Volume 235, number 1,2, 117–121 FEB 06130 August 1988

Purification, composition and Ca^{2+}-binding properties of the monomeric protein of the S-layer of *Thermus thermophilus*

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Received 30 May 1988

The S-layer of *Thermus thermophilus* is apparently formed by a single protein of M_r 100 000 called P100. In the cell envelope of the organism, P100 forms oligomeric complexes of exceptional thermostability in the presence of Ca^{2+}. A simple and rapid method for the purification of P100 is reported. The amino acid composition of the protein was found to be comparable to that of other S-layer proteins. Binding of Ca^{2+} to P100 occurs with a dissociation constant of 50 μM apparently at 12 high-affinity sites per P100 molecule.

Protein purification; Ca^{2+}-binding; S-layer; *(Thermus)*

1. INTRODUCTION

Crystalline surface protein layers (S-layers) are characteristic of certain groups of archaebacteria and eubacteria living under extreme environmental conditions, thereby suggesting that S-layers might be relevant for the adaptation of microorganisms to hostile environments [1,2].

Thermus thermophilus is an extremely thermophilic, Gram-negative eubacterium with a morphologically typical S-layer of hexagonal symmetry, surrounded by an external coat of still uncertain nature [3]. The protein constituting the S-layer has been identified as a protein (P100) of M_r 100000, characterized by its ability to form non-covalent oligomeric complexes, extremely resistant to thermal denaturation in the presence of Ca^{2+}. The oligomeric complexes and P100 itself interact strongly with peptidoglycan, being practically unextractable by the straightforward methods that lead to the purification of other S-layer proteins [1,4]. The properties of P100 suggest that the S-layer of *T. thermophilus* might contribute to the thermostability of its cell envelope. Here, we report on the purification and Ca^{2+}-binding properties of P100.

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2. MATERIALS AND METHODS

2.1. *Strains and conditions of growth*

T. thermophilus strain HB8 [5,6] was obtained from the American Type Culture Collection (ATCC 27634). The organism was routinely grown at 75°C under strong aeration in the rich medium described in [4].

2.2. *Purification of cell envelopes and electrophoretic separation of cell envelope proteins*

Cell envelopes were purified as in [4]. Protein content was measured by the method of Lowry et al. [7].

Cell envelope proteins were analyzed by SDS-PAGE on normal and discontinuous slab gels, as described in [4]. The M_r of the oligomeric complexes derived from P100 was determined in 3.3% acrylamide gels according to Weber et al. [8] using as M_r markers protein standard mixture III (Merck, Darmstadt, cat. no.15126).

2.3. *Ca^{2+}-binding assay*

The affinity of Ca^{2+} to P100 was measured essentially as described by Kawasaki et al. [9]. Aliquots of 5 μl of a solution of P100 (2 mg/ml) in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5, buffer were adsorbed onto nitrocellulose filter disks (Millipore HAWP, 1 cm diameter), which were allowed to dry at room temperature. The filters were then immersed in 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 and thoroughly washed

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00145793/88/$3.50

(30 min, in an orbital shaker at 250 rpm) to eliminate Ca^{2+} bound to P100. To remove EDTA, filters were washed twice in 10 ml of the same buffer without EDTA. Binding of $^{45}Ca^{2+}$ was performed by immersion of the filters in the same buffer containing $^{45}CaCl_2$ (0.35 μCi/μg) at increasing concentrations. After incubation (30 min at 37°C) filters were washed to remove unbound $^{45}Ca^{2+}$, and dried to measure bound radioactivity by liquid scintillation.

Binding of $^{45}Ca^{2+}$ to proteins previously subjected to SDS-PAGE was performed as described by Waissman et al. [10]. Two aliquots (25 μl) of a solution of P100 at 2 mg/ml in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.8, were boiled for 30 min in 10% (w/v) SDS after addition of 1 mM EDTA to one of them, and then subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose paper which was washed in 10 mM Tris-HCl, pH 7.5, and incubated for 30 min at 37°C in the same buffer containing 0.1 mM $^{45}CaCl_2$ (spec. act. 9 μCi/μg). The paper was thoroughly washed in the same buffer without the radioactive compound, dried and subjected to autoradiography to detect labeled proteins.

2.4. *Amino acid analysis*

Samples (2 mg) of purified P100 were hydrolysed in 1 ml of 6 N HCl at 116°C for 16 h in nitrogen-flushed, sealed vials. The amino acid composition was determined in a Kontron 500 automatic amino acid analyzer.

3. RESULTS AND DISCUSSION

3.1. *Purification of P100*

Fig.1 shows the proteins identified by SDS-PAGE of aliquots from fractions at different stages of the purification of P100.

