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Condensation of DNA by a Histone-like Protein in *Escherichia coli*

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Abstract: In *E. coli*, chromosomal DNA associated with proteins is condensed into an organized structure known as nucleoid. Using a nitrocellulose filter binding assay to identify proteins forming nucleoid, a 21 kDa protein was purified from *E. coli*. The molecular weight of the purified protein was 21 kDa on SDS-polyacrylamide gel electrophoresis and 24 kDa on gel permeation chromatography. A molecular weight of 21 kDa on SDS-polyacrylamide gel electrophoresis is unique among known proteins which are believed to be involved in the formation of nucleoid in *E. coli*. The 21 kDa protein nonspecifically binds to both double-stranded and single-stranded DNA. Sedimentation in a sucrose gradient revealed that the protein induced significant condensation of both supercoiled plasmid DNA and linear bacteriophage λ DNA. On the basis of quantitative Western-blot analysis, approximately 40,000 molecules of the protein were estimated to exist in an *E. coli*. The biochemical properties and cellular abundance of the 21 kDa protein suggest that this protein participates in the formation of nucleoid in *E. coli*.

Key words: condensation of DNA, histone-like protein, nucleoid.

Like eukaryotes, prokaryotes should compact their chromosomal DNA within the cell. *Escherichia coli* genome which is composed of 5×10^6 base pairs is packed into an *E. coli* cell with dimensions of 0.75 μm×2 μm (von Meyenberg *et al.*, 1987; Kornberg *et al.*, 1992; Drlica *et al.*, 1987; Pettijohn *et al.*, 1988; Schmid *et al.*, 1990). The volume of the chromosomal DNA must be condensed approximately 1000 fold in nucleoid. According to electron microscopic observations and nuclease digestion studies, the organization of prokaryotic chromosomal DNA in nucleoid is different from eukaryotic DNA. Prokaryotic chromosomal DNA is thought to have two structural features. The first, the DNA is in a topologically constrained state with a specific superhelical density of $\sigma = -0.05$ to 0.08 that is contributed by DNA gyrase and topoisomerase I. Second, the DNA has a condensed conformation due to its interaction with cellular proteins. This condensation is opposed by electrostatic repulsion of negative charges on the phosphates of DNA. As the importance of the positively charged histones in the condensation of eukaryotic chromosomal DNA became clear, the search for prokaryotic histone-like proteins has been attempted. Several DNA binding proteins expected to form nucleoid have been isolated from *E. coli*. Four proteins HU (9.5 kDa), H-NS (15.5 kDa), HLP1 (17 kDa) and H (28 kDa) have emerged as prokaryotic proteins which are most like eukaryotic histones. In addition to these four proteins, IHF (integration host factor, a heterodimer of 11.2 and 10.6 kDa subunits) and FIS (factor for inversion stimulation, 11.2 kDa) appear to play important roles in prokaryotic DNA organization. However, these small, usually basic, and collectively called histone-like proteins fail to satisfy criteria for the formation of nucleoid, genetically and biochemically. The binding of prokaryotic histone-like proteins to DNA is relatively salt sensitive and none of these proteins are as abundant as their eukaryotic counterparts (Pettijohn *et al.*, 1988; Schmid *et al.*, 1990; Broyles *et al.*, 1986). Recent studies have shown unanticipated characteristics of these histone-like proteins, such as properties of DNA bending and roles in illegitimate recombination (Pontiggia *et al.*, 1993; de Vargas *et al.*, 1989; Hulton *et al.*, 1990). Mutation studies suggest that none are essential for growth and survival of *E. coli* (Drlica *et al.*, 1987; Storts *et al.*, 1988). In several aspects, the prokaryotic proteins, which bend DNA and organize nucleoprotein complexes, resemble eukaryotic transcription factors rather than histones. These imply that a set of prokaryotic proteins organizing nucleoid may be more diverse. Other proteins condensing

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chromosomal DNA are needed to be identified.

