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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF VARIANTS OF CHROMOSOMAL PROTEINS FROM PROKARYOTES

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SUMMARY

The separation of variants of chromosomal proteins exhibiting closely related amino acid compositions has been achieved using weak cation-exchange or reversed-phase high-performance liquid chromatography. The purity of the isolated proteins has been ascertained by polyacrylamide gel electrophoresis and in several cases by micro-sequencing.

INTRODUCTION

In the nucleoid of prokaryotes, acid-soluble proteins of molecular weights (M_r) ranging from 7000 to 16 000 daltons were shown to be involved in DNA condensation[1]. In several cases, these proteins are heterodimers constituted of polypeptides of closely related amino acid sequences which are referred to as variants. As an example, the number of sequence differences between variants of the DNA-binding protein II ($M_r \approx 10\,000$ daltons) ranges from only one residue in *Thermoplasma acidophilum*[2] to 27 residues in *Escherichia coli*[3]. Moreover, most of the observed changes are conservative. Highly resolving chromatographic methods are therefore required to separate variants of chromosomal proteins with a view to their characterization by amino acid composition and sequence analyses. Ion-exchange and reversed-phase high-performance liquid chromatography (HPLC) have been shown to be suitable for resolving complex mixtures of basic proteins differing in molecular mass, charge and hydrophobicity such as ribosomal proteins[4–8]. This paper deals with the application of HPLC to the separation of variants of eubacterial and archaebacterial chromosomal proteins.

MATERIALS AND METHODS

HPLC-grade acetonitrile was obtained from Carlo Erba and sequanal grade trifluoroacetic acid (TFA) from Pierce. Water for HPLC was provided by an Elgastat UHQ water apparatus. All other chemicals were proanalysis grade. Saline buffers were filtered through a 0.22-μm Millipore filter.

HPLC separations were performed with Beckman equipment consisting of

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a pair of Model 126 Altex pumps and a Model 167 variable wavelength detector controlled by a Tandon computer.

Preparation of chromosomal proteins

Proteins were prepared by affinity chromatography on a DNA-cellulose column as described in ref. 9 for eubacterial DNA-binding protein II and as in ref. 10 for the protein MC1 from the archaebacterium *Methanothrix soehngenii*. The eubacterial proteins eluted from the DNA-cellulose column were desalted on a Sephadex G-25 column equilibrated and eluted in 10 m*M* hydrochloric acid, and freeze-dried. The protein MC1 was dialysed against water and concentrated in a SpeedVac apparatus.

For cation-exchange chromatography, the proteins were dissolved in 200 μl of 10 m*M* sodium acetate buffer, pH 5.6 containing 8 *M* urea. The variants were separated on a TSK IEX CM-2SW column (250 mm × 4.6 mm) (Beckman) equilibrated in 10 m*M* sodium acetate buffer (pH 5.6) containing 6 *M* urea and eluted with a linear gradient of NaCl in the same buffer. Proteins were then desalted on a reversed-phase column as described in ref. 11.

Reversed-phase chromatography was performed on a Ultrapore C_8 column from Beckman (particle size 5 μm, pore size 300 Å, column size 250 mm × 4.6 mm). The proteins dissolved in 10 m*M* hydrochloric acid were loaded on the column equilibrated in 0.05% TFA in water and eluted with a gradient of acetonitrile in 0.05% TFA.

Analytical gel electrophoreses and amino acid analyses were performed as described in ref. 9.

RESULTS AND DISCUSSION

Cation-exchange HPLC

The separation by ion-exchange HPLC of the two variants 1 and 2 of the DNA-binding protein II from *E. coli* is shown in Fig. 1. In preliminary experiments performed at pH 7.0 with 10 m*M* sodium phosphate, the two variants were coeluted in a single fraction, whereas at pH 6.0 with 10 m*M* sodium phosphate or sodium acetate they were partially resolved (data not shown). The use of 10 m*M* sodium acetate at pH 5.6 gave the best resolution. The separation of these variants appears mainly dependent on the pH and, to a lesser extent, on the nature of the salt used as the buffer. Sequence analyses[3] indicate that the only difference in net charge between the two variants of this protein can be brought by the presence in variant 2 of one histidine residue. Since the pK' of the imidazole group is equal to 6.0, the protonation of the histidine residue probably plays an important role in the retention time of this protein. On the other hand, the presence of 6 *M* urea in the buffer is necessary to dissociate the two polypeptide chains which tightly bind together to form a stable dimer corresponding to the functional state of the protein[12].

