Bacillus subtilis DnaG primase stabilises the bacteriophage SPP1 G*40*P helicase-ssDNA complex

Silvia Ayora[a], Uwe Langer[b], Juan C. Alonso[a,*]

[a] *Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*

[b] *Max-Planck-Institut für molekulare Genetik, Ihnestrasse 73, D-14195 Berlin, Germany*

Received 7 September 1998; received in revised form 7 October 1998

Abstract Purified *Bacillus subtilis* DnaG primase (predicted molecular mass 68.8 kDa) behaves as a monomer in solution. We demonstrate that DnaG physically interacts with bacteriophage SPP1 hexameric helicase G*40*P (G*40*P$_6$) in the absence of ATP. G*40*P$_6$-ATP forms an unstable complex with ssDNA, and by itself carries out ATP-driven translocation along a ssDNA template with low processivity. The presence of DnaG in the reaction mixture increased the helicase activity of G*40*P$_6$ about 3-fold, but not the ATPase activity. The results presented here suggest that the DnaG protein stabilises the G*40*P$_6$-ssDNA complexes.

Key words: Phage biology; SPP1; DNA replication; Replication initiation protein; DNA helicase; DNA primase

1. Introduction

The enzymes at the replication fork of *Escherichia coli* cooperate with one another to coordinate synthesis of both leading and lagging strands [1]. Much of the required coordination could come as a result of protein-protein interaction. The protein-protein complex at each fork consists of the dimeric and asymmetric DNA polymerase III (Pol III) and the DnaB helicase [2,3]. At intervals the DnaG primase binds to the DnaB helicase and synthesises an RNA primer on the lagging strand [4,5]. The replication process is timed by the association and disassociation of DnaG with DnaB, the interaction between Pol III and DnaG, which regulates primer size and the loading of a new β subunit Pol III at the new primer, without releasing from the fork [5,6]. Surprisingly, very little is known about protein-protein interactions within the replisome of the Gram-positive bacteria which are phylogenetically distant from *E. coli*.

SPP1 is a virulent *Bacillus subtilis* linear dsDNA bacteriophage whose replication is independent of the host-encoded replication fork helicase [7,8]. Initiation of SPP1 DNA replication strictly requires phage-encoded gene *38*, *39*, and *40* products (G*38*P, G*39*P and G*40*P) as well as the host DNA primase (DnaG) and DNA polymerase III [7–9]. Although the precise mechanism by which SPP1 and host components interact to promote initiation of theta replication is unknown, it is clear that G*38*P, which interacts with G*39*P, binds specifically to one or both cognate sites (*ori*L and *ori*R) [8,9]. The role of G*39*P, which does not bind DNA, remains to be determined. The G*40*P helicase (predicted molecular mass 49.8 kDa) is a hexamer that exists in different quaternary intermediate states with the two extreme forms sharing a C_3 and a C_6 symmetry [10]. Hexameric G*40*P (G*40*P$_6$) binds DNA in a sequence-independent manner. G*40*P$_6$ binds dsDNA in the absence of any cofactor while it binds with higher affinity ssDNA in the presence of ATP or ATPγS [11]. G*40*P$_6$ shows an ATPase activity that is enhanced in the presence of ssDNA and, to a minor extent, in presence of dsDNA or RNA. G*40*P$_6$ is able to hydrolyse the four rNTPs with similar efficiency, and with a 6–12-fold lower efficiency the four dNTPs. ATP hydrolysis occurs in the presence of Mg^{2+}, to a lesser extent in the presence of Mn^{2+} and Ca^{2+}, but not in the presence of Ni^{2+} or Zn^{2+} [11]. G*40*P$_6$ is a DNA helicase capable of unwinding DNA in a 5′ to 3′ polarity with low processivity [11]. Weise et al. [11] have documented that the addition of *Eco*SSB protein to the G*40*P$_6$ helicase reaction did not enhance the amount of substrate unwound. Furthermore, *Eco*SSB seems to exert a negative effect on G*40*P$_6$ helicase activity.