A basic 21 kDa protein purified from *E. coli* is abundant and condenses bacteriophage λ DNA and plasmid DNA. The biochemical properties of this 21 kDa protein suggest that it may participate in the formation of nucleoid in *E. coli*. Isolation and characterization of nucleoid-forming proteins to reconstruct nucleoids *in vitro* is meaningful to illuminates the processes of replication, transcription, recombination, and repair.

Materials and Methods

Reagents

Sources were as follows: Superdex-75 and Mono S H/R 10/10 were purchased from Pharmacia LKB Biotechnology, Inc. (Uppsala, Sweden); Bradford reagent from Bio-Rad (Richmond, USA); [γ-^{32}P]ATP (6000 Ci/mmol) from Amersham Corp. (Amersham, UK); S-[Methyl-^{3}H]-adenosyl-L-methionine (SAM) (73.4 Ci/mmol) from NEN (Boston, USA). Buffer A contained 25 mM HEPES-KOH (pH 7.6), 2 mM DTT, 0.1 mM EDTA, and 15% glycerol.

Proteins and bacterial strain

Restriction endonucleases, T4 polynucleotide kinase, DNA ligase, and *Hha*I methylase were purchased from New England Biolabs. *E. coli* W3110 (λ^-, *IN*[*rrnD*-*rrnE*]) was used for purification of the 21 kDa protein.

Plasmid DNA

A *Bam*HI recognition sequence was introduced into the nucleotide sequence of 208 to 214 of *oriC* in M13 RE85 (Bramhill *et al*., 1988) using a site directed mutagenesis system (Amersham Corp.). *Bgl*II-*Xho*I fragments (370 bp) isolated from the mutated M13RE85 RF DNA were inserted into the *Bam*HI/*Xho*I site of pBluescript(+) to yield pOriB.

Nitrocellulose filter binding assay

*Bam*HI-cleaved DNA fragments (120 bp) which was isolated from pOriB were dephosphorylated with calf thymus intestinal alkaline phosphatase, and 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Labeled fragments were purified over Sephadex G-50 in TE buffer, when necessary. The reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2 mM DTT, 50 mM KCl, 10% glycerol, 1.5 fmol of ^{32}P-labeled fragments, and the indicated amounts of protein. Incubations were performed at 37℃ for 10 min, then the mixtures were immediately passed through a Millipore HAWP 01300 filter (pore size 0.45 μm, activated by boiling for 5 min). Radioactivities on the filter were counted using a Liquid Scintillation Counter (Pharmacia LKB). One unit of binding activity for the 21 kDa protein were defined as the amount needed to retain 50% of the input DNA fragments.