The chromatogram obtained with the *Azotobacter vinelandii* DNA-binding protein II using the sodium acetate buffer at pH 5.6 is presented in Fig. 2. Variants 1 and 2 were eluted in fractions 1 and 2 respectively. By contrast with *E. coli* variants which are in equimolar amounts, the variant 1 is about twice as abundant as the variant 2 in *A. vinelandii*. The amino acid compositions of variants 1 and 2 present only slight differences except in the amounts of threonine and glutamic acid (Table I). The variants display the same total number of lysine plus arginine and differ by the presence of two histidine residues in variant 2.

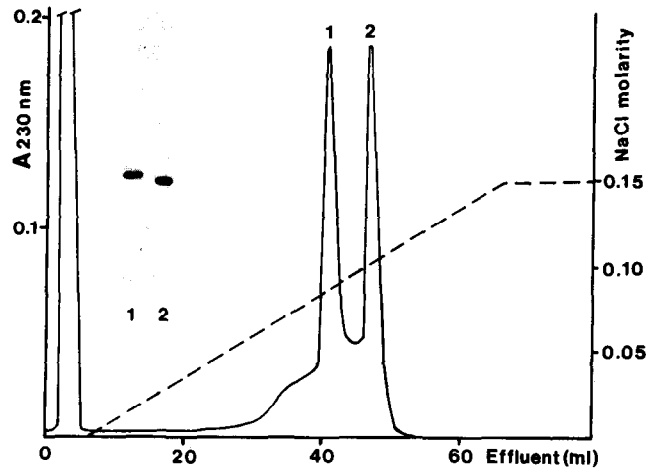

Fig. 1. Separation of the variants of the *E. coli* DNA-binding protein II (2 mg injected) on a TSK IEX CM-2SW column (250 mm × 4.6 mm). ---- = Linear gradient of sodium chloride in 10 m*M* sodium acetate pH 6 containing 6 *M* urea; flow-rate 1 ml/min. Fractions of 1 ml were collected. Insert: polyacrylamide gel electrophoresis (PAGE) of variants 1 (lane 1) and 2 (lane 2) in 0.9 *M* acetic acid containing 6.25 *M* urea and 0.38% Triton X-100. Samples (3 μg) dissolved in 10 m*M* hydrochloric acid, 8 *M* urea, 0.5 *M* 2-mercaptoethanol were run at 22 mA for 3 h at room temperature in the gel containing 17% acrylamide. The gel was stained and destained according to ref. 14.

Reversed-phase HPLC

The DNA-binding protein II from *Synechococcus* PCC 7002 was desorbed from the DNA-cellulose column together with a contaminant protein of $M_r \approx 16000$ daltons. Using a C_8 Ultrapore column eluted with a linear gradient of acetonitrile in 0.05% TFA, the contaminant protein was eluted in fraction 1 whereas the DNA-

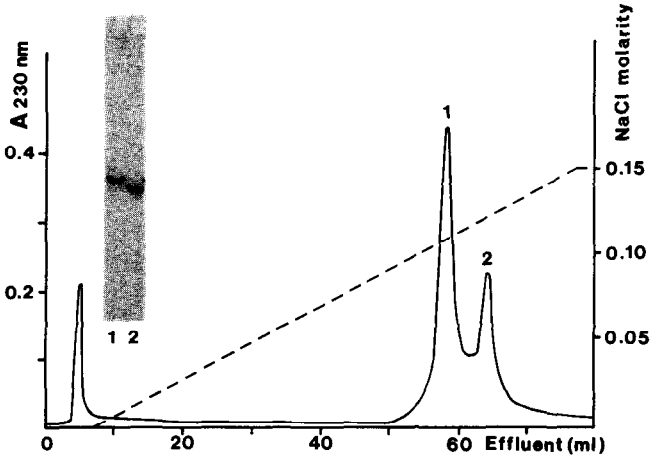

Fig. 2. Separation of the variants of the *A. vinelandii* DNA-binding protein II (2.5 mg injected) on a TSK IEX CM-2SW column (250 mm × 4.6 mm). The protein was chromatographed as indicated in Fig. 1. Insert: PAGE of variants 1 (lane 1) and 2 (lane 2) in 0.9 *M* acetic acid containing 2.5 *M* urea. Preparation of samples and electrophoretic conditions were as in Fig. 1.

