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PURIFICATION OF B. SUBTILIS DNA-BINDING PROTEIN RELATED TO E. COLI HU

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SUMMARY: From vegetative and sporulating chromosomes of B. subtilis, dominant DNA-binding(DB) proteins were purified by KCl-$(NH_4)_2SO_4$ salting out, DEAE-cellulose and DNA-cellulose columns. The DB proteins purified from vegetative and sporulating cells were revealed to have similar mobilities and a unique band by electrophoreses on SDS-urea and acid-urea-Triton gels, respectively. Their amino acid compositions were completely identical. These results indicate that the protein species is generally presented associated to DNA through the growth and sporulation of the bacteria.

INTRODUCTION: It has been reported that isolation and characterization of low molecular weight DNA-binding protein, HU from Escherichia coli(1) and blue-green algae(2). The HU protein displays a resemblance to eukaryotic histone, such as H2B by its amino acid composition, some characteristics(1,3) and forming nucleosome-like structure with circular double-stranded DNA(4). On the other hand, a dominant polypeptide P^{36} with a molecular weight of 36,000 daltons, which remained associated with the vegetative folded chromosomes of Bacillus subtilis after sarcosyl treatment(5), was recently identified as flagellin present as membrane-associated flagella(6). Recently, we reported existence of a dominant protein, which could bind to DNA in the vegetative chromosomes of Bacillus subtilis and had a molecular weight of 6,000 daltons(Nakayama,T. et al., submitted to J. Bacteriol.). For use of the protein as a tool for the in vitro investigation of the ternary structure of folded chromosomes, the present study was started to purify large amounts of the DNA-binding (DB) proteins from the vegetative and sporulating cells.

METHODS: Bacterial strain and Media: B. subtilis 168 wild type was grown at 37°C in a modified Schaeffer medium(2XSG). To obtain maximum synchronized cells, cells were transferred at log phase to a fresh 2XSG

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medium(7). The cells were harvested at late log stage, and T_2, T_4 and T_7 during the sporulation process(T_n is times in hours(n) after T_0, the end of the exponential growth), and then washed with buffer to remove extracellular protease as described previously(8).

Preparation of folded chromosomes from vegetative and sporulating cells. Washed cells(5 g wet weight) were suspended in 100 ml of solution A[10 mM Tris-HCl(pH 8.2), 10 mM NaN_3, 0.1 M NaCl, 10 %(w/v) sucrose and 2 mM phenylmethylsulfonyl fluoride(PMSF)] and 2 ml of solution B[0.1 M Tris-HCl(pH 8.2), 50 mM ethylenediamine tetraacetic acid(EDTA) and 30 mg/ml lysozyme] was added. The lysozyme treatment of the cell suspension was carried out at 0°C for 20 min. After addition of 100 ml of solution C[1 %(w/v) Brij-58, 0.4 %(w/v) sodium deoxycholate, 10 mM EDTA, 2 mM PMSF and 12 mM spermidine], the detergent treatment of the mixture was carried out at 23°C for 20 min for vegetative cells and for 60 min for sporulating cells, and 4.3 M NaCl solution was added to give a final concentration of 0.2 M at 0°C. The lysate was layered on 10 % sucrose in solution A containing 12 mM spermidine, and centrifuged at 24,000 xg for 30 min. The pellets obtained were collected and used as folded chromosomes.

DEAE-cellulose column chromatography. The chromosomes from 38 g (log phase), 24 g(T_2), 20 g(T_4) and 20 g(T_7) wet weight cells were respectively combined. To the pooled chromosome preparations were added KCl and $(NH_4)_2SO_4$ to final concentrations of 1.5 M and 60 % saturation, respectively, and the suspensions were stirred at 0°C for 2.5 h. The pellets obtained by centrifugation at 24,000 xg for 30 min, which contained solid KCl and $(NH_4)_2SO_4$, were suspended in 80 ml of TEG buffer[10 mM Tris-HCl(pH 7.6), 0.1 mM EDTA, 5 % glycerin and 0.5 mM PMSF] and then the suspensions were dialyzed against six changes of 30-fold excess of the buffer for 48 h. The dialyzed fractions were applied to DEAE-cellulose columns which had been equilibrated with TEG buffer and the columns were washed with the buffer. Linear gradient elutions were carried out at a flow rate of 20 ml/h using two chambers which contained 180 ml each of the starting buffer and the buffer containing 0.8 M KCl, respectively, and followed by stepwise elutions with 70 ml of 1.5 M KCl in the buffer. The 3.6-ml fractions were collected and absorbance at 280 nm and 260 nm was measured, respectively.