We have purified the *B. subtilis* DnaG primase. Unless otherwise stated, in the following the products are of *B. subtilis* origin. In this report we describe the physical interaction between DnaG and G*40*P$_6$, and show that the presence of DnaG increased the helicase activity of G*40*P$_6$ about 3-fold, but did not modify the ATPase activity of the G*40*P$_6$ helicase. The results presented here suggest that the DnaG protein stabilises the G*40*P$_6$-ssDNA complexes.

2. Materials and methods

2.1. Bacterial strains, plasmids and bacteriophages

The *E. coli* strains JM103 [12] and BL21DE3 [13] were used. The pT712-borne *dna*G gene, pBT217, was described previously [14]. The bacteriophages M13mp18 [15] and its derivative lacking the 33-bp *Eco*RI-*Hin*dII DNA segment (M13mp18-Δ*Eco*RI-*Hin*dII) [11] were used.

2.2. DNA manipulations

Bacteriophage M13mp18 and M13mp18-Δ*Eco*RI-*Hin*dII replicative forms and viral DNA were prepared as described [12]. The amount of DNA is expressed as mol of nucleotides.

Oligonucleotides which were used to test helicase activity have been previously reported [11].

The helicase substrate to test processivity was constructed as described by Weise et al. [11].

2.3. Enzymes and reagents

The protease inhibitor PMSF was from Boehringer Mannheim and IPTG (isopropylthiogalactoside) was from Calbiochem. Sephadex G-100, heparin-Sepharose, protein A Sepharose and Superose 12 were from Pharmacia, phosphocellulose was from Whatman and Affi-Gel-10 was from Bio-Rad.

ATP, and ATPγS were purchased from Boehringer Mannheim. The nucleotides were dissolved as concentrated stock solutions at pH 7.0 and their concentration was determined spectrophotometrically.

*Corresponding author. Fax: (34) (91) 585 4506.
E-mail: jcalonso@cnb.uam.es

PII: S0014-5793(98)01337-4

2.4. Protein purification

G*40*P from bacteriophage SPP1 was purified as previously described [11] and is expressed as mol of protein hexamers. *B. subtilis* DnaG was overexpressed from plasmid pBT217 in *E. coli* as described for G*40*P (see [11]). The cells were resuspended in buffer A (50 mM Tris-HCl pH 7.5, 0.1% sucrose) containing 1% Brij 58 and 1 M NaCl and lysed by several rounds of freezing and thawing (dry ice-ethanol: 37°C). After centrifugation, the supernatant, containing the *E. coli* DnaB (*Eco*DnaB) and *Eco*DnaG proteins, was discarded and the pellet containing DnaG was washed with buffer A containing 50 mM NaCl and 2 M urea. DnaG was solubilised in buffer A containing 50 mM NaCl and 3 M urea (fraction I) (Fig. 1, lane 2). Fraction I was loaded onto a phosphocellulose column, and eluted with a step gradient with buffer A containing 50–150 mM NaCl and 3 M urea (fraction II) (Fig. 1, lane 3). DnaG was loaded onto a heparin-Sepharose column, and eluted from the column with buffer A containing 200 mM NaCl and 3 M urea. The eluted DnaG protein (fraction III) was more than 95% pure as judged by SDS-PAGE (Fig. 1, lane 4). The DnaG was further purified by gel filtration chromatography using a Superose 12 in buffer A containing 150 mM NaCl and 3 M urea (~99% pure). DnaG was refolded by dialysis against buffer A containing 150 mM NaCl, and decreasing the amounts of urea in a stepwise manner and finally concentrated 10-fold with PEG 6000 and dialysed against buffer A containing 150 mM NaCl. Glycerol was added to a final concentration of 50% and samples were stored at −20°C. DnaG concentration was determined using a molar extinction coefficient of 41 120 M^{-1} cm^{-1} at 280 nm and is expressed as mol of protein monomers. The NH_2-terminal amino acid sequence of DnaG was determined with an automated Edman degradation in a pulsed-liquid phase sequencer (model 476, Applied Biosystems).