A suspension of purified cell envelopes (10 ml, 30 mg/ml protein) (fig.1, lane 1) was centrifuged (30000 × g, 15 min), resuspended in 50 mM NH_4 acetate, 1 mM EDTA, pH 7.4 (acetate buffer) (fig.1, lane 2), and then incubated for 30 min at 60°C after addition of Triton X-100 at a final concentration of 1% (w/v). Solubilized material was removed by centrifugation (30000 × g, 15 min) (fig.1, lane 3) and the pellet was washed three times by repeated cycles of resuspension in acetate buffer

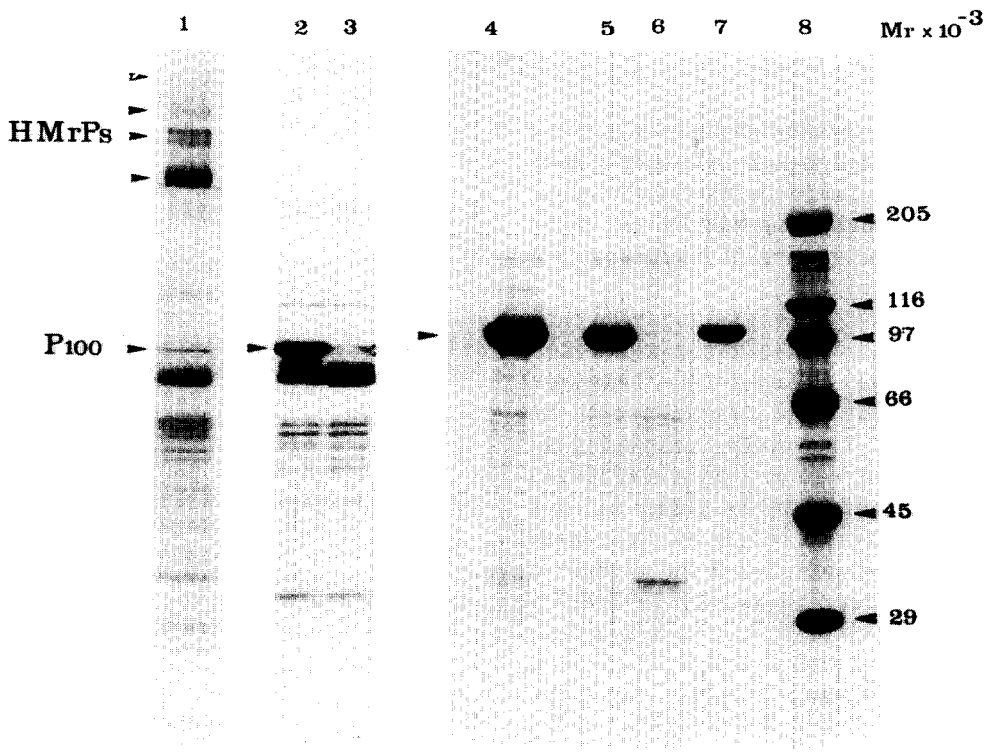

Fig.1. Purification of P100. Fractions obtained at relevant stages of the purification of P100 (see text), were subjected to SDS-PAGE on discontinuous 6/11% acrylamide gels. (1) Total envelope proteins (HMrPs, oligomeric forms of P100); (2) total envelope proteins treated with EDTA; (3) Triton X-100-soluble fraction; (4) Triton X-100-soluble material after lysozyme digestion; (5) CM-Sephadex, unretained material; (6) CM-Sephadex, retained proteins; (7) purified P100 (50 μg protein loaded on gel); (8) M_r markers: myosin (205000), β-galactosidase (116000), phosphorylase b (97000), bovine albumin (66000), egg albumin (45000), carbonic anhydrase (29000).

(40 ml) and centrifugation as above. The pellet of the last centrifugation was resuspended in 20 ml acetate buffer, treated with lysozyme (200 μg/ml, 16 h at 37°C) and centrifuged as above. The pellet was resuspended in 1 ml of 1% (w/v) Triton X-100 in acetate buffer, pH 6.8, and incubated for 30 min at 60°C. Degradation of peptidoglycan by lysozyme made P100 easily extractable with Triton X-100, and upon centrifugation (30000 × g, 15 min), it was quantitatively recovered in the soluble fraction (fig.1, lane 4). The fraction enriched in P100 was diluted 1:10 in acetate buffer, pH 6.0, and subjected to ion-exchange chromatography, first on CM-Sephadex C-25-120 where P100 eluted in the flow-through (fig.1, lane 5) but most contaminating proteins were retained (fig.1, lane 6), and then on DEAE-Sephadex A-25, where P100 was retained. After washing with acetate buffer, pH 6.0, the column was eluted with a linear gradient of NaCl (0–1 M) in the same buffer. P100 eluted at 0.6 M NaCl. Finally, the concentration of NaCl in the fraction enriched in P100 was raised to 2 M, causing precipitation of the protein. The precipitate was washed three times by resuspension in water and centrifugation as above. The last pellet was lyophilized and stored at −20°C until use. SDS-PAGE of the product followed by Coomassie blue staining indicated that P100 was purified to homogeneity according to this criterion (fig.1, lane 7).

