulting plasmid was named as pETTY (Fig. 1A) and used for transformation of *E. coli* BL21(DE3). His-tagged *B. thermoglucosidarius HrcA* (His-HrcA) was produced in the transformant cells by induction with isopropyl- β -D-thiogalactopyranoside (0.5 mM) and purified essentially according to the protocol supplied by Clontech (Palo Alto, Calif.) under denaturing conditions in the presence of 6 M urea with metal affinity chromatography. All procedures were done at 4°C unless otherwise stated. After passing through the affinity resin, the eluate containing pure His-HrcA in the presence of 6 M urea was extensively dialyzed against 20 mM Tris-HCl-5 mM EDTA (pH 7.5) containing 3 M urea. The protein concentration was then adjusted to 200 μ g/ml with dialyzing buffer. To 1-ml aliquots, 50 μ g of plasmid DNA (pUC119) dissolved in dialyzing buffer (50 μ l) was added and then extensively dialyzed against 20 mM Tris-HCl-5 mM EDTA (pH 7.5). The dialysate was ultracentrifuged at

100,000 \times g for 15 min, and the supernatant was saved as a final sample. Plasmid DNA used for renaturation was purified by ultracentrifugation to equilibrium in cesium chloride-ethidium density gradients (18).

Band shift assay. The CIRCE-containing probe was a 330-bp DNA fragment carrying the region upstream of the start codon of *B. thermoglucosidarius hrcA* and synthesized by PCR with primers 1 and HrcA-5 (CGGGATCCAAAATAA GCAGTTGG, corresponding to amino acid residues 5 to 10 of *B. thermoglucosidarius HrcA*). The probe used as nonspecific DNA was a 340-bp fragment carrying *B. thermoglucosidarius dnaK* from amino acid residues 505 to the stop codon (26). The nonspecific DNA probe was generated by PCR with primers DnaK505 (CGGGATCCGGAAAGAGCGGCAGAACTC) and DnaK-Rev (CGGGATCCTTATTTGTTGTGCTCTTTAC). Each probe was internally labeled by PCR in the presence of 60 kBq of [α -³²P]dCTP. The mixture for binding assay reactions contained binding buffer (20 mM Tris-HCl-5 mM EDTA [pH 7.5]), 0.2 μ g of labeled probe, and 1.0 μ g of purified His-HrcA. Binding was performed as described before (12, 28). The samples were loaded onto a 7% polyacrylamide gel and run at 20 mA with 1 \times Tris-borate-EDTA buffer (18). After polyacrylamide gel electrophoresis (PAGE), gels were wrapped with Saran Wrap and exposed to X-ray film for 3 to 15 h at room temperature.

Light scattering photometry. Light scattering was carried out using a Shimadzu RF5300PC fluorescence spectrophotometer equipped with a temperature-controlled cell holder and a magnetic stirrer. The excitation and emission wavelengths were set at 450 nm; the excitation and emission slits were set at 5 nm. The data were sent to a computer every 20 s. The reaction was started by adding 25 μ g of purified His-HrcA (0.1 ml) to a solution (2.9 ml) containing the appropriate amount of DNA in 20 mM Tris-HCl-5 mM EDTA (pH 7.5). Purified *B. thermoglucosidarius DnaK* (26) and GroEL (unpublished result) proteins used for the assay were prepared from *E. coli* MV1184 harboring plasmids carrying the *B. thermoglucosidarius dnaK* and *groEL* genes, respectively.

Protein concentration assay. Protein concentration was determined by the Bradford method (3) to eliminate false-positive signals associated with urea.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB039833.