2.5. Protein affinity chromatography

Protein-protein interactions were assayed by affinity chromatography. DnaG or BSA proteins (1.5 μM) were covalently cross-linked to Affi-Gel-10 (0.5 ml) resin as recommended by the manufacturer (Bio-Rad). G*40*P (0.2 μM) was loaded onto both affinity columns, which were equilibrated in buffer B (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100) containing 50 mM NaCl. Columns were washed with 10 column volumes of buffer B containing 50 mM NaCl. Bound fractions were eluted with 10 volumes of buffer B containing 1 M NaCl. Fractions of 100 μl were collected and analysed by SDS-PAGE.

Antibodies against G*40*P were coupled to a protein A-Sepharose column as recommended by the supplier (Pharmacia). DnaG (0.2 μM) was incubated alone or in the presence of G*40*P (0.2 μM) at 30°C for 5 min in buffer B containing 50 mM NaCl and then loaded onto the anti-G*40*P-protein A-Sepharose column (50 μl column) equilibrated with the same buffer. The columns were then washed with 10 column volumes of buffer B containing 50 mM NaCl, and the protein(s) eluted with 1 M NaCl. Fractions were analysed by SDS-PAGE.

2.6. Molecular mass determination

The native molecular mass of DnaG was determined by gel filtration FPLC using a Superose 12 column (Pharmacia). The chromatography was carried out in buffer A containing 150 mM NaCl at a flow rate of 0.4 ml/min, and the A_{280} was recorded. A standard curve of K_{av} versus $\log_{10}$ of relative mobility was determined as recommended by Pharmacia. Protein standards were obtained from Pharmacia (ovalbumin, 44 kDa; albumin, 67 kDa; aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa and thyroglobulin, 670 kDa).

2.7. Biochemical assays

ATPase activity was determined by measuring the amount of phosphate set free upon hydrolysis as previously described using poly(dA) (150 μM in nucleotides) as effector [11].

For helicase activity the reaction was incubated for 30 min at 30°C in buffer C (50 mM Tris-HCl pH 7.5, 12 mM $MgCl_2$, 5 mM ATP, 50 μg/ml BSA, 1 mM DTT) with 50 nM of G*40*P_6 and 50 nM of DnaG in a 20 μl volume. Different helicase substrates (3 μM, concentration referring to nucleotides) were tested (specific activity 2000 cpm/fmol). The reaction was stopped by addition of 5 μl of stopping solution (100 mM EDTA, 2% SDS in DNA loading buffer, [12]) and subsequently loaded onto a 1% agarose gel. Gels were run and dried prior to autoradiography.

DNA binding assays with various amounts of DnaG, G*40*P_6 or both proteins and M13 ssDNA were used. The DNA (20 μM in nucleotides) was incubated at 30°C for 5 min in buffer C containing 50 mM NaCl in a total volume of 20 μl. Samples were transferred to ice and 4 μl of a solution containing 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added. Binding reactions were analysed in 0.8% agarose gel in 0.5×TBE buffer [9].

3. Results and discussion

3.1. Purification and physical properties of B. subtilis DnaG primase

Plasmid pBT217, which contains the *dna*G gene downstream of a T7 promoter, was introduced into *E. coli* strain BL21(DE3)/pLysS, [13] and the *dna*G gene [16] was overexpressed by the addition of IPTG and rifampicin as previously described [11]. The DnaG (formerly termed DnaE) polypeptide, which is toxic to *E. coli* cells [14], accounts for about