Purification of the 21 kDa protein

E. coli W3110 was grown to late-log phase, harvested, and frozen as described (Hwang *et al*., 1990). Thawed cell paste (350 g) was diluted to 0.7 l with 25 mM HEPES-KOH (pH 7.6), 1 mM EDTA, and 2 mM DTT, followed by addition of 3 M KCl to 0.25 M, 1 M spermidine-HCl to 20 mM, and 10 mg/ml lysozyme to 0.4 mg/ml. The suspension was incubated at 37℃ for 5 min and then frozen in liquid nitrogen. Cells, thawed at 8℃, were lysed and centrifuged at 40,000 rpm in Ti 45 rotor at 4℃ for 30 min (L70 Ultracentrifuge, Beckman). The supernatant (fraction I) was precipitated by addition of ammonium sulfate (0.27∼0.37 g/ml). The precipitate was solubilized in buffer A, cleared by centrifugation (fraction II), dialyzed against a conductivity of 0.2 M KCl, and loaded on a phosphocellulose column (bed volume, 80 ml) equilibrated with 0.2 M KCl in buffer A. The column was washed with 200 ml of 0.2 M KCl in buffer A and the protein was eluted with a linear gradient of 400ml of 0.2 to 0.6 M KCl in buffer A. Fractions with DNA binding activity, eluted in a peak between 0.32 to 0.4 M KCl, were pooled (fraction III), dialyzed against 50 mM KCl in buffer A, and applied to a fast-flow S column (bed volume, 5 ml), equilibrated with 50 mM KCl in buffer A, and washed with 50 mM KCl in buffer A. The activity was eluted with a linear gradient of 50 ml of 50 to 1000 mM KCl in buffer A. The activity was eluted at about 0.5 M KCl. The active fractions were pooled (fraction IV) and dialyzed against 50 mM KCl in buffer A until the conductivity was the same as the dialysis buffer. Fraction IV was then applied to a single-stranded DNA cellulose column (bed volume, 4 ml) equilibrated with 50 mM KCl in buffer A, and washed with 50 mM KCl in buffer A. DNA binding activity was eluted with a linear gradient of 30 ml of 50 to 1000 mM KCl in buffer A. The activity was eluted at about 470 mM KCl. The active fractions were pooled (fraction V) and dialyzed against 50 mM KCl in buffer A until the conductivity was the same as the dialysis buffer, applied to a heparin-agarose column (bed volume, 2 ml) equilibrated with 50 mM KCl in buffer A, then washed with 50 mM KCl in buffer A. DNA binding activity was eluted with a linear gradient of 30 ml of 50 to 700 mM KCl in buffer A. The activity was eluted at about 400 mM KCl (fraction VI). The active fractions were pooled and dialyzed against 50 mM KCl in buffer A until the conductivity was the same as the dialysis buffer, applied to Mono S column

(FPLC) equilibrated in 50 mM KCl in buffer A, and washed with 50 mM KCl in buffer A. DNA binding activity was eluted with a linear gradient of 30 ml of 50~500 mM KCl in buffer A. The activity was eluted at about 250 mM KCl (fraction VII).

Preparation of polyclonal antibody

Polyclonal antibody against the purified 21 kDa protein was prepared by immunizing a Male Sprague-dawley rat. 50 μg of protein mixed with complete Freund's adjuvant was subperitonealy injected, and a booster injection was administered with 50 μg of protein suspended in incomplete Freund's adjuvant three weeks after the primary injection. Antiserum was collected 7 days after the booster injection.

Sucrose gradient sedimentation

Bacteriophge λ DNA and plasmid pBluescript were labeled with *Hha*I methylase and [^{3}H] S-adenosyl-methionine. Samples were prepared as for filter binding assays except that tritium labeled DNA was used. Samples were placed on 4.3 ml of a 5 to 20% sucrose gradient and centrifuged at 40,000 rpm in Beckman SW60 rotor for 2 h at 4℃. Separation of DNA was detected by counting radioactivity after fractionation into 30 to 35 tubes of a similar volume.

Results and Discussion

Purification of the 21 kDa protein

In the course of purification of possible regulatory factors for *E. coli* chromosomal DNA replication, we encountered a nonspecific DNA binding protein, very abundant and basic, with a molecular weight of 21 kDa on SDS-polyacrylamide gel electrophoresis. This molecular weight is distinct from the known nucleoid-forming proteins in *E. coli* (Schimid *et al.*, 1990). Purification of the 21kDa protein was performed using nitrocellulose filter binding and gel mobility-shift assays. Phosphocellulose, fast-flow S, single-stranded DNA cellulose, heparin agarose, and finally mono S chromatographic columns were used (Table 1). The ammonium sulfate precipitate of soluble lysate (fraction II) was dialyzed and applied to a phosphocellulose column. The DNA binding activity was eluted as a peak at 0.32 to 0.4 M KCl. The fractions were pooled and applied onto fast-flow S column. DNA binding activity was eluted at 0.2 M to 0.3 M KCl. The active fractions from the fast flow S column was dialyzed and applied to a single-stranded DNA cellulose column. Active material was eluted at 0.4 M KCl. The most effective step was heparin-agarose chromatography which efficiently separated the 21 kDa protein from other ptoteins. By mono S chromatography, the 21 kDa protein was puri-

