In vitro DNA binding of the archaeal protein Sso7d induces negative supercoiling at temperatures typical for thermophilic growth

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ABSTRACT

The topological state of DNA in hyperthermophilic archaea appears to correspond to a linking excess in comparison with DNA in mesophilic organisms. Since DNA binding proteins often contribute to the control of DNA topology by affecting DNA geometry in the presence of DNA topoisomerases, we tested whether the histone-like protein Sso7d from the hyperthermophilic archaean *Sulfolobus solfataricus* alters DNA conformation. In ligase-mediated supercoiling assays carried out at 37, 60, 70, 80 and 90°C we found that DNA binding of increasing amounts of Sso7d led to a progressive decrease in plasmid linking number (Lk), producing negative supercoiling. Identical unwinding effects were observed when recombinant non-methylated Sso7d was used. For a given Sso7d concentration the DNA unwinding induced was augmented with increasing temperature. However, after correction for the overwinding effect of high temperature on DNA, plasmids ligated at 60–90°C exhibited similar σ values at the highest Sso7d concentrations assayed. These results suggest that Sso7d may play a compensatory role in vivo by counteracting the overwinding effect of high temperature on DNA. Additionally, Sso7d unwinding could be involved in the topology changes observed during thermal stress (heat and cold shock), playing an analogous role in crenarchaeal cells to that proposed for HU in bacteria.

INTRODUCTION

Small, abundant and usually basic proteins with DNA binding affinities are widespread in all organisms. By analogy with eukaryotic histones, bacterial proteins bearing these properties are often referred to as histone-like, although they are not generally sequence homologues (1,2). In the domain Archaea, up to four distinct phylogenetic groups of DNA binding proteins have been identified. Three of them have been characterized in the euryarchaea (kingdom Euryarchaeota), which have considerable sequence similarity and similar 3-dimensional structures to eukaryotic histones (3,4). In the other archaeal branch, the crenarchaea (kingdom Crenarchaeota), true histone homologues have not been detected. Instead, the existence of three classes of DNA binding proteins with molecular weights of ~7, 8 and 10 kDa has been reported (5). Sso7d is the major component of the 7 kDa class (a group of closely related proteins of different basicity) from the hyperthermophile *Sulfolobus solfataricus*. This abundant and extremely basic protein has been purified and sequenced (6) and is highly similar to the Sac7 proteins from *Sulfolobus acidocaldarius* (7,8). The physical properties of Sso7d have been studied in detail and its 3-dimensional structure has been elucidated using NMR spectroscopy (9). The protein behaves unambiguously as a monomer and consists of a triple-stranded anti-parallel β-sheet onto which an orthogonal double-stranded β-sheet is packed, a topology very different from histones. Sso7d has a strong binding affinity for dsDNA, which probably predominates in vivo, since ssDNA and ssRNA binding are weak under physiological salt conditions (9). A model of the Sso7d–DNA complex involving interaction of the triple-stranded β-sheet with the DNA major groove has been proposed (10). As with many other DNA binding proteins, Sso7d protects DNA from denaturation (9). Interestingly, some lysine residues are methylated in vivo and a possible involvement of this methylation in the heat shock response has been proposed (9). However, no significant differences in protein thermal stability and binding to dsDNA have been detected between methylated and non-methylated Sso7d, and the role of this post-transcriptional modification remains enigmatic (11).

To date the cellular functions of Sso7d have not been clearly established. Apart from protection against DNA melting, Sso7d could be involved in DNA compaction, but how it may condense DNA has not been determined. A putative phosphate binding site was identified, leading to the proposal of Sso7d acting as a molecular chaperone with ATPase activity (12). Also, putative RNase activity with a narrow substrate specificity (13) and the promotion of homologous ssDNA fragment annealing (14) have been proposed. The actual occurrence of all these putative functions deserves further analysis.

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Much more information about the roles of small DNA binding proteins in eukaryotes and bacteria has been accumulated. Their functions are generally related to DNA compaction and local alterations in DNA geometry that may influence transcription, replication or recombination (1,2,15–17). Many of these proteins are involved in the generation or maintenance of supercoiling levels. Core histones constrain negative DNA supercoiling in eukaryotes which is, in turn, generated by DNA wrapping around histones in the presence of topoisomerases (18,19). The linker histone H1 can affect DNA geometry, leading to the generation of negative supercoiling in topological assays (20). In bacteria, purified HU in the presence of a topoisomerase also induces a decrease in DNA linking number (21,22). HU could help to restrain negative supercoiling in bacteria in a dynamic way (23).