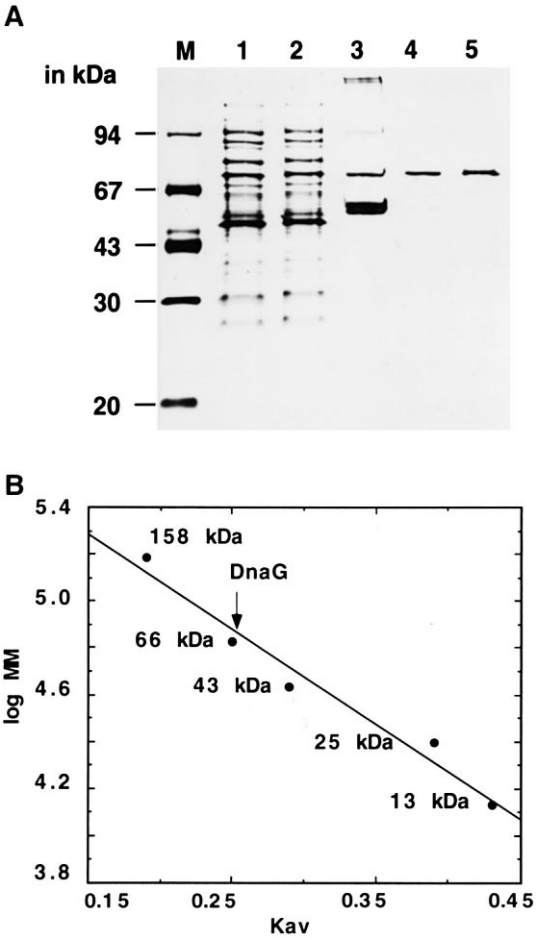

Fig. 1. Purification and molecular mass determination of the DnaG protein. A: Coomassie blue-stained 12.5% SDS-polyacrylamide gel. 2 μl of the sample was loaded at each lane. M, molecular mass standard in kDa; lane 1, supernatant of 1 M NaCl 3 M urea; lane 2, supernatant of high speed centrifugation in 50 mM NaCl 3 M urea; lane 3, phosphocellulose 120 mM NaCl 3 M urea elution; lane 4, heparin-Sepharose 200 mM NaCl, 3 M urea elution; lane 5, DnaG after Superose 12 in 150 nM NaCl, 3 M urea. B: Molecular mass determination of purified DnaG. Gel filtration chromatography was carried out using a Superose 12 column on a Pharmacia FPLC apparatus. Calibration graph of molecular mass against K_{av} of several protein standards (closed circles) for a Superose 12 column using the same buffer conditions as described for DnaG (buffer A containing 150 mM NaCl). The arrow indicates DnaG.

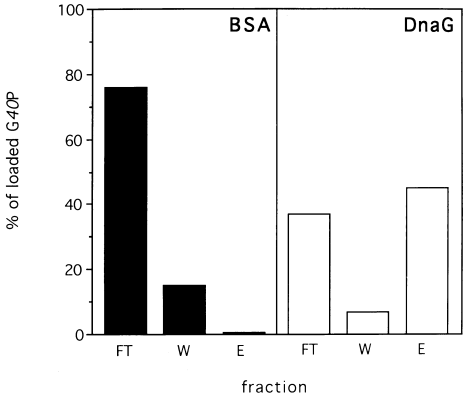

Fig. 2. DnaG-G*40*P$_6$ interaction. DnaG or BSA as control were immobilised in Affi-10 columns. Equal amounts of G*40*P$_6$ were then loaded on both matrices. The flow-through fractions (FT) were collected. The beads were washed (W) with 10 column volumes of buffer B containing 50 mM NaCl and the proteins were eluted (E) from the columns with 10 column volumes of buffer B containing 1 M NaCl. Aliquots of the fractions were analysed by SDS-PAGE and the G*40*P bands were quantified by densitometric scanning. The percentage of G*40*P$_6$ in the fraction with respect to the amount of loaded protein is given.

0.6% of total protein mass. Under these experimental conditions a yield of about 0.15 mg of DnaG protein per litre of induced culture was obtained.

The overproduced 69 kDa polypeptide was insoluble. The DnaG protein aggregates could, however, be dissolved in the presence of 3 M urea. This property was exploited in our purification scheme to release unwanted proteins and the remaining chromosomal DNA.

Identification of the final product as DnaG protein was confirmed by sequencing the amino-terminus of the purified protein. The sequence of the first 15 NH_2-terminal residues of the purified protein is in perfect agreement with the prediction from the nucleotide sequence of the *dna*G gene [16], except for the Met residue of the initiator codon which was absent in the purified protein. No traces of *Eco*DnaG and *Eco*DnaB proteins were present in our DnaG preparation (data not shown).