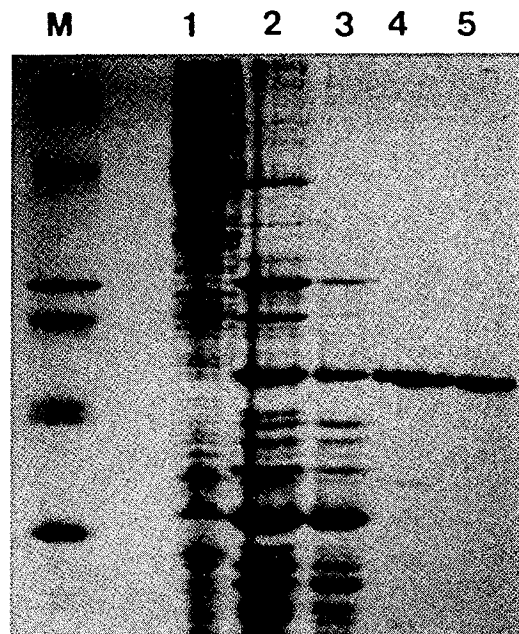


Fig. 1. Purification of the 21 kDa protein. Fractions were analyzed on a 15% SDS-polyacrylamide gel and visualized by coomassie brilliant blue staining. Lane 1 contained 1.5 μl of fraction II. Lanes 2 to 5 contained 70 U of fraction III, V, VI and VII, respectively. Molecular weight markers (M) were albumin bovine, 66 kDa; albumin egg, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen (bovine pancreas), 24 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa.

Table 1. Purification of 21 kDa protein.

Fraction	Volume (ml)	Protein (mg)	Activity (U/10^{-3})	Specific activity (U/10^{-3}/mg)	Step-yield (%)	Purification (fold)
I. Lysate	109	1537	–	–	–	–
II. Ammonium sulfate	12	792	–	–	–	–
III. Phosphocellulose	124	17.4	10.3	0.59	100	1
IV. Fast flow S	7	7	23.8	3.4	231	5.7
V. Single-stranded DNA cellulose	4	3.4	16	4.7	67.2	7.9
VI. Heparin-agarose	1.5	1.13	12	10.7	75	18
VII. Mono S	1	0.42	11.5	27.4	96	46

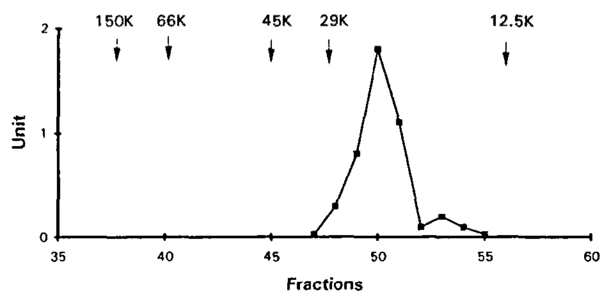


Fig. 2. Gel filtration of the 21 kDa protein The 21 kDa protein (fraction VII) in 0.2 ml was loaded onto superdex-75 gel permeation column of FPLC equilibrated with buffer A containing 0.1 M KCl and eluted at a flow rate of 0.5 ml/min. Fractions (0.25 ml) were collected and the DNA binding activities in 3 μl were detected in nitrocellulose filter binding assay. Molecular weight markers were alcohol dehydrogenase, 150 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; cytochrome C, 12.4 kDa.