Indeed, HU deficiency can be suppressed by gyrase mutations, which further supports its participation in regulation of bacterial (negative) supercoiling (24). In mesophilic euryarchaea the histone-like protein MC1, found in *Methanosarcinaeae*, also induces DNA bending, negative supercoiling and compaction (25). In contrast, in hyperthermophilic euryarchaea containing archaeal histones, positive supercoiling around the histone core is induced (26). This would apparently correlate with the fact that thermococcal plasmids are relaxed to positively supercoiled (27,28). However, in hyperthermophilic crenarchaea of the order Sulfolobales, lacking archaeal histones, plasmid DNA is also found to be from relaxed to positively supercoiled (28). Clearly, studies about the effects of their histone-like proteins on DNA geometry are lacking.

Supercoiling levels can change in bacteria depending on growth phase and a variety of environmental factors (29,30). In particular, heat and cold shocks cause transient shifts in plasmid superhelicity, producing a linking increase during heat shock (31) and a linking decrease during cold shock (32). Although DNA topoisomerases, specifically gyrase, are known to participate in superhelical changes during thermal stress in bacteria (33), some DNA binding proteins could additionally be involved. For instance, HU-defective *Escherichia coli* mutants exhibit excessive plasmid DNA relaxation after heat shock and, therefore, a role for HU in maintaining negative supercoiling under thermal stress has recently been proposed (34). Also, bacterial H-NS is overexpressed during cold shock (35), although a direct effect of this protein on supercoiling has not been clearly demonstrated (36).

In hyperthermophilic archaea, DNA supercoiling also changes depending on growth phase and during thermal stress (28). Therefore, it could be reasonably hypothesized that Sso7d plays a role in heat or cold shock-induced superhelical changes as well.

In the present report we demonstrate that the *Sulfobolas* DNA binding protein Sso7d affects DNA geometry at different temperatures compatible with growth, generating negative supercoiling levels in topological assays as a consequence. We hypothesize that Sso7d-induced unwinding of DNA could play a role analogous to that proposed for HU in bacterial cells during thermal stress.

### MATERIALS AND METHODS

#### Purification of native and recombinant Sso7d

Methylated Sso7d and recombinant non-methylated Sso7d were purified from *S.solfataricus* and *E.coli* respectively, as described elsewhere (9,11). Two independent preparations were obtained and used for the present studies in order to assure reproducibility.

#### Preparation of plasmid DNA

pTZ18R (37) was purified from an overnight 250 ml culture of *E.coli* JM109 cells harbouring this plasmid using the system Nucleobond AX-500 (Macherey-Nagel, Düren). Nicked DNA was prepared by limited digestion of pTZ18 with DNase I (0.068 U/µg DNA) in the presence of 0.3 mg ethidium bromide/ml for 1 h at 30°C, as described (38). After addition of EDTA, pH 8.0, to 3 mM in order to stop the reaction, DNA was immediately purified by phenol/chloroform extraction and ethanol precipitation. Plasmid DNA prepared in this way contained no supercoiled forms and only a small proportion of linear DNA.

#### Ligase-mediated supercoiling assays

We used T4 ligase (Boehringer Mannheim) for the reactions performed at 37°C and Pfu DNA ligase (Stratagene) for the reactions carried out at 60–90°C. Aliquots of 1 or 2 µg nicked pTZ18 were mixed with increasing amounts of Sso7d in a 50 µl final volume of 1X ligase buffer (supplied by the manufacturers, without modification of Mg²⁺ concentration, 10 mM) and incubated for 10 min at the corresponding temperature. The only exceptions were samples to be ligated at 90°C, which were incubated at room temperature for 10 min in order to prevent denaturation of nicked DNA. Subsequently 4 U of T4 or Pfu ligase were added and ligation was allowed to proceed for 1 h at 37, 60, 70, 80 or 90°C. Samples were then chilled on ice and immediately extracted with phenol/chloroform and re-extracted with chloroform after dilution with TE, pH 8.0, to 200 µl plus addition of NaCl to 1 M and SDS to 1% final concentration. Samples containing Sso7d concentrations near to saturation were additionally treated with 0.1 µg/ml proteinase K for 1 h at 37°C and then mixed with hot phenol, incubated at 65°C for 20 min and extracted prior to the phenol/chloroform step. After ethanol precipitation DNA was resuspended in TE and analysed by 2-dimensional gel electrophoresis.