TABLE I

AMINO ACID COMPOSITIONS OF VARIANTS OF DNA-BINDING PROTEIN II FROM EUBACTERIA

Results are expressed as the number of residues per mol of protein. Numbers in parentheses are the nearest integers. Values for *Synechococcus* 7002 variants are from ref. 15.

Amino acid	*A. vinelandii*		*Synechococcus 7002*	
	Variant 1	*Variant 2*	*Variant 1*	*Variant 2*
Asp	9.1 (9)	7.9 (8)	8	7
Thr	4.2 (4)	8.3 (8)	6	6
Ser	3.9 (4)	4.1 (4)	5	6
Glu	5.3 (5)	8.7 (9)	12	11
Pro	3.8 (4)	2.1 (2)	5	5
Gly	9.1 (9)	9.2 (9)	7	7
Ala	18.0 (18)	14.0 (14)	12	11
Val	8.0 (8)	6.3 (6)	9	8
Met	0.7 (1)	0.7 (1)	4	4
Ile	6.7 (7)	5.1 (5)	4	4
Leu	5.2 (5)	7.0 (7)	3	3
Phe	3.0 (3)	3.0 (3)	4	4
His	0 (0)	1.8 (2)	1	2
Lys	8.7 (9)	9.7 (10)	11	10
Arg	4.0 (4)	3.1 (3)	4	6
Total	90	91	94	95

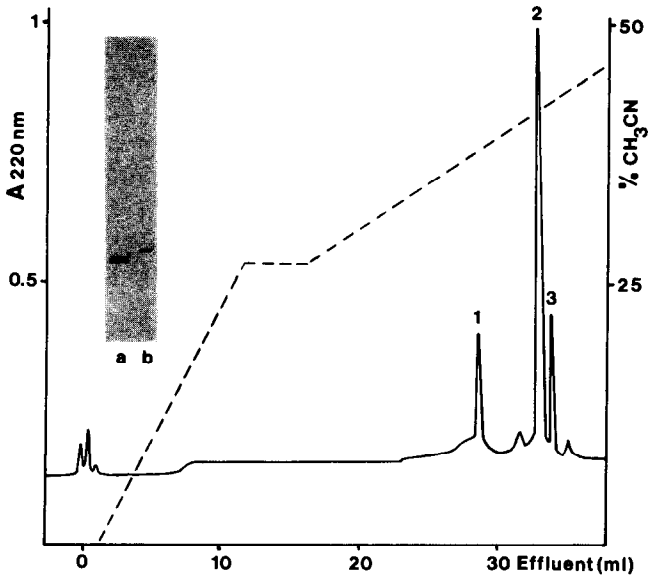

Fig. 3. Separation of variants of *Synechococcus* DNA-binding protein II (0.3 mg injected) on an Ultrapore C_8 column (250 mm × 4.6 mm). --- = Gradient of acetonitrile in 0.05% TFA, flow-rate 1 ml/min. Fractions of 0.5 ml were collected. Insert: PAGE of variants 1 (lane a) and 2 (lane b) in 0.9 *M* acetic acid containing 6.25 *M* urea. Preparation of samples and electrophoretic conditions were as in Fig. 1.

