Degradation of mutant initiator protein DnaA204 by proteases ClpP, ClpQ and Lon is prevented when DNA is SeqA-free

Monika SLOMINSKA*†, Anne WAHL*, Grzegorz WEGRZYN† and Kirsten SKARSTAD*[1]

*Department of Cell Biology, Institute for Cancer Research, Montebello, 0310 Oslo, Norway, and †Department of Molecular Biology, University of Gdansk, Kladki 24, 80-822 Gdansk, Poland

A mutant form of the *Escherichia coli* replication initiator protein, DnaA204, is unstable. At low growth rates, the *dnaA204* mutant cells experience a limitation of initiator protein and grow with reduced initiation frequency and DNA concentration. The mutant DnaA protein is stabilized by the lack of SeqA protein. This stabilization was also observed in a *dam* mutant where the chromosome remains unmethylated. Since unmethylated DNA is not bound by SeqA, this indicates that DnaA204 is not stabilized by the lack of SeqA protein by itself, but rather by lack of SeqA complexed with DNA. Thus the destabilization of DnaA204 may be due either to interaction with SeqA–DNA complexes or changes in nucleoid organization and superhelicity caused by SeqA. The DnaA204 protein was processed through several chaperone/protease pathways. The protein was stabilized by the presence of the chaperones ClpA and ClpX and degraded by their cognate protease ClpP. The *dnaA204* mutant was not viable in the absence of ClpY, indicating that this chaperone is essential for DnaA204 stability or function. Its cognate protease ClpQ, as well as Lon protease, degraded DnaA204 to the same degree as ClpP. The chaperones GroES, GroEL and DnaK contributed to stabilization of DnaA204 protein.

Key words: chaperone, *Escherichia coli*, initiation of replication, methylation, protease, sequestration.

INTRODUCTION

In wild-type *Escherichia coli* cells, initiation of DNA replication is tightly coupled with cell growth. In slowly growing *dnaA204* (Ts) mutant cells, the initiator protein (DnaA) concentration is 2- to 3-fold lower and the mass at initiation 2- to 3-fold higher than in the wild-type, whereas a rapidly growing mutant has wild-type levels of DnaA and essentially normal initiation mass [1–3]. This effect is due to instability of the mutant protein which is degraded with a half-life of approx. 1 h. The degradation leads to a deficiency of DnaA204 protein in slowly growing cells, which in turn leads to an increased initiation mass [3]. The degradation is in part prevented by the absence of the SeqA protein [3]. The SeqA protein binds to Dam-methylated DNA, especially hemi-methylated, and also to fully methylated DNA [4–8]. It interacts with newly replicated DNA in such a way that foci can be observed by immunofluorescence microscopy [9]. SeqA prevents reinitiation of newly initiated origins as well as premature primary initiation [1,10–12]. This protein has the capacity to change the topology of DNA [13,14] and has been found to act as a transcriptional activator [8]. It has been suggested that the DnaA204 protein may be stabilized by interaction with DNA and that the absence of SeqA facilitates this interaction [3].

In the present study, we investigate the basis of the stabilization of DnaA204 by lack of SeqA, and also attempt to determine which chaperones and proteases influence degradation of the DnaA204 protein.

EXPERIMENTAL

Bacterial strains and plasmids

E. coli K-12 strains and plasmids used in the present study are listed in Tables 1 and 2 respectively.

Growth conditions

Bacteria were cultivated in Luria–Bertani (LB) medium at 30 °C. For cultivation of the KA460 and KA459 strains, the medium was supplemented with 50 μg of thymine/ml of medium. Bacterial mass was monitored by measuring the attenuance D at 600 nm.

Measurement of protein degradation

Chloramphenicol (200–800 μg/ml; Sigma) was added to exponentially growing cells (D_{600} 0.15 or 0.3) to inhibit protein synthesis. Samples were taken at 0, 0.5, 1, 2, 3.5, 5 and 24 h for determination of DnaA concentrations as described below.

Preparation of cell extract

Bacteria were harvested by centrifugation at 4 °C. The cells were resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 8.0), containing 1 % (w/v) SDS and 8 % (v/v) glycerol and boiled for 5 min. Then, total protein concentration was determined by a colorimetric assay (bicinchoninic acid kit from Pierce). Using the protein concentration information obtained, the extract was further diluted in the same buffer to a concentration appropriate for the subsequent Western-blot analysis. Finally, a volume of sample buffer (containing 4 % SDS, 2.5 M 2-mercaptoethanol and Bromophenol Blue) corresponding to 15 % of the total volume was added, and the samples boiled for 2 min.