RESULTS

Cloning of *B. thermoglucosidarius hrcA*. Since the *dnaK*, *grpE*, and *dnaJ* genes were cloned from *B. thermoglucosidarius* KP1006 together with the C terminus of the *hrcA* gene in the same operon (26), we tried to clone the N terminus of the *hrcA* gene and its 5'-flanking region, using colony hybridization on the genomic library of various restriction enzyme-digested fragments. However, we were unable to obtain the fragment

encompassing the desired region of the *hrcA* gene. This had also been the case for *B. stearothermophilus hrcA* (13). Thus, we switched to a method based on cloning by PCR, essentially as used for *B. stearothermophilus hrcA*. By this PCR-based method, a 1.1-kbp DNA fragment was amplified as a single band. The fragment was cleaved with *EcoRI*, and the larger fragment was ligated to the vector plasmid pUC119 at the *EcoRI* site (pTY-1 [Fig. 1A]). The entire DNA sequence of the cloned fragment in pTY-1 was determined. As expected, the fragment contained the region coding for the N terminus of *B. thermoglucosidarius hrcA* and 300 bp of its 5'-flanking region. The coding region further upstream of *hrcA* showed high sequence similarity to the *hemN* gene of *B. subtilis* (9). This indicates that the gene organization is identical to that found in *B. subtilis* MB11 (10) and *B. stearothermophilus* NUB36 (7, 13). Between the stop codon of the *hemN* gene and the start codon of the *hrcA* gene, there were 83-bp-long intervening sequences. A set of vegetative promoters (TTGACA for the -35 region and TACGTT for the -10 region) and a genetic element consisting of a pair of 9-bp inverted repeats separated by 9-bp spacers were also found in the intervening region (Fig. 1B). The inverted repeats here are the CIRCE element of *B. thermoglucosidarius* KP1006, which precisely matches the CIRCE elements of *B. subtilis* MB11 (29) and *B. stearothermophilus* NUB36 (13).

The predicted amino acid sequence of the putative *B. thermoglucosidarius* HrcA protein implied that HrcA consists of 344 amino acids and exhibits 63.1, 92.4, and 29.4% identity with the HrcA proteins of *B. subtilis* MB11, *B. stearothermophilus* NUB36, and *B. japonicum* 110*spc4*, respectively. As it has been reported that *B. stearothermophilus hrcA* can complement *B. subtilis hrcA* deletion (13), it seems highly likely that *B. thermoglucosidarius hrcA* would also be able to complement a *B. subtilis hrcA* mutant. In gross features, the *B. thermoglucosidarius* KP1006 *dnaK* operon genes resemble that of *B. stearothermophilus* NUB36.

Purification and renaturation of *B. thermoglucosidarius* HrcA. Although intact *B. thermoglucosidarius* HrcA was over-expressed in *E. coli* cells, the HrcA protein was hardly detected in the soluble cell-free fraction by sodium dodecyl sulfate (SDS)-PAGE analyses. The band for the HrcA protein was seen at the position of the predicted molecular mass (39 kDa) only for the whole-cell fraction, not for the cell extract (data not shown). This indicates that *B. thermoglucosidarius* HrcA can be expressed but is almost entirely insoluble. This characteristic also applies for HrcA proteins from other bacteria. Thus, we modified the *B. thermoglucosidarius hrcA* gene to add a six-His tag at its N terminus, using pET15-b plasmid. His-HrcA was then purified to homogeneity by metal affinity chromatography under denaturing conditions containing 6 M urea (Fig. 2A). After purification, the effects of stepwise decreases in the urea concentration were determined. Results from these analyses indicated that His-HrcA could be hardly solubilized in less than 3 M urea.

To renature purified His-HrcA in the absence of urea, various additives, such as glycerol, detergents, and plasmid DNA, were added at different concentrations to the His-HrcA solution containing 3 M urea, followed by dialysis. Eventually, we found that DNA served to solubilize His-HrcA in the absence of urea. The addition of additives other than DNA caused the

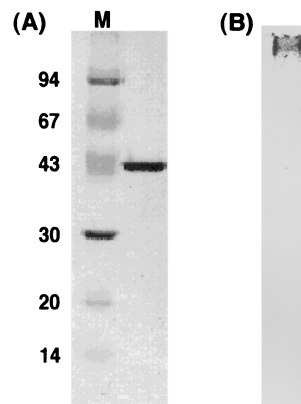


FIG. 2. (A) SDS-PAGE pattern of *B. thermoglucosidarius* HrcA purified by metal chromatography; (B) native-PAGE pattern of *B. thermoglucosidarius* HrcA renatured with nonspecific DNA (pUC119). The gel concentrations used were 10% for SDS-PAGE and 7.5% for native PAGE. Each lane contained 2 μ g of *B. thermoglucosidarius* HrcA protein. Molecular masses of the protein markers (M) in panel A are indicated in kilodaltons.

