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Flagellin parts acquiring a regular structure during polymerization are disposed on the molecule ends

A.S. Kostyukova, M.G. Pyatibratov, V.V. Filimonov and O.V. Fedorov

Institute of Protein Research, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR

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Flagellins of two *Escherichia coli* strains have been investigated by limited proteolysis and scanning microcalorimetry. It has been shown that a monomer flagellin consists of two parts: a central one cooperatively melting, rather resistant to proteases, and the other without a stable tertiary structure and thus easily degrading terminals. Just these terminals acquire a regular structure during polymerization. Core fragments of the two strains have been isolated and characterized.

Flagellin; Limited proteolysis; Microcalorimetry; (*Escherichia coli*)

1. INTRODUCTION

Bacterial flagellar filaments are constructed from subunits of flagellin, a globular protein [1]. It is known that the secondary structures of flagellin in the monomer and polymer forms are different [2]. Previously we have suggested that a part of the monomer flagellin polypeptide chain acquires a regular form only in the process of polymerization [3]. To determine the disposition of this part in the molecule we have carried out a comparative study of two *E. coli* strain flagellins by limited proteolysis with different proteases and scanning microcalorimetry.

2. MATERIALS AND METHODS

Two strains of *E. coli*, K12J62 and B38, from the All-Union Collection of Microorganisms (Moscow) were used. The culturing conditions and the technique of polymer flagellin preparation have been described previously [4]. Monomer flagellin was prepared by heating polymer solutions at 65°C for 3 min. The protein concentration was determined from the nitrogen content [5]. The purity of proteins was checked by SDS-PAGE [6].

Limited proteolysis by trypsin, chymotrypsin and thermolysin (Sigma) was done in a phosphate buffer containing 0.15 M NaCl and 10 mM phosphate, pH 7.0, at 21°C. Aliquots for electrophoresis were taken at definite time periods. Proteolysis was terminated by addition of an equal volume of the electrophoretic sample buffer. The kinetics of the proteolysis was tested by SDS-PAGE. Cleavage of flagellin with CNBr was performed according to [7].

Tryptic fragments of *E. coli* B38 flagellin were prepared by addition of a trypsin solution (0.5 mg/ml) to monomer flagellin (5 mg/ml). The protein/enzyme weight ratio was 100:1. Trypsinolysis was terminated after 10 min by addition of an equimolar amount of trypsin inhibitor from soybean (Serva). The mixture of tryptic fragments was transferred to 20 mM Tris-HCl, pH 8.0, by gel filtration on a PD-10 column (Pharmacia). Fractionation of fragments was done with the FPLC system (Pharmacia) on an anion-exchange mono Q column by gradient elution (0.05–0.22 M NaCl in 20 mM Tris-HCl, pH 8.0). The purity of the fragments in each fraction was checked electrophoretically.

Tryptic fragments of flagellin from the K12J62 strain were obtained by treating the monomer flagellin under the same conditions. Trypsinolysis was terminated after 3 min. Fractionation was carried out by the Uniform system for preparative electrophoresis (LKB) in 10% polyacrylamide gel in the absence of SDS using a column 21 mm in diameter at 400 V. The mixture of tryptic fragments was layered on the column in an electrophoretic sample buffer without mercaptoethanol and SDS.

Calorimetric measurements were carried out according to [4].

Correspondence address: O.V. Fedorov, Institute of Protein Research, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR

Abbrevations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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3. RESULTS AND DISCUSSION

Molecular masses of the flagellins from *E. coli* K12J62 and B38 were 56 and 59 kDa, respectively. Their amino acid content was in general similar to that of other flagellins, though there was some difference between the flagellins of these two strains, e.g. in the content of phenylalanine, proline and histidine.

These flagellins were subjected to limited proteolysis by different proteases (thermolysin, chymotrypsin, trypsin). The following regularities in the proteolysis were revealed for both the flagellins: (i) polymer flagellins were resistant to protease treatment; (ii) a monomer flagellin is sequentially cleaved into several smaller fragments one after another. At the first stages a rapid degradation of terminal regions occurred resulting in the formation of a rather stable fragment (figs 1 and 2). Since the easily degraded regions are equally accessible for proteases with a very different specificity, it is natural to presume that these regions belong to that part of the polypeptide chain which acquires a regular structure only during polymerization.