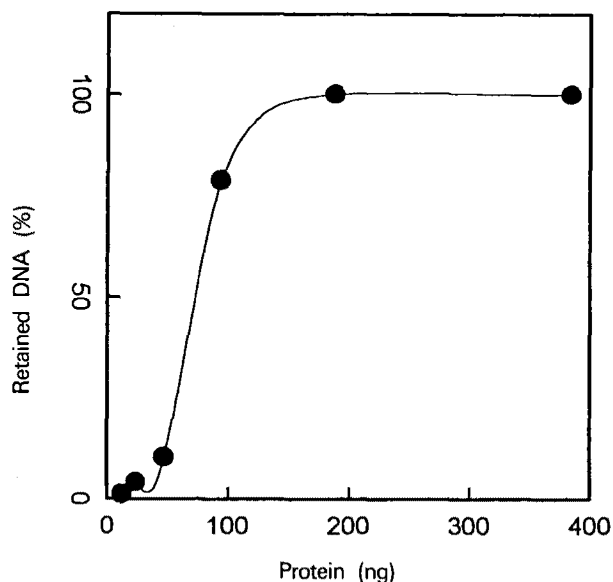
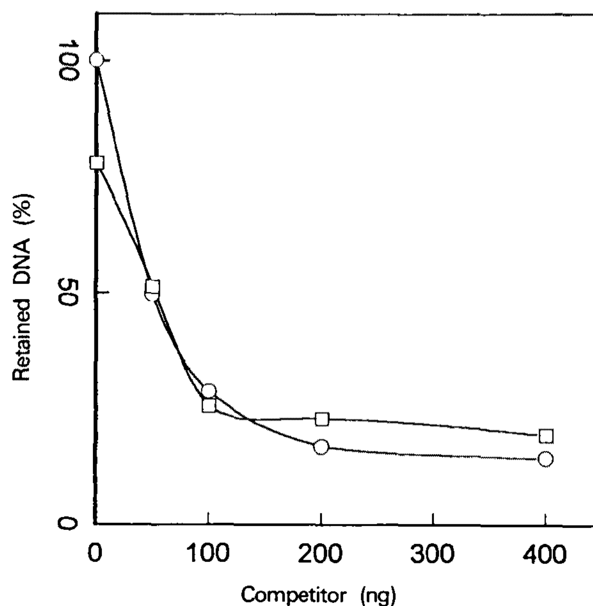


Fig. 3. Binding of the 21 kDa protein to DNA The indicated amounts of 21 kDa protein were titrated in nitrocellulose filter binding assay.

fied to near homogeniety. The molecular weight of 21 kDa was determined on SDS-polyacrylamide gel electrophoresis (Fig. 1). Upon gel filtration of Superdex-75 column of FPLC, the molecular weight of the native protein was determined as 24 kDa by comparison with molecular weight markers (Fig. 2). Taken together with 21 kDa on SDS-polyacrylamide gel electrophoresis, 21 kDa protein behaves as a monomer in *E. coli*. Overall purification of the 21 kDa protein from fraction III was about 46 fold (Table 1). The activities in the crude lysate (fraction I) and the ammonium sulfate fraction (fraction II) could not be reliably assayed. Neither nuclease nor topoisomerase activity was detected in the final fraction. The isoelectric point of 21 kDa protein



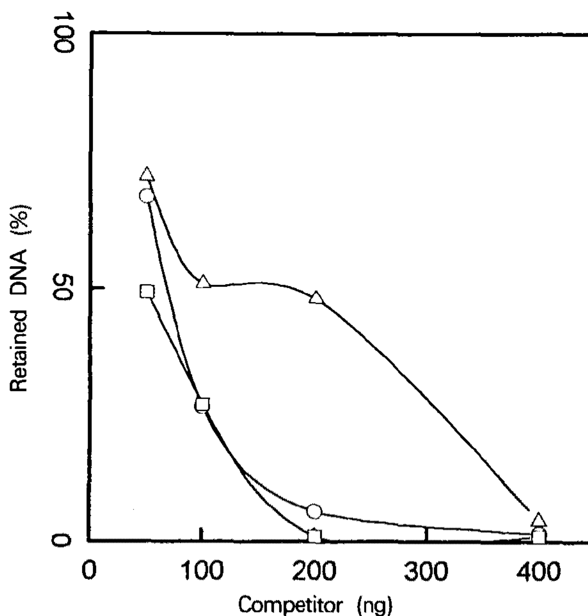


Fig. 4. Nonspecific binding of the 21kDa protein to DNA 200 ng of 21kDa protein and the indicated amount of nucleic acid were added to the nitrocellulose filter binding assay. (A) ○, pOriB; and □, pBluescript. (B) ○, calf thymus DNA; □, M13 single-stranded DNA; and △, total RNA from mouse thymus.

was about 9.5 (Data not shown).