formation of visible aggregates rapidly after the start of dialysis. In contrast, the addition of more than 50 μ g of plasmid DNA (pUC119, 3.2 kbp) to 200 μ g of purified His-HrcA in 1 ml of 20 mM Tris-HCl-5 mM EDTA-3 M urea (pH 7.5) effectively renatured His-HrcA. We note that the DNA added should be dissolved in advance in the same buffer (20 mM Tris-HCl, 5 mM EDTA, 3 M urea [pH 7.5]) to prevent slight aggregation of HrcA. Furthermore, effective plasmid DNA is not restricted to pUC119; any other vector plasmid DNAs that are also nonspecific for HrcA are as effective for His-HrcA renaturation as pUC119. However, plasmid pTY-1, which contains the CIRCE element, was much more effective than nonspecific plasmid DNAs. The amount of plasmid DNA added could be decreased to less than 30 μ g. Moreover, we investigated the His-HrcA renaturation with sonicated plasmid DNA. When DNA fragments prepared by sonication of plasmid pUC119 (resulting lengths were 200 to 500 bp) were used, more than 200 μ g DNA was necessary for renaturation of the same amount of His-HrcA indicated above. The increase in the requirement for DNA may be due to excessive disruption of plasmid DNA by sonication. At any rate, these results suggest that the His-HrcA protein binds to nonspecific DNA that is favorable for renaturation and that binding of the protein to DNA containing the CIRCE element is much stronger than that with nonspecific DNA. The native-PAGE pattern (Fig. 2B) showed that HrcA renatured with DNA hardly migrated, presumably due to the apparent increase of molecular weight by binding to plasmid DNA, which has a rather high molecular mass (3.2 kbp = 2.1×10^6 Da). The native-PAGE pattern was independent of whether DNA used for HrcA solubilization contained the CIRCE element. In addition, it should be noted that the His-HrcA solution does not precipitate from solution for more than several weeks at 4°C.

Renatured HrcA protein binds to the CIRCE element without urea and GroEL. To provide evidence that HrcA proteins bind to the CIRCE element, excessive amounts of HrcA proteins from *B. stearothermophilus* NUB36 (12) and *B. subtilis* 168 (28) were used for the band shift assay for the DNA

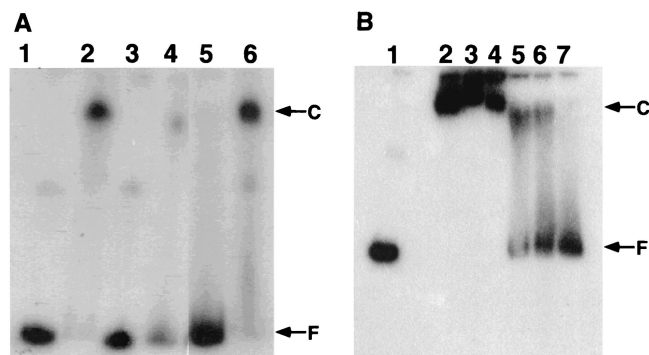


FIG. 3. (A) Band shift assays of the CIRCE probe with *B. thermoglucosidarius* HrcA renatured with nonspecific DNA (pUC119). Positions of the radiolabeled free DNA probe (F) and HrcA-probe complex (C) are marked. *B. thermoglucosidarius* HrcA samples contained no urea. Lane 1, labeled CIRCE probe (0.2 μ g) alone; lane 2, labeled CIRCE probe (0.2 μ g) with renatured HrcA (0.1 μ g); lane 3, labeled nonspecific probe (0.2 μ g) alone; lane 4, labeled nonspecific probe (0.2 μ g) with renatured HrcA (0.1 μ g); lane 5, same as lane 2 with nonlabeled CIRCE probe (1 μ g); lane 6, same as lane 2 with nonlabeled nonspecific probe (1 μ g). (B) Thermostability analyses of HrcA and probe complex by band shift assays. The binding reaction of *B. thermoglucosidarius* HrcA to the CIRCE probe was done at room temperature for 10 min; then each mixture was incubated at the indicated temperature for 1 min. Immediately after heat shock treatment, the samples were loaded into wells of a polyacrylamide gel. Lane 1, labeled CIRCE probe (0.2 μ g) alone; lanes 2 to 7, the labeled CIRCE probe (0.2 μ g) with renatured HrcA (0.1 μ g); lane 2, no heat shock treatment after the binding reaction (control); lanes 3 to 7, heat shock treatment at 40, 50, 60, 65, and 70°C, respectively. Positions of the radiolabeled free CIRCE probe (F) and HrcA-probe complex (C) are marked.