Determination of DnaA protein concentration

A fixed amount of cell extract (determined as described above) and DnaA standards (purified DnaA protein) were subjected to SDS/PAGE [12 % (w/v) gel]. The proteins were transferred on to a PVDF membrane by semi-dry blotting. The membrane was probed with anti-DnaA rabbit antibodies using standard procedures [15]. Detection was with ECF (enhanced chemifluores-

Abbreviations used: LB, Luria–Bertani.

[1] To whom correspondence should be addressed (e-mail kirsten.skarstad@labmed.uio.no).

Table 1 ***E. coli*** **K-12 strains**

Strain	Relevant genotype	Reference/Construction
CM735	*metE46*, *trp-3*,*his-4*,*thi-1*, *galK2*, *lacY1* or *lacZ4*,*mtl-1*, *ara-9*, *tsx-3*, *ton-1*, *rps-8*, or *rps-9*, s*upE44*, λ^-	[30]
CM746	As CM735 but *dnaA204*	[30]
EBO185	*seqA10*, *dnaA204*	[3]
EBO208	*dam13*::cm, *seqA10*, *dnaA204*	This work
GM2927	*dam13*::cm	[11]
BM747	As CM746 but *dam13*::cm	This work, P1 GM2927
SG22007	*clpP1*::cat	[31]
SG12064	*clpQ*::cat	[26]
KA459	*thyA*, *thr*, *trpE9829*(Am), *ilv*,*tyrA*(Am) *metE*, *deo*, *supF6*(Ts), *hslU1*::minitet (*clpY*)	[27]
KA460	*thyA*, *thr*, *trpE9829*(Am), *ilv*,*tyrA*(Am) *metE*, *deo*, *supF6*(Ts), *lon*::Tn10	[27]
SG22080	*clpX1*::kan	[32]
SG22099	clpA*319*::Δkan	[33]
MC4100	*clpB*::kan	[34]
BM234	*dnaK756 zaa*::Tn10	[35]
BM235	*dnaJ259 thr*::Tn10	[35]
BM233	*grpE280 zfh*::Tn10	[35]
BM275	*groEL44*	[36]
BM276	*groES619*	[36]
BM748	As CM746 but *clpP1*::cat	This work, P1 SG22007
BM749	As CM746 but *clpQ*::cat	This work, P1 SG12064
BM750	As CM746 but *lon*::Tn10	This work, P1 KA460
BM751	As CM746 but *clpX1*::kan	This work, P1 SG22080
BM752	As CM746 but *clpA319*::Δkan	This work, P1 SG22099
BM753	As CM746 but *clpB*	This work, P1 MC4110
BM754	As CM746 but *dnaK756 zaa*::Tn10	This work, P1 BM234
BM755	As CM746 but *dnaJ259 thr*::Tn10	This work, P1 BM235
BM756	As CM746 but *grpE280 zfh*::Tn10	This work, P1 BM233
BM757	As CM746 but *groEL44*	This work, P1 BM275
BM758	As CM746 but *groES619*	This work, P1 BM276
BM759	As CM746 containing pACYC177	This work, by transformation
BM760	As CM746 containing pMOR6	This work, by transformation
BM761	As CM746 containing pFH2102	This work, by transformation
BM762	As CM746 containing pMAK7	This work, by transformation

Table 2 **Plasmids**

Plasmids	Properties	Reference
pACYC177	*ori*p15A, amp^R, kan^R	[37]
pMOR6	*ori*p15A, *datA*, amp^R	[24]
pFH2102	*ori*pBR322, amp^R	[38]
pMAK7	*ori*pBR322, *plac*(A1/04/03)-*seqA*, amp^R	[38]

cence; Amersham Biosciences). The membranes were scanned on a STORM840 (Molecular Dynamics) and quantified using ImageQuant software (Molecular Dynamics). DnaA standard curves were made and the DnaA concentrations in the cell extracts were determined by comparison with the standard curves.

Calculation of the half-life of DnaA204

Half-lives of the DnaA protein were estimated by plotting the protein determinations logarithmically as a function of time after chloramphenicol addition and determining the slope of the curve.