DnaG, which has a M_r of 69 000 in SDS-PAGE, consists of 603 amino acid residues, corresponding to a molecular mass of 68 799 Da deduced from the nucleotide sequence of the *dna*G gene [16]. Purified DnaG was loaded onto a Superose 12 column equilibrated in buffer A containing 150 mM NaCl, and eluted from this column as a single peak corresponding to M_r 69 000 (Fig. 1), showing that the protein is a monomer in solution.

3.2. The DnaG primase interacts with the G40P$_6$ helicase

All primases, whether from bacteriophage, viral, bacterial, or eukaryotic sources, catalyse the template-directed de novo synthesis of oligoribonucleotides on ssDNA in the presence of a replication fork helicase [1]. The specific recognition sequence used by DNA primases of Gram-positive bacteria to initiate oligoribonucleotide synthesis and the mechanism to assemble a stable primosome are poorly understood. To study a possible interaction between the host-encoded monomeric DnaG and the SPP1 G*40*P$_6$ helicase, a protein affinity column was employed. DnaG (1.5 μM) or bovine serum albumin (BSA) (1.5 μM), as non-specific control, were immobilised on an Affi-Gel-10 matrix, and then G*40*P$_6$ was loaded on the immobilised protein matrices. As shown in Fig. 2, about 50% of G*40*P$_6$ was specifically retained in the DnaG primase affinity column. Most of the G*40*P$_6$ retained in the BSA-Affi-Gel-10 column eluted in the wash fraction, whereas the G*40*P$_6$ protein retained in the DnaG-Affi-Gel-10 column eluted mostly in the 1 M NaCl fraction. The same results were observed when 0.5 mM ATP or 0.5 mM ATPγS was present in the reaction mixture (data not shown).

To analyse further the interaction between DnaG and G*40*P$_6$, polyclonal antibodies raised against G*40*P were immobilised in a protein A-Sepharose column. The DnaG protein (0.2 μM) in buffer B containing 50 mM NaCl was not retained on the anti-G*40*P-protein A-Sepharose matrix. DnaG (0.2 μM) was then incubated with G*40*P$_6$ (0.2 μM) at 30°C for 5 min in buffer B containing 50 mM NaCl and loaded onto an anti-G*40*P-protein A-Sepharose column (50 μl column). G*40*P was completely retained in the column, whereas about 60% of the DnaG protein was retained on the anti-G*40*P-protein A-Sepharose column. When G*40*P protein concentration was