fragment containing the CIRCE element. However, these reaction mixtures still contained 0.5 M urea and/or GroEL to prevent further aggregation of HrcA. Therefore, we carried out band shift assays to examine whether *B. thermoglucosidarius* HrcA renatured with DNA can efficiently interact with the CIRCE element under in the absence of urea and GroEL.

His-HrcA protein was incubated at room temperature with a 330-bp DNA fragment containing the CIRCE element and end labeled with 32 P. As seen in Fig. 3A, His-HrcA protein (0.10 μ g, 2.4 pmol) retarded the entire probe DNA fragment (0.20 μ g, 0.92 pmol) containing the CIRCE element (lane 2). If it is assumed that the renatured protein is 100% active, the molar ratio of HrcA and DNA would be 2.6:1. A lower amount of His-HrcA protein failed to induce a complete band shift for the probe. This result indicates that two to three HrcA molecules bind to the fragment. As reported previously, HrcA protein renatured by GroEL and ATP retarded at most only approximately 0.3 equivalents of the probe fragment (12). Therefore, the His-HrcA protein used here was demonstrated to be renatured to a more functional state and thus to interact with the fragment containing the CIRCE element more efficiently. On the other hand, His-HrcA protein retarded the nonspecific probe DNA with poor efficiency (lane 4). In competition assays (lanes 5 and 6), the excess amount of nonlabeled probe DNA containing the CIRCE element significantly inhibited the interaction of His-HrcA with the labeled specific probe DNA, while nonlabeled nonspecific probe DNA slightly affected retardation of the probe DNA. These results indicated

that His-HrcA protein interacts far more strongly with DNA containing the CIRCE element than with any other DNA.

Next we examined the thermostability of His-HrcA bound to the probe DNA containing the CIRCE element. After formation of a complex of His-HrcA and the probe DNA at room temperature, the mixture was incubated at various temperatures (40, 50, 60, 65 and 70°C) for 1 min. Then the reaction mixtures were immediately applied to the gel (Fig. 3B). Although His-HrcA continued to bind to the specific probe DNA after heat treatment below 50°C, the binding complex of His-HrcA and the probe diminished in quantity after a temperature shift to above 60°C. At 70°C, the interaction of His-HrcA and the probe DNA was abrogated, and no band retardation was observed. Similar results were observed in the case of the upshift to 70°C even after formation of a complex of His-HrcA and the probe at 50°C.

Light scattering assays revealed that DNA prevents HrcA from aggregation without GroEL. To confirm the effect of DNA on renaturation of *B. thermoglucosidarius* HrcA, we investigated the light scattering change of His-HrcA solution containing DNA. Light scattering increases with time if the protein is aggregated. As seen in Fig. 4A, plasmids pUC119 and pTY-1 suppressed the increase of light scattering. Addition of 50 μ g of pUC119 (7.4 nM) or 15 μ g of pTY-1 (1.66 nM) to 25 μ g of His-HrcA protein (0.20 μ M) prevented HrcA aggregation. The stationary level of light scattering was dependent on the amount and species of DNA added. Amounts of DNA lower than those indicated above could suppress aggregation, but the stationary level of light scattering was elevated. However, the level of light scattering did not increase even if the reaction time was extended. These results indicate that HrcA has a stronger interaction with the CIRCE element than with nonspecific DNA and that the HrcA-DNA complex is stable under these conditions.