#### Gel electrophoresis

Agarose gels (1%, Indubiose A37NA; IBF, France) were prepared in TBE buffer (39). Electrophoreses were performed at 25°C in the same buffer. Unless otherwise stated, no intercalating agent was required for the first dimension, running conditions were 0.5 µg/ml chloroquine, 1.5 V/cm for 20 h (first dimension) and 3 µg/ml, 1 V/cm for 20 h (second dimension). After extensive washing in water to eliminate the chloroquine, gels were stained with ethidium bromide (1 µg/ml). Polaroid photographs were taken under UV light. Stained gels were additionally photographed using the Sony UVP Image Store 5000 system and images were stored using the program Adobe Photoshop 3.0. Densitometric band analysis was performed using the GelScan v.1.1 program (Y.Zivanovic, IGM, France).

#### Determination of linking difference (ΔLk) and specific linking difference (σ)

ΔLk was estimated by the band counting method, with a relative precision of ±0.5 (40). The specific linking difference, σ, was determined using the equation \( \sigma = \Delta Lk/Lk_0 \) (41). A correction...
factor to account for the effect of temperature, from 25°C (electrophoresis) to the temperature at which the different ligase-mediated reactions were carried out, was applied in some cases for comparative purposes. For this we used the value $-0.011^{\circ}C/bp$ as an estimation of the rotation angle of the DNA double helix with temperature (27,42,43).

**RESULTS AND DISCUSSION**

Since Sso7d exhibits strong binding affinity for dsDNA under physiological conditions (9), we wanted to test if this binding has an effect on DNA supercoiling at temperatures typical for growth. *Sulfolobus solfataricus* can grow between 60 and 87°C (44,45). Therefore, we decided to test induction of supercoiling at different temperatures covering that range, namely 60, 70, 80 and 90°C. For this purpose we carried out ligase-mediated supercoiling assays using the highly thermostable Pfu ligase (Stratagene). This enzyme is active over a wide temperature range, from 45 to >80°C, with a half-life of >60 min at 95°C. As a mesophilic control of the effects of Sso7d on DNA we also carried out an identical set of reactions at 37°C using T4 ligase. The availability of thermophilic ligases makes these assays advantageous over topoisomerase-mediated relaxation assays, in which negatively supercoiled DNA is currently mixed with a DNA binding protein and topoisomerase I. Furthermore, ligase-mediated assays are less affected by ambiguities caused by potential inhibitory effects of the tested protein on topoisomerase activity, since putative inhibition of ligation would not alter the final distribution of topoisomers, but only the amount of ligated product (20).

The assay consists of ligation of nicked plasmids in the absence and presence of a DNA binding protein. The ligation products in the absence of protein should generate a Boltzmann distribution of topoisomers, corresponding to the relaxed state at each temperature (42). However, if binding of the tested protein has any effect on the geometry (twist and/or writhe) of the DNA molecule a change in the distribution of topoisomers will be observed after ligation of the nicks and protein removal. In our case we incubated nicked pTZ18 (2880 bp) with increasing amounts of purified partially methylated Sso7d (7.4 kDa) at each of the above mentioned temperatures for 10 min before adding T4 or Pfu ligase. For every set of reactions, protein:DNA ratios used were 0, 1:70, 1:50, 1:30, 1:15 and 1:4 (molecules SSo7d:DNA bp). The concentration 1:4 corresponded approximately to saturation, which was previously reported to be 1:6 (9). After ligation and phenol/chloroform extraction to remove proteins, pTZ18 topoisomers were first fractionated by 1-dimensional electrophoresis in order to observe induction of supercoiling. Indeed, an increase in topoisomer mobility with increasing Sso7d concentration was observed (not shown). Samples that had been incubated at 90°C, but not those incubated at 80°C, were degraded, most likely due to denaturation of nicked DNA before ligation. Therefore, the ligase at 90°C were repeated after preincubation of pTZ18/Sso7d mixtures at room temperature. Induction of plasmid supercoiling was also observed (not shown).