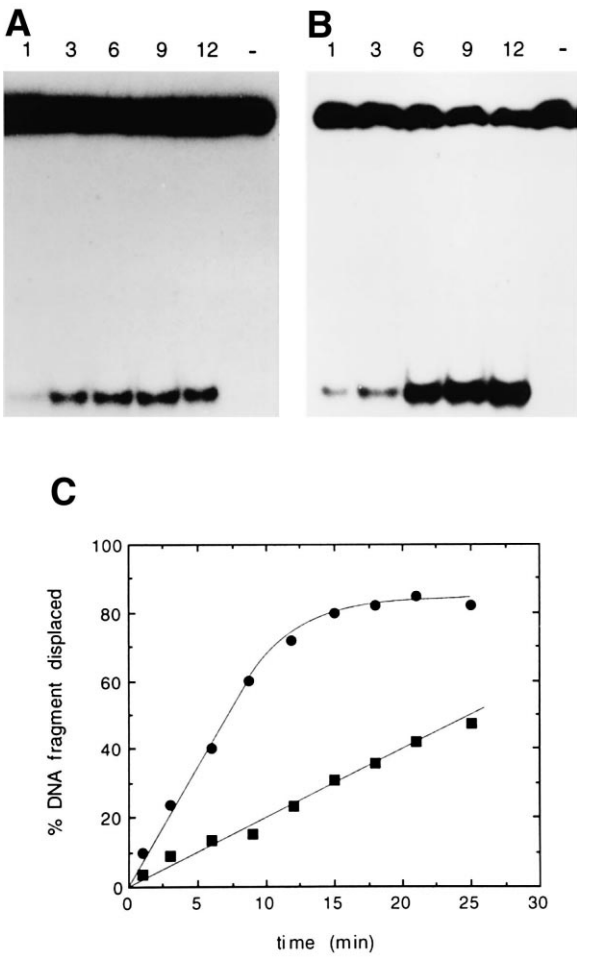

Fig. 3. Effect of DnaG on the helicase activity of G*40*P$_6$. Standard helicase assay mixtures of 200 μl containing G*40*P (50 nM) (squares) or G*40*P (50 nM) and DnaG (50 nM) (circles) were assembled at 4°C. At the times indicated, 20-μl aliquots were taken, processed and analysed for helicase activity as described in Section 2. A: Helicase assay in the absence of DnaG. B: Influence of DnaG on G*40*P helicase activity. The numbers 1, 3, 6, 9 and 12, in parts A and B, indicate the incubation time in minutes. The minus denotes the absence of the protein(s). In C, the data plotted are the average of three independent experiments.

increased to 0.4 μM and the amount of DnaG protein (0.2 μM) was maintained, all the DnaG protein was retained on the anti-G*40*P-protein A-Sepharose matrix (data not shown).

These result suggests that a direct interaction between monomeric DnaG and G*40*P_6 (~1:6 primase-helicase complex) takes place in the absence of DNA and a nucleotide cofactor. Recently it has been shown that the *Eco*DnaG primase is physically and functionally associated with *Eco*DnaB helicase [4,5,17]. Unlike the bacterial DnaG primases of *B. subtilis* (this report) and *E. coli* origin [17] that form stable complexes with the replication fork helicase in the absence of DNA and energy cofactor, only the ATP-activated T4 gp41 helicase binds to the T4 gp61 primase-DNA complex [18].

3.3. The interaction of DnaG with G40P_6 enhances the helicase activity of G40P

Under our experimental conditions the amount of DNA fragments of different length displaced in the helicase assay depends on the amount of G*40*P_6 added to the reaction, classifying G*40*P_6 as a concentration-dependent helicase. Could other replication proteins enhance the processivity of G*40*P_6? *Eco*DnaB in vitro also shows low processivity [19], which increases in the presence of other replication proteins. The rate of DNA unwinding by *Eco*DnaB in vitro is greatly enhanced in the presence of *Eco*DnaG and *Eco*SSB, *Eco*DNA gyrase, and *Eco*DNA pol III [2,20,21].

As *B. subtilis* DnaG has been shown to interact with G*40*P_6 on an affinity column, we tested whether the interaction of both proteins affects the unwinding reaction catalysed by G*40*P_6. Time course experiments in the absence or in the presence of DnaG were performed (Fig. 3A,B). As shown in Fig. 3C, the addition of DnaG at a ratio of one monomer of DnaG per hexamer of G*40*P increased the fraction of the 50 nt long DNA segment displaced about 3-fold. The same results are obtained when an excess of DnaG was added to the reaction mixture, further suggesting that it is a 1:6 complex. Under these conditions the presence of CTP, GTP and UTP (at 65 μM final concentration) does not increase the helicase activity of G*40*P_6.

An enhancement of G*40*P_6 helicase activity was also observed when substrates with longer annealed regions were employed (data not shown). The presence of DnaG (50 nM) did not markedly increase the processivity of G*40*P_6, because when the unwinding reaction was challenged with a 20-fold excess of cold helicase substrate, the rate of unwinding remained constant when compared to that of G*40*P_6 alone. Furthermore, under the same conditions such an increase in helicase activity is not concomitant with an increase in the ATPase activity of G*40*P_6 when assayed either in the presence or in the absence of ssDNA. Under our experimental conditions, the increase of G*40*P_6 translocation on the DNA template in the presence of DnaG could be caused by stabilising the binding of G*40*P_6 to ssDNA.