It has been reported that GroEL modulates HrcA activity (12). Therefore, *B. thermoglucosidarius* GroEL was used in place of DNA to examine the effect of GroEL on renaturation of HrcA. As shown in Fig. 4B, GroEL but not other proteins (bovine serum albumin and DnaK) prevented aggregation of HrcA. However, light scattering for HrcA in the presence of GroEL increased slightly and did not show a stationary level like that in the presence of DNA. This indicates that the complex of HrcA and GroEL is not so stable and that the potential function of GroEL to prevent aggregation of HrcA might be insufficient compared with DNA.

Next we tested the thermostability of the HrcA-DNA complex in a light scattering assay. His-HrcA renatured with plasmid pTY-1 containing the CIRCE element was incubated in a cuvette maintained at various temperatures (15, 40, 50, 60, 65, and 70°C) (Fig. 4C). No increase in light scattering was observed during extended incubation at 15°C, while only a slow increase was detected during incubation for several minutes below 50°C. However, the increase became faster with the elevation of temperature. At 70°C, HrcA protein was mostly aggregated within 750 s, while some portions of the protein were still soluble below this temperature. This result, which is consistent with that obtained by band shift assays (Fig. 3B), suggested that heat treatment above the optimum growth temperature range for *B. thermoglucosidarius* KP1006 may subject the cells to heat stress, and hence HrcA is easily denatured