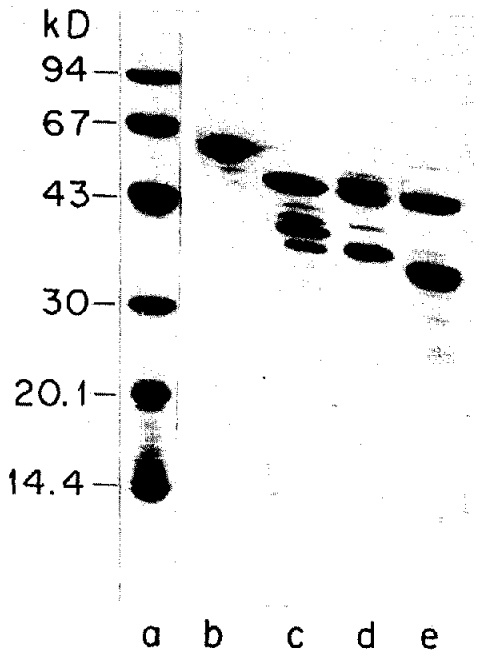

Fig.1. Results of limited cleavage of monomer flagellin B38 by different proteases (lanes): marker proteins (a); strain B38 flagellin (b); trypsin treatment (c): cutting time is 15 min, the protein/enzyme ratio is 200:1; chymotrypsin treatment (d): 30 min at 20:1; thermolysin treatment (e): 15 min at 200:1.

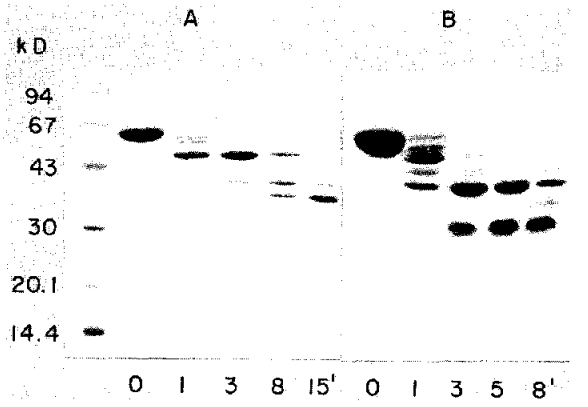

Fig.2. Time course of trypsinolysis of monomer flagellins from different *E. coli* strains. The protein/enzyme ratio is 100:1. (A) Strain B38 flagellin; (B) strain K12J62 flagellin.

Further we have isolated preparative amounts of the first protease-resistant intermediate and also one of the later intermediates for both strains.

The *E. coli* K12J62 flagellin fragments had molecular masses of 44 and 34 kDa, those of *E. coli* B38 fragments were 50 and 37 kDa, and were designated as FK44, FK34, FB50 and FB37, respectively. While fragments FK44 and FB50 were electrophoretically pure, preparations of FK34 and FB37 had a minor component of a lower molecular mass (fig.3).

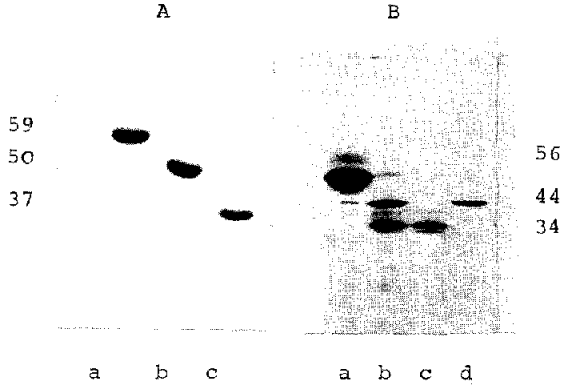

Fig.3. Electrophoretic analysis of fragment purity. (A) Strain B38 flagellin (a), FB50 fragment (b), FB37 fragment (c). (B) Strain K12J62 flagellin (a), mixture of its tryptic fragments before isolation (b), FK34 fragment (c), FK44 fragment (d).