We have previously shown that in absence of ATP, the apparent equilibrium constant (K_{app}) of the G*40*P_6-ssDNA complexes was estimated to be approximately 1 μM and the presence of 1 mM ATP increased the affinity of G*40*P_6 for naked ssDNA (K_{app} 330 nM) about 3.5-fold [11]. When ATPγS (1 mM) is used instead of ATP, the K_{app} for the G*40*P_6-ssDNA complex decreased about 20-fold (K_{app} 14 nM) when compared to the absence of ATP [11]. It is likely, therefore, that the hydrolysis of ATP leads to dissociation of the ssDNA-G*40*P_6 complex. Considering that the G*40*P_6 DNA binding affinity is enhanced by the presence of a non-hydrolysable analogue such as ATPγS, it is likely that DnaG could fulfil a similar function as ATPγS by keeping the helicase on the DNA track during ATP hydrolysis. This hypothesis is supported by DnaG binding preferentially to ssDNA (K_{app} 40 nM) and by a mobility shift assay where we observed that DnaG recruits G*40*P_6 on ssDNA (data not shown).

We could observe an enhancement of the G*40*P_6 helicase activity by DnaG in the absence of both SSB and ribonucleotides other than ATP, which is required for helicase activity, so that synthesis of primers by DnaG is not required for the observed enhancement of helicase. In this respect the G*40*P_6 helicase differs from the *Eco*DnaB helicase (see [22]).

Acknowledgements: This research was partially supported by Grants PB 96-0817 from DGCYT, 06G/004/96 from CAM and BIO4-CT98-00106 to J.C.A. We are very grateful to T.A. Trautner for his continuous interest in this project, to F.W. Studier for providing bacterial strains, and to V. Kruft for performing the sequencing of DnaG protein.

References

[1] Kornberg, A. and Baker, T.A. (1992) DNA Replication, 2nd edn., W.H. Freeman, New York.
[2] Kim, S., Dallmann, H.G., McHenry, C.S. and Marians, K.J. (1996) Cell 84, 643–650.
[3] Yuzhakov, A., Turner, J. and O'Donnell, M. (1996) Cell 86, 877–886.
[4] Tougu, K., Peng, H. and Marians, K.J. (1994) J. Biol. Chem. 269, 4675–4682.
[5] Tougu, K. and Marians, K.J. (1996) J. Biol. Chem. 271, 21391–21397.
[6] Zechner, E.L., Wu, C.A. and Marians, K.J. (1992) J. Biol. Chem. 267, 4054–4063.
[7] Burger, K.J. and Trautner, T.A. (1978) Mol. Gen. Genet. 166, 277–285.
[8] Pedré, X., Weise, F., Chai, S., Lüder, G. and Alonso, J.C. (1994) J. Mol. Biol. 236, 1324–1340.
[9] Missich, R., Weise, F., Chai, S., Lurz, R., Pedré, X. and Alonso, J.C. (1997) J. Mol. Biol. 270, 50–64.
[10] Barcena, M., San Martin, C., Weise, F., Ayora, S., Alonso, J.C. and Carazo, J.M. (1998) J. Mol. Biol. (in press).
[11] Weise, F., Ayora, S. and Alonso, J.C. (1998) (submitted).
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Handbook, Vol. 1–3, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[13] Studier, F.W. (1991) J. Mol. Biol. 219, 37–44.
[14] Langer, U. (1991) Diplomarbeit, Freie Universität, Berlin.
[15] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[16] Wang, L.F., Price, C.W. and Roi, H.D. (1985) J. Biol. Chem. 260, 3368–3372.
[17] Lu, Y.B., Ratnakar, V.A.L., Mohanty, B.Y. and Bastia, D. (1996) Proc. Natl. Acad. Sci. USA 93, 12902–12907.
[18] Dong, F., Gogol, E.P. and von Hippel, P.H. (1996) J. Biol. Chem. 270, 7462–7473.
[19] Matson, S.W. and Bean, D.W. (1995) Methods Enzymol. 262, 389–405.
[20] Lohman, T.M. and Bjornson, K.P. (1996) Annu. Rev. Biochem. 65, 169–214.
[21] Matson, S.W. and Kaiser-Rogers, K.A. (1990) Annu. Rev. Biochem. 59, 289–329.
[22] LeBowitz, J.H. and McMacken, R. (1986) J. Biol. Chem. 261, 4738–4748.