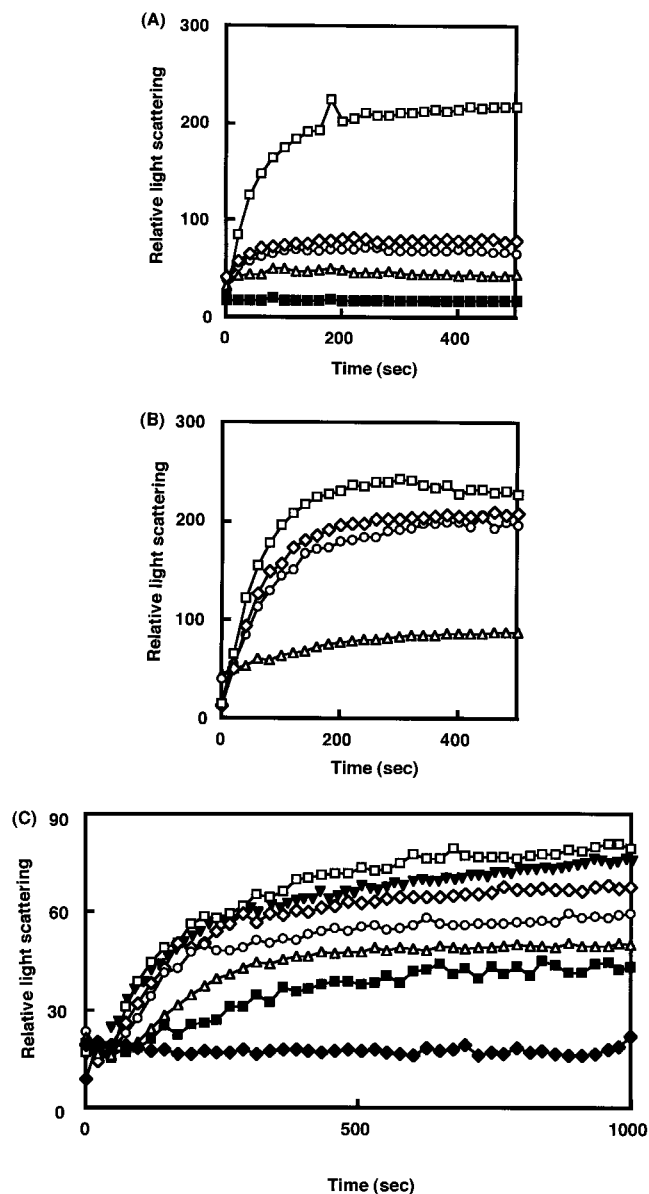


FIG. 4. Light scattering patterns of *B. thermoglucosidasius* HrcA in the presence of DNA (A) and other proteins (B) and effect of temperature on light scattering patterns (C). Incubation was at 15°C throughout the experiments in panels A and B. (A) 25 μ g of *B. thermoglucosidasius* HrcA (final concentration, 0.2 μ M) in 0.1 ml of 20 mM Tris-HCl-5 mM EDTA-3 M urea (pH 7.5) was added to the solution (2.9 ml) as follows: no DNA (\square), 50 μ g of plasmid DNA pUC119 (\diamond), 100 μ g of pUC119 (\circ), 20 μ g of plasmid DNA pTY-1 containing the CIRCE element (\triangle), and 50 μ g of pTY-1 (\blacksquare). (B) The solution contained HrcA (final concentration, 0.2 μ M) alone (\diamond) or with bovine serum albumin (0.2 μ M; \square); *B. thermoglucosidasius* DnaK (0.2 μ M; \circ) or *B. thermoglucosidasius* GroEL (0.2 μ M; \triangle). (C) The solution (3.0 ml) containing HrcA (25 μ g) and plasmid DNA pTY-1 (20 μ g) was preincubated with stirring at 15°C for 3 min; then the cuvette was transferred into the cell holder of a fluorescence spectrophotometer set at 15°C (\blacklozenge), 40°C (\blacksquare), 50°C (\triangle), 60°C (\circ), 65°C (\diamond), or 70°C (\square). In the experiment to investigate the effect of *B. thermoglucosidasius* GroEL (\blacktriangledown), the GroEL protein (final concentration, 0.2 μ M) was added to the solution mixture in the cuvette, which was then transferred into the cell holder set at 70°C. Data collection always started when the cuvette was transferred into the cell holder.

above that temperature. Furthermore, we examined whether GroEL prevents the aggregation of HrcA in the presence of DNA at 70°C (Fig. 4C). The presence of GroEL had no protective effect against the aggregation of HrcA.

DISCUSSION

This work presents a convenient method to renature and solubilize *B. thermoglucosidasius* KP1006 HrcA with DNA. Previous attempts to perform in vitro studies for bacillary HrcA proteins have been impeded by the insolubility of the protein (12, 19). The presence of the denaturing agent urea and the chaperonin GroEL, which are somehow effective for renaturation of *B. stearrowthermophilus* HrcA, prevents direct analysis of the native nature of HrcA repressors (12). Therefore, using *B. thermoglucosidasius* KP1006 HrcA, which has features similar to those of *B. stearrowthermophilus* HrcA, we devised a method to renature and solubilize the protein in the absence of urea and GroEL. During assays with various reagents, we found that DNA can efficiently renature and solubilize *B. thermoglucosidasius* HrcA in a functional state that is free from urea and GroEL. The method utilizes the inherent capacity of *B. thermoglucosidasius* HrcA to bind to DNA.

Early work on DNA-dependent renaturation of an insoluble DNA-binding protein was reported by Egan and Schleif (5), who used sheared salmon sperm or calf thymus DNA in large excess to AraC and RhaS proteins. In contrast, we found here that widely available and nonspecific plasmid DNA can promote the refolding of *B. thermoglucosidasius* HrcA in quantitative amounts. We also found that DNA sheared by sonication (to about 200 to 500 bp in length) was less effective for renaturation, probably due to undetectable damage to the DNA that we prepared. While any species of DNA can renature *B. thermoglucosidasius* HrcA, DNA containing the CIRCE element is far more effective for renaturation than nonspecific DNA. This finding was also confirmed by the band shift and light scattering assays. Many DNA-binding proteins in addition to HrcA tend to aggregate during purification processes (4, 6). The types of protocols represented by our method using plasmid DNA and the work reported by Egan and Schleif (5) should prove useful for eliminating or minimizing solubility problems during the process of solubilization and renaturation of insoluble DNA-binding proteins.