DNA-cellulose column chromatography. The DEAE-cellulose active fractions were applied to DNA-cellulose columns at a flow rate of 6 ml/h. After washing with 50 ml of the buffer until absorbance at 280 nm and 260 nm was less than 0.01 unit, respectively, linear gradient elutions were carried out from 0 M to 0.5 M at a flow rate of 10 ml/h using two chambers which contained 90 ml each of the buffer, and washings with 30 ml of 1.5 M KCl in the buffer were carried out. The 2.6-ml fractions were collected and absorbance at 280 nm and 260 nm was measured.

SDS-urea polyacrylamide gel electrophoresis. The method of Wu and Bruening(9) was used at 15 % acrylamide.

Acid-urea-Triton polyacrylamide gel electrophoresis. The method of Hamana and Iwai(10) was used at 15 % acrylamide, 2.2 % Triton X-100 and 6.25 M urea.

Amino acid analysis. The purified proteins were dialyzed against eight changes of 100-fold excess distilled water and lyophilized. The dried materials were hydrolyzed with 6 N HCl at 110°C for 24 h and 48 h. Amino acid analysis was carried out with a Hitachi 835 Amino Acid Analyzer. Tryptophan was determined after hydrolysis with 4 N methanesulfonic acid at 110°C for 24 h.

RESULTS: DEAE-cellulose column chromatography. The elution profiles of the chromosomal components of vegetative, T_2, T_4 and T_7 cells are

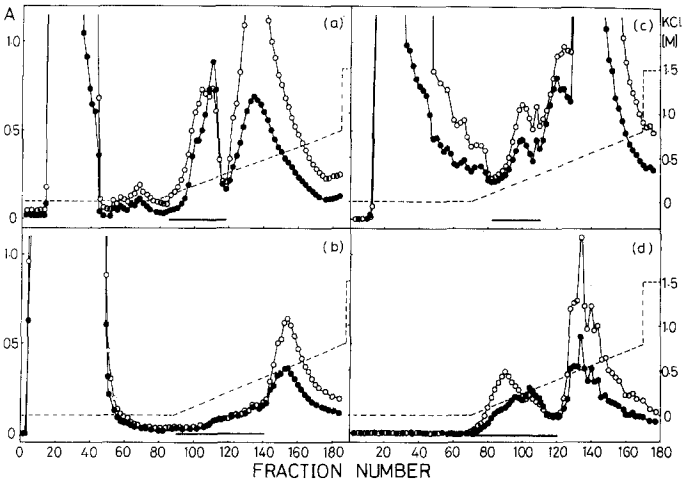

Fig.1. Elution patterns from DEAE-cellulose columns of the components of vegetative and sporulating chromosomes. The KCl-$(NH_4)_2SO_4$ precipitated fractions of vegetative(a), T_2(b), T_4(c) and T_7(d) cells were respectively applied to DEAE-cellulose columns(1.8 x 20 cm) and linear gradient elutions of KCl were carried out as shown in METHODS. Absorbance at 280 nm and 260 nm is represented by closed and open circles and KCl concentration by the broken lines.

presented in Fig.1(a), (b), (c) and (d), respectively. Although the elution patterns were different from each other, DNA-binding activities were eluted in all cases at 0.1 M to 0.3 M KCl as reported previously. The active fractions indicated by hatched bars were respectively pooled and dialyzed against TEG buffer.

DNA-cellulose column chromatography. The dialyzed DEAE-cellulose active fractions were respectively applied to calf-thymus DNA-cellulose columns. As shown in Fig.2(a), (b), (c) and (d), the DB proteins of vegetative, and T_2, T_4 and T_7 cells were eluted at about 0.1 M KCl from the affinity columns, respectively.