In order to determine the direction of supercoiling induced, samples obtained after ligation in the presence of 0, 1:70, 1:50 and 1:30 Sso7d molecules/bp were subjected to 2-dimensional electrophoresis. As can be observed in Figure 1A, in the absence of an intercalating agent during the first dimensions, all topoisomer distributions became more negative (occupying the left part of the arches) with increasing concentrations of Sso7d. The greatest differences in linking number compared with the control relaxed state ($\Delta$Lk) were observed with the highest Sso7d concentrations (1:30), as expected. At this Sso7d concentration identification of major topoisomers was difficult in the absence of a DNA intercalating agent to enhance resolution. Therefore, these samples were additionally electrophoresed in 1-dimensional gels in the presence of chloroquine to determine the respective topoisomer distribution centers at different temperatures (not shown). A representative 2-dimensional gel in the presence of chloroquine for samples incubated at 80°C is shown in Figure 2. Maximal ALk differences ($\Delta$Lk$_{1:30}$ – $\Delta$Lk$_0$) were similar at all assay temperatures (–5/-6 topoisomers) (Figs 1B and 2), although slightly increased at 90°C (–7 topoisomers). However,
as observed in 2-dimensional gels, the distribution of topoisomers in the absence of protein when ligations were carried out at increasingly higher temperatures is not centered around 0, but around negative topoisomers (Fig. 1). This is due to the effect of the temperature shift from the assay temperature to the electrophoresis temperature (25°C). A decrease in temperature tends to increase the twist, leading to a decrease in writhe (supercoiling) of covalently closed DNA molecules. Therefore, nicked-sealed
Figure 2. Resolution of negatively supercoiled topoisomers generated in ligase-mediated assays at 80°C in the presence of methylated and non-methylated Sso7d. (A) Increasing quantities of Sso7d (indicated as molecules Sso7d per bp plasmid) were assayed as in Figure 1 and the respective topoisomer distributions resolved by 2-dimensional electrophoresis. Chloroquine at 0.5 µg/ml was added for electrophoresis in the first dimension. (B) Quantitation of topoisomer distributions (see legend to Fig. 1).

molecules that were relaxed in the absence of Sso7d at high temperature become negatively supercoiled at room temperature.

Samples obtained by incubation at protein:DNA ratios of 1:15 led to still more negative supercoiling (topoisomers migrated further) in 1-dimensional gels (not shown). However, it was difficult to identify major topoisomers, since a significant loss of DNA after phenol/chloroform extraction occurred. This phenomenon was even more emphasized when the protein:DNA ratio was 1:4, when only trace amounts of nicked and linear pTZ18 remained. This is probably due to tight binding of Sso7d to DNA at high protein concentrations, where the compact DNA–protein clusters are inaccessible to proteinase and stable in phenol/chloroform. It was only after incubation with hot phenol (see Materials and Methods) that we recovered most DNA for topoisomer analysis. At 80°C and Sso7d concentrations near saturation (one Sso7d molecule/10 bp plasmid) a ΔLk of –7 topoisomers was observed (Fig. 2).

In order to compare the relative variations in linking number independently of temperature effects and also of plasmid size we estimated the specific linking differences (σ) of major topoisomers generated in all the assays. Estimated errors were ±0.002. In a first approach we estimated relative specific linking differences against the protein:plasmid ratios up to one Sso7d molecule/30 bp plasmid. For this we normalized σ values so that σ = 0 corresponded to control reactions without protein, i.e. relaxed plasmids at each temperature (Fig. 3A). As can be observed, at increasing temperatures progressive Sso7d binding induces larger differences in plasmid superhelicity, which is reflected in a more pronounced slope of the respective curves. Although not plotted in the curve, at 80°C and near protein:DNA saturation (1:10 Sso7d:bp plasmid) standardized σ reached its lowest value, –0.047. Subsequently we plotted the corresponding σ values after applying a correction factor to account for the physical effect of higher temperatures on the DNA molecule (see Materials and Methods) (Fig. 3B). As can be observed, the curves obtained at 60, 70 and 80°C are practically superimposable. At 90°C progressive Sso7d binding induces a slightly larger difference in plasmid superhelicity, whereas the curve at 37°C remains in the negative part of the graph. At 80°C and near protein:DNA saturation (1:10 Sso7d:bp plasmid) σ would be –0.029. Two interesting aspects can be noted in Figure 3B. First, the overall differences between the curve at 37°C and the others reflect the different topological states of plasmid DNA at those temperatures, which correlate with the differences found in vivo (27,28). Secondly, although higher temperatures decrease the twist and increase positive supercoiling, addition of increasing amounts of Sso7d attenuate this effect. Thus, the 1:30 samples exhibit very similar σ between 60 and 90°C, in remarkable contrast to the differences observed between normalized values (Fig. 3A). This suggests a ‘buffering’ role for Sso7d under growth conditions, which would counteract the overwinding effect of temperature.

Assuming that all the protein is bound to DNA in our assays and taking into account concentrations up to near saturation (one Sso7d/10 bp plasmid), an estimate of the unwinding angle induced by binding of Sso7d at 80°C can be made. The difference in superhelical turns induced by one Sso7d molecule can be inferred from the least squares line slope in Figure 4, where major topoisomer distributions are plotted against Sso7d:DNA ratios.
Figure 3. Variation in the specific linking differences induced by incubation with increasing amounts of Sso7d in supercoiling assays at different temperatures. (A) Relative specific linking differences after normalization of $\sigma$ values ($\sigma = 0$ in the absence of protein). (B) Specific linking differences after correction for temperature effects on DNA topology (see text).