Nonspecific binding of the 21 kDa protein to DNA, and condensation of bacteriophage λ DNA and plasmid DNA

Binding of the 21 kDa protein to DNA was examined on nitrocellulose filter binding assay (Fig. 3). A constant amount of ^{32}P-labeled 120 bp *Bam*HI fragment isolated from a plasmid pOriB was mixed with increasing amounts of the 21 kDa protein, then DNA-

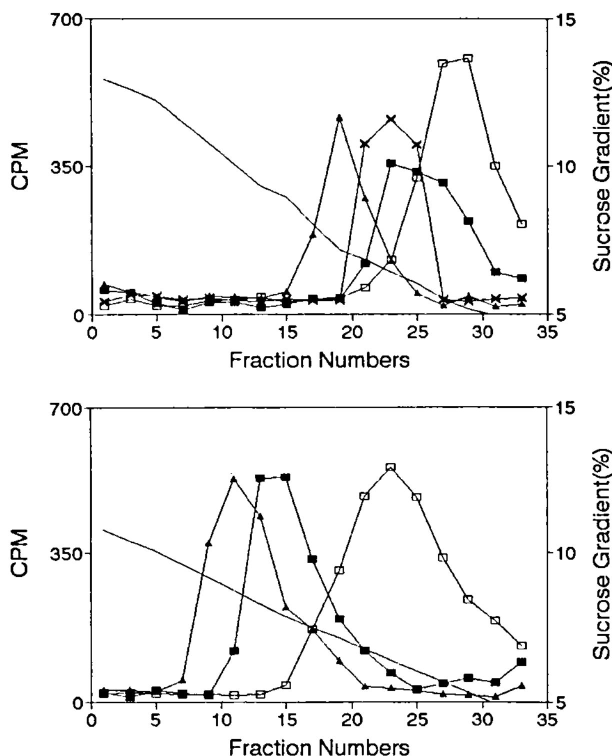


Fig. 5. Condensation of DNA by the 21 kDa protein. 100 ng of ^{3}H-labeled plasmid pOriB supercoiled DNA (12000 cpm) (A) or 200 ng of ^{3}H-labeled bacteriophage λ linear DNA (about 7000 cpm) (B) was incubated without (□) or with 2 μg (■) and 4 μg (△) of 21 kDa protein in the nitrocellulose filter binding reaction. 2 μg of 21 kD protein (x) was incubated without DNA. After 10 min incubation at 37℃, the samples were diluted with 100 μl of 5% sucrose in the same buffer. The samples (120 μl) were layered on the top of linear 5~20% sucrose gradient in the same buffer. Sedimentation was performed at 40,000 rpm at 4℃ for 2 h in a SW60 rotor with L-70 ultracentrifuge, Beckman. The samples were collected from the bottom of tube. DNAs were detected by counting radioactivity and the density of sucrose was determined by refractometer. 21 kDa protein was detected by nitrocellulose filter binding assay.

protein complex retained on nitrocellulose filter was detected by using liquid scintillation counter. The sigmoidal appearance of the binding may indicate a cooperativity in the binding process.

Nonspecific binding of the 21 kDa protein to DNA was determined by competition of various DNAs or RNA with the ^{32}P-labeled 120 bp *Bam*HI fragment in nitrocellulose filter binding assay (Fig. 4). Plasmid pOriB contains the 120 bp *Bam*HI fragment, which fragment was used for probe in nitrocellulose filter binding assay, inserted in a vector pBluescript. Plasmid pOriB and pBluescript equally competed to the 120 bp fragment, indicating that the binding of 21 kDa protein is not specific to the 120 bp *Bam*HI fragment (Fig. 4A). The competition rate of calf thymus double-stranded DNA was similar to that of M13 single-stranded DNA (Fig. 4B). However, RNA did not compete efficiently. Thus, the 21 kDa protein nonspecifically binds to double-stranded and single-stranded DNA with similar affinities.