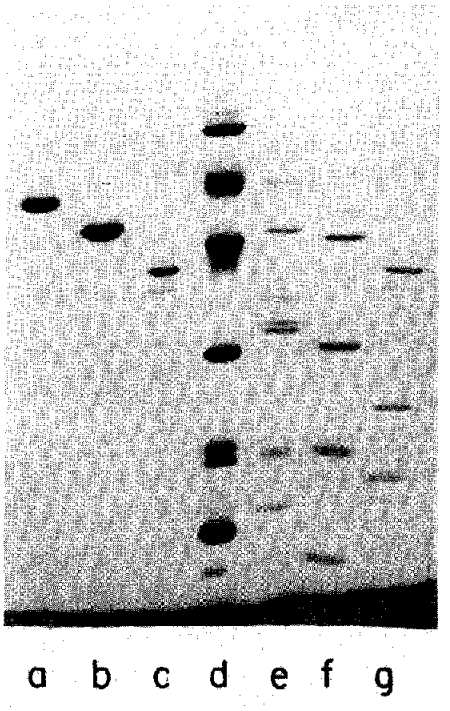

Fig.4. Results of CNBr digest. Lanes: (a) strain B38 flagellin; (b) FB50 fragment; (c) FB37 fragment; (d) marker proteins (see legend to fig.1); (e–g) corresponding CNBr digests.

We have performed an electrophoretic comparison of the number and molecular masses of fragments obtained by CNBr digest of flagellins and their tryptic fragments. It has been shown that flagellins and the first tryptic fragments have only a single identical CNBr digest band; and the second tryptic fragments do not have any CNBr digest bands coinciding with those of the first fragments (fig.4). This can occur only if the first and the second tryptic fragments are formed by sequential splitting from both ends of the polypeptide chain.

Scanning microcalorimetry [8] was used to estimate the number and approximate dimensions of cooperative blocks (domains) in flagellins and their fragments. Despite the differences in qualitative thermodynamic parameters (heat effect, stability, etc.) observed for representatives of the two strains, the following common features were revealed (fig.5): (i) the melting curves of the monomer and its large fragment coincide within experimental error under identical conditions; (ii) the small fragment has a remarkably lower melting enthalpy in comparison with the large fragment and the monomer, though the stability of all three is practically the same.

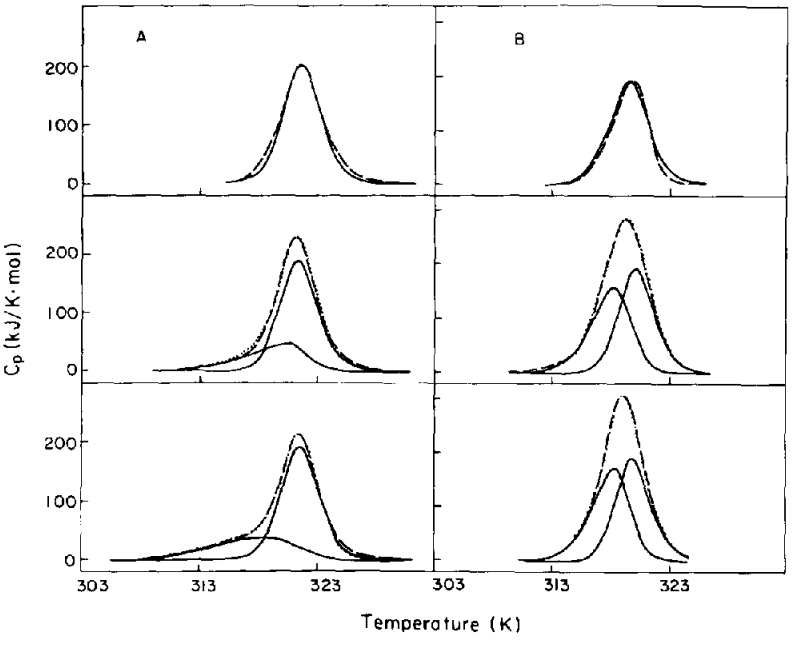

Fig.5. Temperature dependence of C_p excess heat absorption for monomer flagellin (bottom), large fragment (middle) and small fragment (top). (– – –) Experiment; (···) sum of the peaks; (---) peaks. (A) Strain K12J62; (B) strain B38.

A thermodynamic analysis performed as described in [9] has shown that the small fragments FK34 and FB37 melt as a single cooperate block, whereas there are at least two such blocks in the large fragments FK44 and FB50 and in monomers. Hence, from calorimetric data it follows that the terminal fragments do not have a stable tertiary structure in monomer and explains high accessibility to proteases.

Thus, it has been demonstrated that a monomer flagellin consists of two parts: a central one rather resistant to proteases without a rigid structure and therefore terminal parts that easily degrade.

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