For a plasmid size of 2880 bp this corresponds to an unwinding angle of $12.7^\circ$ per Sso7d molecule bound. In agreement with our results, Agback et al. (submitted) have recently analysed the structure of a complex formed by two Sso7d monomers bound to a 12 bp oligomer by NMR spectroscopy, showing that Sso7d unwinds the DNA so that the helical periodicity is $12.9 \pm 0.5$ at 50°C. An unwinding of $12.7^\circ$ at 80°C would correspond to $-11$ bp/turn at 50°C, if we consider a rotation of $0.011^\circ/\text{C/bp}$ (27,42,43). Although both independent results confirm the unwinding capacity of Sso7d, differences in the helical periodicities estimated are probably due to the different lengths of the substrate DNAs, to the different DNA:protein ratios used and to the assumption in our assay that all protein is plasmid bound. Indeed, protein aggregation might occur at high concentrations, as suggested by electron microscopy studies (not shown). Recently the crystal structure of DNA–Sac7d complexes has been analysed. Sac7d binds to the minor groove and sharply kinks DNA, which is associated with DNA unwinding (46).

In vivo Sso7d is subject to $\varepsilon$-monomethylation at five of the 14 lysine residues of the molecule to varying extents (6). A putative role of methylation in the heat shock response has been postulated, since increased methylation is observed with higher growth temperature (9). However, after determination of the Sso7d DNA binding surface it appeared that the residues subject to methylation were the side chain lysines that remained exposed to the solvent, the remaining lysines being buried or close to the interface of the Sso7d–DNA complex (10). This, together with the fact that the pK$_a$ of the lysine side chain is little affected by methylation (47), suggested that the binding affinities of methylated and non-methylated (recombinant) Sso7d were similar. Indeed, this was recently shown by thermodynamic studies of the DNA binding reaction (48). Moreover, no detectable differences in melting temperature or unfolding enthalpy between both protein states were found, indicating that methylation is not related to protein thermal adaptation (11). Nevertheless, since heat or cold shock strongly alters levels of plasmid supercoiling (28), since Sso7d methylation appears to vary with growth temperature and since native Sso7d binding can induce changes in supercoiling when a cutting–resealing activity is present, we decided to analyse whether the non-methylated protein had a different effect on induction of supercoiling. For that, the topoisomer distributions obtained in Pfu ligase-mediated supercoiling assays using nicked pTZ18 incubated at 80°C with increasing amounts of Sso7d, either methylated or recombinant (non-methylated), were compared. As shown in Figure 2, no significant differences were detected in the unwinding capability of non-methylated Sso7d, as expected from previous structural studies. In Sulfolobus strains, changes in plasmid topology are observed during growth and thermal shock (28). Although the precise mechanisms of DNA supercoiling regulation are unknown in these organisms, Sso7d binding could participate in homeostatic control of DNA superhelicity due to its unwinding capacity. Continuous growth at 60–65°C is paralleled by plasmid $\sigma$ values down to $-0.011$ (28; López-García, unpublished results) and during cold shock from 80 to 65°C native plasmid $\sigma$ values transiently reach as low as $-0.014$ or less. It is plausible that Sso7d binding accounts for at least part of this negative supercoiling, especially since Sulfolobus apparently lacks a gyrase introducing negative superturns in DNA. Indeed, Sso7d appears to be a cold shock protein (Knapp, unpublished results). Also, a putative role
in homeostatic recovery of lower linking differences after prolonged heat shock seems likely. In hyperthermophilic archaea, heat shock triggers an immediate sharp increase in plasmid supercoiling, from basal relaxation or slightly positive $\sigma$ values up to high positive supercoiling ($\sigma$ around +0.03), followed by recovery of lower levels of supercoiling (28). Interestingly, a similar phenomenon can be observed in mesophilic bacteria, where heat shock generates a transient increase in $L_k$ (here seen as a relaxation or decrease in negative supercoiling). The demonstration that E.coli mutants lacking $HU$ are not able to recover higher levels of negative supercoiling during heat shock suggested that $HU$ is responsible for maintaining the negative supercoiling of DNA against thermal stress, thus contributing to E.coli thermotolerance (34). These parallels suggest that Sso7d is a functional analog of the bacterial $HU$ protein, which would represent a more eukaryote-like system.

Finally, although DNA binding proteins are undoubtedly essential for DNA homeostasis, the dynamic role of archaeal topoisomerase activity during the life of the cell in changing environments, as well as topoisomerase interaction with such proteins, has still to be elucidated to understand this exquisite example of DNA structural regulation in all its complexity.

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REFERENCES