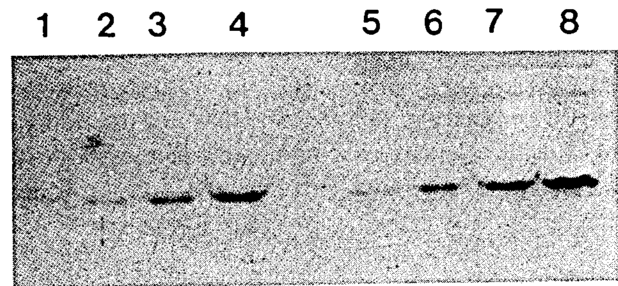


Fig. 6. Cellular abundance of the 21 kDa protein *E. coli* W3110, was grown in LB medium at 37℃. At the OD. of 0.5 at 600 nm, cells were collected by centrifugation at 8,000 rpm in a Centrikon 8.24 rotor for 10 min, washed with cold TE buffer, collected by centrifugation, and resuspended in TE buffer to an OD of 4.0. The resuspended cells (1, 2, 4, 8 μl) were mixed with SDS-polyacrylamide gel electrophoresis sample buffer (10 mM Tris-HCl (pH 6.8), 1% SDS, 0.1 M dithiothreitol, 10% glycerol, and 0.005% bromophenol blue) to 20 μl and boiled for 2 min followed by electrophoresis through a 15% SDS-polyacrylamide gel with known amount of 21 kDa proteins as standards. The separated proteins were transferred to nitrocellulose membrane and analyzed by western blot. 21 kDa proteins were visualized with rat anti-21 kDa protein, horseradish peroxidase-conjugated goat anti-rat IgG (Sigma). The contents of the 21 kDa proteins were determined by comparison with the known amounts of the proteins using Ultrascan tm XL densitometer (Pharmacia LKB). The cellular abundance of the proteins was calculated assuming that an OD value of 1 at 600 nm represents 8×10^{8} cells/ml (Sambrook *et al.*, 1989). Lanes 1 to 4 contained 25, 50, 100 and 200 ng of purified 21 kDa protein, respectively. Lanes 5 to 8 contained 1, 2, 4 and 8 ml of the OD of 4 at 600 nm, respectively.

^{3}H-labeled supercoiled plasmid DNA (Fig. 5A) or linear bacteriophage λ DNA (Fig. 5B) was incubated with the 21 kDa protein, and subjected to sedimentation through a 5 to 20% sucrose gradient (Fig. 5). Compared to DNA alone and the 21 kDa protein alone, an increasing amount of 21 kDa induced sedimentation of DNA with a higher density. The increase in the density of DNA-21 kDa protein complex indicates the condensation of DNA by association with 21 kDa protein.

Abundance of the 21 kDa protein

E. coli W3110 was grown to late-log phase and collected. The number of 21 kDa protein molecules in a cell was determined by western-blot analysis using antiserum raised against the 21 kDa protein in a rat (Fig. 6). Compared to the known amount of purified 21 kDa protein, the amount of 21 kDa protein in a known number of cells was quantified and converted to the number of molecules in a cell. Approximately 40,000 molecules of the 21 kDa protein were estimated to exist in a cell of *E. coli*, indicating that 21 kDa protein is as abundant as other histone-like proteins in *E. coli* (Schimid *et al.*, 1990).

The 21 kDa protein resembles other histone-like proteins in *E. coli*: (i) nonspecific binding to DNA; (ii) cellular abundance, estimated as 40,000 monomers per cell; (iii) basicity; and (iv) condensation of DNA. Therefore, the 21 kDa protein may contribute to the formation of highly organized structure of *E. coli* chromosome.

Acknowledgement

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