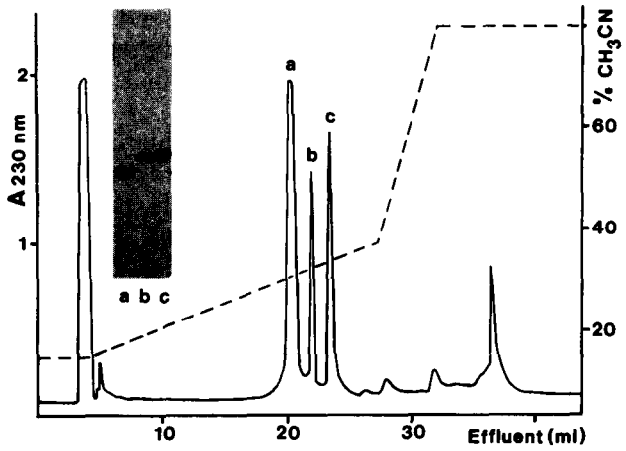

Fig. 4. Separation of variants of the protein MC1 from the archaebacterium *Methanothrix soehngenii* on an Ultrapore C_8 column (250 mm × 4.6 mm). The crude fraction eluted from the DNA-cellulose was concentrated in a Speedvac apparatus and injected. --- = Gradient of acetonitrile in 0.05% TFA; flow-rate 1 ml/min. Fractions of 0.5 ml were collected. Insert: PAGE of variants a (lane a), b (lane b) and c (lane c) in 0.9 *M* acetic acid containing 2.5 *M* urea. Preparation of samples and electrophoretic conditions were as in Fig. 1.

TABLE II

AMINO ACID COMPOSITIONS OF THE VARIANTS OF *Methanothrix soehngenii* PROTEIN MC1

Results are expressed as mol per 100 mol. n.d. = Not determined.

Amino acid	*MC1a*	*MC1b*	*MC1c*
Asp	7.2	8.7	9.1
Thr[a]	3.5	3.6	2.6
Ser[a]	0.0	2.3	2.5
Glu	10.6	12.3	13.0
Pro	6.0	6.0	6.6
Gly	9.2	9.4	8.9
Ala	10.4	11.8	8.5
Cys	0.0	0.0	0.0
Val	7.1	6.0[b]	5.8
Met	1.1	2.1	2.2
Ile	6.8	5.9[b]	7.4
Leu	4.9	5.0	5.9
Tyr	0.0	1.1	0.0
Phe	3.5	3.5	4.4
His	1.9	1.2	1.1
Lys	18.5	12.6	13.3
Arg	9.3	8.5	8.7
Trp	n.d.	n.d.	n.d.
Total	100.0	100.0	100.0

[a] Values obtained by linear extrapolation to zero hydrolysis time.
[b] 72-h Hydrolysis values.

binding protein II was resolved in two variants obtained in fractions 2 and 3 (Fig. 3). The high resolution of the column is demonstrated by the separation of these variants which exhibit similar numbers of amino acid residues with aromatic or bulky aliphatic side chains (Table I)[13].

The chromosomal protein MC1 isolated from the archaebacterium *Methanothrix soehngenii* is a complex constituted of three variants named a, b and c which were eluted from the Ultrapore C_8 column in fractions 1, 2 and 3 respectively (Fig. 4). The three variants have about the same molecular size ($M_r \approx 11\,000$ daltons). From their electrophoretic migration in acid–urea polyacrylamide gel (see insert, Fig. 4), variants b and c have a similar charge whereas variant a is more basic. The three variants were separated in a single step according to their hydrophobicities (Table II). Microsequence analyses of the three polypeptides clearly show that they are obtained in pure form and are structurally related to each other, which indicates that they represent variants of the same protein. Taking into account the low amount of protein available and the complexity of the DNA-cellulose fraction from which the protein MC1 variants were prepared, purification of the three variants in a single step shows the high efficiency of the method.

CONCLUSIONS

Variants of chromosomal proteins isolated from different prokaryotic organisms have been separated by ion-exchange or reversed-phase HPLC. The results demonstrate the efficiency of these methodologies for the separation of variants exhibiting only slight differences in amino acid compositions within each bacterial strain.

Previous chromatography of *E. coli* DNA-binding protein II on carboxymethyl-cellulose gave similar results[3] but required a much longer time (1 week *versus* 2 h) and six times as much mobile phase as does HPLC. Moreover, the sensitivity of HPLC is suitable to identify variants when low amounts of proteins are available and/or when the stoichiometry of one out of the variants is very low.

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