RESULTS AND DISCUSSION

DnaA204 protein is stabilized in *seqA* and *dam* deletion strains

Cells were grown in rich medium and the half-life of the DnaA204 protein was determined by immunoblotting of samples withdrawn at time intervals after inhibition of further protein synthesis with chloramphenicol (see the Experimental section and Figure 1). In accordance with previous results [3], the DnaA204 protein was stabilized in the absence of SeqA. In the present study, where cultures were grown in LB medium, the half-life increased from 1.4 to 15 h (Figure 1, Table 3), whereas in the work of Torheim et al. [3] where cultures were grown in minimal medium with glucose and casamino acids (commercial casein hydrolysate), the half-life of DnaA204 increased from 1 to 3 h.

SeqA protein in the cell can be visualized by immunofluorescence microscopy as foci representing supercomplexes of SeqA with newly replicated, hemimethylated DNA [9,16], and may play a role in restructuring the nucleoid after replication [13,14,16]. SeqA is also capable of binding to areas of fully methylated DNA, namely *oriC* and certain other areas [5–8,12], but not with unmethylated DNA. In strains lacking the Dam methylase, the SeqA protein is not found associated with DNA, and sequestration, as well as the DNA organization by SeqA, do not seem to function [17,18].

It was suggested that the stabilization of DnaA204 protein in the absence of SeqA might be due to an increased opportunity for DnaA204 to bind to DNA [3]. If so, the DnaA204 protein should also be stabilized in the absence of Dam methylase since SeqA protein cannot bind unmethylated DNA. The *dam13* allele was transduced into the *dnaA204* strain and the stability of DnaA204 protein measured (Figure 1, Table 3). The half-life of DnaA204 was increased approx. 10-fold in the absence of Dam methylation. This indicates that the DnaA204 protein is

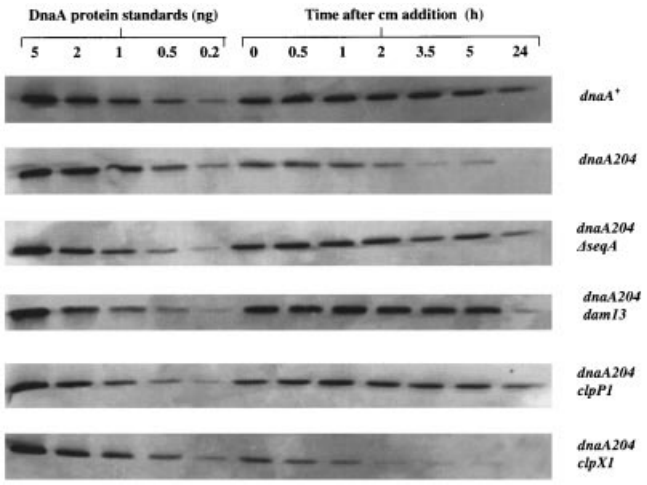

Figure 1 Stability of the DnaA protein in the strains

CM735 (*dnaA*$^+$), CM746 (*dnaA204*), EBO185 (*dnaA204*, *seqA10*), BM747 (*dnaA204*, *dam13*), BM748 (*dnaA204*, *clpP1*), BM751 (*dnaA204*, *clpX1*). Bacteria were grown exponentially at 30 °C in LB medium and treated with chloramphenicol at zero time. Samples were withdrawn between 0 and 24 h and separated by SDS/PAGE (12% gel) and analysed by Western blotting, using antiserum against DnaA protein. Purified DnaA protein was used as a standard.

Table 3 DnaA204 protein is stabilized in *dam* and *seqA* mutant strains

Strain	Doubling time (min)	Half-life of DnaA204 (h)
*dnaA*204	50	1.4
*dnaA*204*seqA*10	71	15
*dnaA*204*dam*13	250	11
*dnaA*204*seqA10dam*	85	17

not stabilized by the lack of SeqA protein by itself, but by the absence of SeqA from the DNA. This suggests that the destabilization of DnaA204 by SeqA reported previously [3], is not a direct effect of the SeqA protein acting, for instance, as a co-protease or chaperone. The fact that a similar stability of DnaA204 was found in the double mutant, *seqAdam*, shows that there was no additive effect arising due to the absence of both enzymes (Table 3).