The band shift assays shown in Fig. 3A provide a clue to the molecular relationships between *B. thermoglucosidasius* HrcA and the CIRCE element. Judging from the molecular ratio obtained (2.6:1), two or three *B. thermoglucosidasius* HrcA molecules bound to the CIRCE element. Previous studies on bacillary HrcA proteins failed to determine the molecular relationships with the CIRCE element since a large excess amount of HrcA was necessary for band shift assays due to the extreme insolubility (11, 12, 28). *B. thermoglucosidasius* HrcA is more likely to have such an oligomeric structure since DNA-binding proteins tend to be oligomeric (4, 27). In contrast to our observation, Ohta et al. visualized the binding of presumably monomeric *S. aureus* HrcA to the CIRCE element by atomic force microscopy (16). The low identity (31.2%) between *B. thermoglucosidasius* HrcA and *S. aureus* HrcA may explain the difference in the subunit structures of the proteins. More stoichiometric analysis is required to elucidate the pre-

cise molecular relationship. In addition, the results of light scattering assays (Fig. 4B) demonstrated that DNA containing the CIRCE element can keep *B. thermoglucosidasius* HrcA in the folded state more effectively and stably than GroEL. This suggests that the fate of HrcA molecules present in a cell should be dependent on whether the protein molecules bind to the CIRCE element under nonstress conditions.

While preparing this report, we became aware of a report that *B. japonicum* HrcA remained in a soluble form upon overproduction and during purification processes (11). The identity between *B. japonicum* HrcA and *B. thermoglucosidasius* HrcA was as low as 29.4% despite their common function to bind to the CIRCE element. We do not know the reason for the comparatively high solubility of *B. japonicum* HrcA. However, the fact that an excess amount of *B. japonicum* HrcA versus DNA was necessary for a complete band shift indicates that the repressor protein may precipitate from solution over time. By contrast, *B. thermoglucosidasius* HrcA solubilized with DNA does not precipitate for several weeks at 4°C.

How can we explain the implication of HrcA binding to nonspecific DNA? Two plausible explanations may be proposed based on two different viewpoints. From the viewpoint of repression, HrcA may possess two modes of binding to DNA. The first mode would be preliminary binding to nonspecific DNA, which would serve to assist the second mode binding to the CIRCE element for negative control of heat shock genes. This seems reasonable since there are only two copies of the CIRCE element in the genomic DNA of *B. subtilis* (15), while nonspecific DNA is abundant in a cell. From the viewpoint of protein refolding, nonspecific DNA may act as a ligand to promote refolding of HrcA. In this case, the DNA would prevent correctly folded HrcA from unfolding and aggregating just like chaperonin GroEL (12).

The thermostability of the *B. thermoglucosidasius* HrcA-DNA complex containing the CIRCE element was examined in band shift and light scattering assays. Its thermostability should be crucial in investigating whether HrcA plays a role as a thermosensor for chaperone expression. It is natural that *B. thermoglucosidasius* HrcA exists on genomic DNA in cells to repress gene expression by binding to the CIRCE element under nonstress conditions. Therefore, it seems reasonable to assume that *B. thermoglucosidasius* HrcA renatured with DNA containing the CIRCE element *in vitro* would be comparable to the native form of the repressor *in vivo*. As seen in Fig. 3B and 4C, *B. thermoglucosidasius* HrcA is stable below 50°C during a few minutes of incubation, but much of the protein is denatured by treatment above 65°C. The susceptibility of *B. thermoglucosidasius* HrcA at higher temperatures suggests that the protein may be detached from the CIRCE element after denaturation caused by heat stress. The susceptibility is not affected by the presence of GroEL (Fig. 4C), as seen in yeast α -glucosidase (8). However, the denaturation of HrcA in our experiments was mostly irreversible since thermally denatured HrcA failed to re-form the complex with probe DNA upon incubation at the lower temperature (data not shown). This irreversibility of denaturation suggests that HrcA inactivated by heat stress is less likely to be regenerated by chaperonin GroEL (Fig. 4C), and *de novo* HrcA synthesis should be required when cells have recovered. This implies that *B. thermoglucosidasius* HrcA may act as a thermosensor, and the thermal

inactivation of HrcA protein may affect heat stress gene expression as an alternative. Most probably, *de novo* HrcA synthesis and effective refolding of newly synthesized HrcA by GroEL are key for initiation of the negative control of heat shock genes (28).

We suggest that HrcA may be a thermosensor to affect the expression of heat shock genes, while the overall flow of negative gene regulation is dependent on how HrcA can be effectively refolded by GroEL. We plan to analyze more native features of *B. thermoglucosidasius* HrcA and determine the basis of its physical interaction with GroEL.

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