Polyacrylamide gel electrophoresis. SDS-urea and acid-urea-Triton X-100 gel electrophoreses of the purified proteins were carried out at 15 % acrylamide, respectively and the electrophoretic patterns are shown in Fig.3. As shown in Fig.3(A), the proteins purified from four forms of the folded chromosome at log phase, and T_2, T_4 and T_7 had the dominant

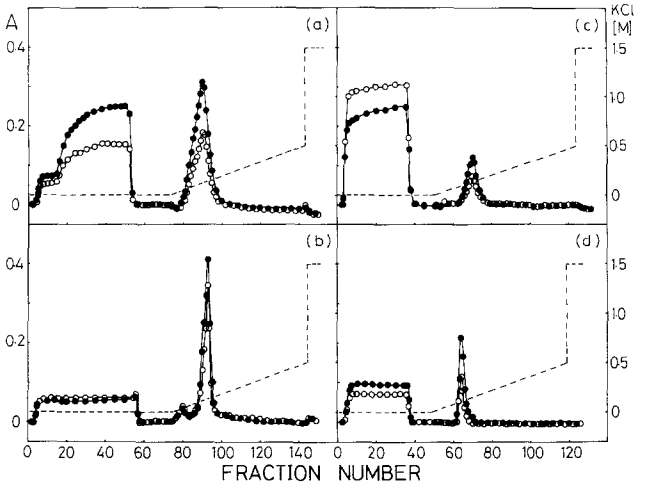

Fig.2. Elution patterns from DNA-cellulose columns of the DEAE-cellulose active fractions. The DEAE-cellulose active fractions of vegetative(a), T_2(b), T_4(c) and T_7(d) cells were respectively applied to DNA-cellulose columns(0.9 x 12 cm) and linear gradient elutions of KCl were carried out as shown in METHODS. Absorbance at 280 nm and 260 nm is represented by closed and open circles and KCl concentration by the broken lines.

protein bands and their mobilities were completely identical. The molecular weight was 6,000 daltons as reported previously(Nakayama,T. et al., submitted to J. Bacteriol.). It was discussed that E. coli HU protein had two subspecies, α and β, were revealed by electrophoresis on acid-urea-Triton gels under the conditions that separated histone subspecies, although they were presented as a unique band in SDS-urea or

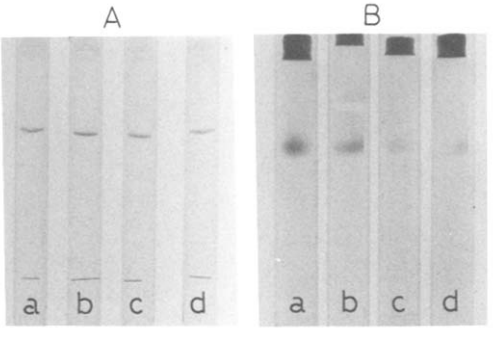

Fig.3. Polyacrylamide gel electrophoretic patterns. SDS-urea gel electrophoresis(A) and acid-urea-Triton gel electrophoresis(B) were carried out at 15 % acrylamide as shown in METHODS.
a, log phase; b, T_2; c, T_4; d, T_7.

in acid-urea gels(4). Therefore, B. subtilis DB proteins were also examined by electrophoresis on acid-urea-Triton gels. As shown in Fig.3(B), the DB proteins from vegetative and sporulating cells had dominant bands with identical mobility.

Amino acid composition. Amino acid composition of the DB proteins is shown in Table I. The number of proline residues could not be determined since an unknown material contaminated the proline peak when amino acid analysis was carried out. Amino acid composition of the DB proteins from four stages, log phase, T_2, T_4 and T_7 during the sporulation process was completely identical. About a half of the residues in the combined total of Asx and Glx would be amide forms because the proteins can be thought as neutral or slightly basic from the elution patterns from DEAE-cellulose columns and electrophoretic patterns, and about 15 ammonium residues were recovered as presented in the proteins when amino acid analysis was carried out(data not shown).

DISCUSSION: The vegetative DB protein was tentatively called as polypeptide P^6 in the previous paper since it had a molecular weight of 6,000 daltons when analyzed on SDS-urea polyacrylamide gel(Nakayama,T. et al., submitted to J. Bacteriol.). The result was confirmed in this paper. From the number of amino acid residues, a minimum molecular weight of 9,000 can be calculated for the DB protein, a value somewhat different from that of 6,000 obtained from SDS-urea polyacrylamide gel electrophoretic measurement. A similar discrepancy between the molecular weight determinations deduced from the analysis and from electrophoretic mobility study has already been reported in the case of egg white lysozyme, which on gel, behaves generally as less than 9,000-dalton protein(5 and our unpublished data), whereas the known molecular weight is 14,500. These phenomena probably due to the fact that B. subtilis DB protein and lysozyme are arginine-rich proteins, respectively.