Both SeqA and DnaA proteins seem to be capable of forming higher-order nucleoprotein complexes with DNA. At *oriC*, DnaA forms an initiation structure which includes approx. 200 bp of DNA [19] harbouring five primary DnaA-binding sites (dnaA boxes) and an AT-rich area of single-strand interaction [20]. Part of this sequence also harbours binding sites required for the formation of a higher-order SeqA structure [5]. The SeqA–DNA structure seems to be important for proper organization of newly replicated DNA not only at *oriC*, but throughout the chromosome. It is possible that the SeqA–DNA structure may displace some of the bound DnaA when the DNA becomes hemimethylated [21]. DnaA protein binds to numerous dnaA boxes around the chromosome [22,23], but whether it is capable of forming higher-order structures at sites other than *oriC* is not known. It is likely that not only DnaA204 situated at the origin is stabilized by the absence of SeqA, but also the DnaA204 bound elsewhere on the chromosome. The results reported in the present study suggest that the mutant DnaA204 protein becomes susceptible to degradation on interaction with either SeqA–DNA complexes or by a more indirect mechanism as a result of changes in DNA topology organized by SeqA.

Table 4 Stability of DnaA204 in the presence of extra DnaA-binding sites or in the presence of excess SeqA protein

Strain	Gene cloned on plasmid	Doubling time (min)	Half-life of DnaA204 (h)
*dnaA*204/pACYC177	–	48	1.3
*dnaA*204/pMOR6	*datA*	63	1.2
*dnaA*204/pFH2102	–	45	1.3
*dnaA*204/pMAK7	*seqA*	45	0.8

The stability of DnaA204 was not affected by providing extra *datA* sites

DnaA protein binds to 9-mer binding sites (dnaA boxes) [23], whereas SeqA binds to two or more hemimethylated GATC sequences if situated with a correct spacing in the DNA helix [4]. Even though the two proteins do not have common binding sites, it is possible that SeqA organizes the DNA in such a way that binding of DnaA204 is destabilized. If so, it is reasonable to suppose that the destabilization of DnaA204 might be less effective if extra DNA harbouring dnaA boxes, but lacking GATC sequences, were provided. The *dnaA204* strain was therefore transformed with a *datA*-containing plasmid pMOR6. The *datA* sequence contains several dnaA boxes binding DnaA especially strongly [22], but contains few GATC sequences. We found that the concentration of DnaA204 protein in this strain was approx. 2-fold higher than in the control, carrying only the vector pACYC177 (results not shown). This is consistent with the expectation that more DnaA must be produced to bind the DnaA boxes in the extra *datA* copies [22,24], and could be achieved because the *dnaA* promoter is autoregulated. There was however no significant improvement of the stability of the DnaA204 protein under these conditions (Table 4). This result indicates that all DnaA204 protein in the cell becomes susceptible to degradation at least once during the cell cycle, irrespective of whether it is bound to *oriC*, *datA* or other sites. This susceptibility to degradation might, however, still occur on interaction with SeqA–DNA complexes.

The results of the present study also indicate that wild-type DnaA protein interacts with SeqA–DNA complexes at a certain point in the cell cycle. The wild-type protein does not seem to be susceptible to degradation, but the enhancement of degradation of DnaA204 by SeqA may indicate that a conformational change of the DnaA protein is induced by interaction with SeqA–DNA complexes, and may suggest a direct interaction between DnaA and SeqA, not only at *oriC*, but also at other binding sites around the chromosome.

Overproduction of SeqA protein leads to further destabilization of DnaA204

To investigate whether an increase in the cellular content of SeqA would lead to further destabilization of the Dna204 protein, the *dnaA204* strain was transformed with a *seqA* containing plasmid pMAK7, from which SeqA could be expressed after induction with isopropyl β-D-thiogalactoside. Cells were grown with 0.1 mM isopropyl β-D-thiogalactoside for four generations before chloramphenicol addition and measurement of DnaA204

Table 5 The DnaA204 protein is stabilized in protease mutants and destabilized in chaperone mutants

Strain	Doubling time (min)	Half-life of DnaA204 (h)
dnaA204	50	1.4
dnaA204 clpP	270	19
dnaA204 clpQ	140	15
dnaA204 lon	62	14
dnaA204 clpA	105	0.7
dnaA204 clpX	93	0.6
dnaA204 clpY	Not viable	–
dnaA204 dnaK756	67	0.7
dnaA204 dnaJ259	64	1.4
dnaA204 grpE280	56	2.2
dnaA204 groEL44	56	0.8
dnaA204 groES619	61	0.5
dnaA204 clpB	55	1.2

Table 6 Mutations that cause stabilization of DnaA204 protein suppress the temperature sensitivity of the *dnaA204* mutant

+ and − indicate growth and no growth on LB plates after overnight incubation respectively.