Table I. Amino Acid Composition*

	DB protein								HU protein		Calf-thymus Histone**			
	Veg		T_2		T_4		T_7		*E.coli*(1)	Blue-green algae(2)	H2A	H2B	H3	H4
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h						
Asx	18.4	19.1	18.4	18.5	18.5	18.1	18.1	18.6	8.9	8.0	6.2	5.0	3.7	4.9
Thr	6.0	6.0	6.1	5.9	6.1	6.1	6.1	6.0	6.8	6.0	3.9	6.4	7.4	6.8
Ser	9.1	7.8	9.0	8.2	8.7	8.9	8.9	8.1	4.5	8.1	3.1	10.4	3.7	1.9
Glx	5.4	5.7	5.1	5.1	4.9	5.3	5.3	5.1	9.5	8.1	9.3	8.7	11.1	5.8
Pro									2.6	4.5	3.9	4.9	4.4	1.0
Gly	11.5	11.3	11.8	11.8	11.4	11.7	11.7	11.4	7.2	7.5	10.9	5.9	5.2	16.5
Ala	11.4	11.1	10.9	10.9	10.8	10.8	10.8	11.0	18.5	11.5	13.2	10.8	13.3	6.8
Cys	0.3	0.4	0.4	0.5	0.5	0.4	0.4	0.4	0	0	0	0	1.5	0
Val	5.1	5.6	5.2	5.4	5.2	5.2	5.2	5.4	7.4	10.0	6.2	7.5	4.4	8.7
Met	1.3	0.9	1.2	1.1	1.1	1.1	1.1	1.0	1.2	2.5	0	1.5	1.5	1.0
Ile	4.9	5.3	4.3	5.2	5.0	5.0	5.0	5.2	6.6	3.3	4.7	5.1	5.2	5.8
Leu	7.3	7.5	7.5	7.4	7.2	7.4	7.4	7.5	6.9	3.4	12.4	4.9	8.9	7.8
Tyr	2.2	2.5	2.2	2.3	2.3	2.3	2.3	2.4	0	0	2.3	4.0	2.2	3.9
Phe	2.1	2.6	2.4	2.6	2.5	2.6	2.6	2.7	3.3	4.0	0.8	1.6	3.0	1.9
Lys	5.7	5.3	5.1	5.1	5.2	5.1	5.1	5.1	11.1	14.0	10.9	14.1	9.6	10.7
His	0.9	1.0	0.9	0.9	0.9	0.8	0.8	0.8	0.9	1.5	3.1	2.3	1.5	1.9
Trp	0.0		0.0		0.0		0.0		0	0	0	0	0	0
Arg	9.4	8.8	9.3	9.1	9.5	9.2	9.2	9.2	4.7	5.4	9.3	6.9	13.3	13.6

* indicated by mol %.

** from " Atlas of protein sequence and structure ", Vol. 5, Suppl. 2(1976), Dayhoff, M. O. National Biomedical Foundation, Washington, D.C.

Two explanations for the results obtained in this paper can be considered: one is that the DB protein species from the vegetative and sporulating cells is completely identical. The other possibility is that the protein contains at least two subspecies like to E. coli HU protein(4,11) and molar ratio of the subspecies is constant during the growth and sporulation by T_7, but they could not be separated from each other by electrophoreses on SDS-urea and acid-urea-Triton gels. It needs further studies to determine whether the DB protein is a unique species or has some subspecies like to E. coli HU. Comparison of amino acid composition indicates that the composition variability is found between the three proteins, E. coli HU, blue-green algae HU and B. subtilis DB as shown in Table I. It was discussed that E. coli HU protein resembled the eukaryotic histone H2B in its amino acid composition(1) and the variability between E. coli and blue-green algae HU was as low as that found among eukaryotic histones, H2A and H2B(2). B. subtilis DB protein, whose functional property was almost similar to that of E. coli HU protein (Nakayama,T. et al., manuscript in preparation), resembled eukaryotic histones H3 or H4 in its amino acid composition as shown in Table I.

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