Relevant genotype	30 °C	42 °C	43 °C
dnaA204	+	−	−
dnaA204 seqA10	+	+	−
dnaA204 dam13	+	+	−
dnaA204 clpP1	+	+	−
dnaA204 clpQ	+	+	−
dnaA204 lon	+	+	−

protein stability. The overproduction of SeqA was approx. 3-fold relative to the control strain (results not shown) and the protein half-life was reduced to 0.8 h (Table 4). The result shows that the degradation of the DnaA204 protein may be increased with increasing SeqA concentrations.

DnaA204 protein is stabilized in cells lacking proteases ClpP, ClpQ or Lon and destabilized in cells lacking chaperones

To determine which of the known cellular proteases [25,26] are responsible for the degradation of DnaA204, double mutants were constructed by transduction of mutant alleles into the *dnaA204* strain (see the Experimental section). Considerable stabilization of DnaA204 with approx. 10-fold increase in half-life was found in strains with the mutant genes *clpP*, *clpQ* and *lon* (Table 5), indicating that all three systems for quality control and degradation of proteins are capable of recognizing mutant DnaA. In strains with mutations in the ATPase subunits ClpA or ClpX, which recognize, denature and present proteins to the ClpP protease subunit [25], the half-life of DnaA204 protein was reduced by approx. 50 % (Table 5). This suggests that the chaperone-like denaturation ability of these proteins also promotes correct refolding of DnaA204 and release from the ClpAP or ClpXP complex without degradation. Thus the ClpAP or ClpXP interaction with the mutant protein may lead to either unfolding and degradation, or refolding and salvage of the protein. Alternatively, the ClpA-ATP and ClpX-ATP subunits might be acting independently of ClpP.

The same dual chaperone/protease function may hold for ClpYQ. Absence of ClpQ led to considerable stabilization of DnaA204 (Table 5). A situation in the absence of ClpY could not be tested, however, because the double-mutant *dnaA204 clpY* strain could not be constructed. Despite several trials, we were not able to obtain viable *dnaA204 clpY* transductants, whereas analogous double mutants bearing the *dnaA204* allele and a mutation in other *clp* genes could be easily constructed by the same procedure (Table 1). This indicates that ClpY might be especially important for DnaA204 stability or function. A putative interaction between ClpY and the DnaA46 mutant protein has been observed previously, but in this case, the *clpY* null mutant suppressed the temperature sensitivity of *dnaA46* [27].

Other cellular chaperones which assist in *de novo* folding of polypeptides and refolding after heat shock [28] were found to affect the degradation of DnaA204. In strains with the mutant alleles *dnaK756*, *groEL44* or *groES619*, the stability of DnaA204 was reduced by approx. 50 %, whereas in strains with mutant allelles *dnaJ259*, *grpE280* or *clpB* the stability was not affected (Table 5).

Attempts to purify the DnaA204 protein were not successful because degradation of this protein on cell lysis was very rapid. Experiments with purified wild-type DnaA protein showed that this protein was not degraded by the ClpXP protease [29], irrespective of whether the protein was in the active ATP-, or inactive ADP-bound form or whether supercoiled *oriC* plasmid and SeqA protein were present (results not shown).

Mutations which cause stabilization of DnaA204 protein suppress the temperature sensitivity of the dnaA204 mutant

The mutant strains in which the DnaA204 protein was found to be stabilized (Table 5) were grown at 30, 42, and 43 °C. Mutations in genes *clpP*, *clpQ*, *lon*, *seqA* and *dam* suppressed the temperature sensitivity of the *dnaA204* mutant at 42 °C, but not at 43 °C (Table 6). The suppression of *dnaA204* temperature sensitivity by the *seqA*10 mutation was in accordance with the previous findings [10]. The results indicate that the degradation of DnaA204 protein contributes to the temperature sensitivity of the *dnaA204* strain and that suppression of the degradation therefore also suppresses the temperature sensitivity.

We are grateful to Susan Gottesman, Tsutomu Katayama, Hiroshi Nakai, Igor Konieczny and Krzystof Lieberek for providing strains and purified protein, to Alicja Węgrzyn and members of the Skarstad laboratory for discussions, and to Erik Boye for reading the manuscript. This work was supported by an EMBO short term fellowship no. 9563 (to M.S.), by the Norwegian Research Council (K.S.), the Norwegian Cancer Society (K.S.), Anders Jahre's Foundation for the Promotion of Science (K.S.), the Polish State Committee for Scientific Research (project no. 3 P04A 029 22) and the Foundation for Polish Science (subsidy 14/2000 to G.W.).

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Received 24 July 2002/5 December 2002; accepted 13 December 2002
Published as BJ Immediate Publication 13 December 2002, DOI 10.1042/BJ20021161