Table 1

Amino acid composition of P100

Amino acid	%
Arginine	3.18
Alanine	16.44
Aspartic acid + asparagine	13.07
Cystine	N.D.
Glutamic acid + glutamine	11.96
Glycine	5.24
Histidine	0.7
Isoleucine	2.19
Leucine	10.23
Lysine	4.15
Methionine	0.69
Phenylalanine	4.24
Proline	N.D.
Serine	8.26
Threonine	6.07
Tryptophan	N.D.
Tyrosine	5.62
Valine	7.32

3.2. *Amino acid composition of P100*

As shown in table 1, P100 had a high proportion of acidic (25%) and hydrophobic (46.2%) amino acid residues, a strongly reduced proportion of sulfur-containing amino acids (0.69%) and completely lacked proline, tryptophan and cysteine. These features as well as the values of the

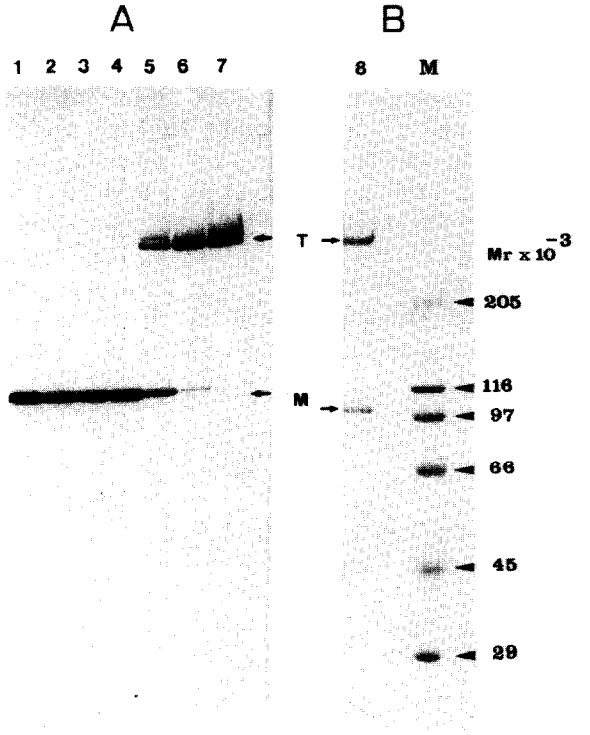

Fig.2. Effect of Ca^{2+} on the thermostability of oligomeric complexes of purified P100. A sample (1 mg) of lyophilized P100 was washed in 1 ml of 1 mM EDTA, 10 mM Tris-HCl, pH 7.8, by resuspension (P100 was insoluble in this buffer) for 45 min to remove contaminating Ca^{2+}. Washed protein was recovered by centrifugation (30000 × g, 30 min) and solubilised in 0.1% (w/v) Triton X-100, 10 mM Tris-HCl, pH 7.8, at a concentration of 1 mg/ml. Aliquots of 30 μl were incubated (5 min at 30°C) in the presence of $CaCl_2$ at different concentrations, boiled (15 min) in 10% (w/v) SDS, 2% (v/v) β-mercaptoethanol and subjected to SDS-PAGE. [$CaCl_2$]: (1) zero, (2) 0.01 mM, (3) 0.1 mM, (4) 0.5 mM, (5) 1 mM, (6) 5 mM, (7) 10 mM. (B) An aliquot of purified P100 treated as in lane 4 of (A) was subjected to SDS-PAGE (lane 8) along with the M_r markers described in fig.1 (lane M), to give a reference of the relative mobilities of the monomeric and oligomeric forms of P100.

hydrophobicity index (Hϕ = 953 cal/residue) and discriminant function (Z = 0.196) [11] were very similar to those reported for the S-layer proteins of most microorganisms analysed so far, in particular to that of *Deinococcus radiodurans* [1,12].

3.3. *Formation of thermostable oligomeric complexes by purified P100*

Previous work indicated that in the cell envelope P100 was apparently able to form non-covalent oligomers which, in the presence of Ca^{2+}, were virtually stable in boiling 10% (w/v) SDS, 2% (v/v) β-mercaptoethanol. Removal of Ca^{2+} destabilized the complexes, which dissociated into monomeric subunits when heated to 70–75°C in the solution described above [4]. To study the response of purified P100 to changes in Ca^{2+} concentration, lyophilized P100 was washed in 1 mM EDTA, 10 mM Tris-HCl buffer, pH 7.8, to remove contaminating Ca^{2+} before being solubilized in 1% Triton X-100, 10 mM Tris-HCl, pH 7.8. Aliquots were treated with $CaCl_2$, boiled in 10% SDS and then subjected to SDS-PAGE. The results (fig.2) confirmed that purified P100 was able to form stable complexes upon addition of $CaCl_2$ at a concentration (1–5 mM) comparable to that required for the stabilization of the complexes in the cell envelope [4]. The estimated M_r of the complexes, 310000 as determined by the method of Weber et al. [8] (not shown), suggests that purified P100 exclusively forms trimers as thermostable oligomers, a result that contrasts with its ability to form, in addition, higher-order oligomers in the cell envelopes (cf. fig.1, lane 1 and fig.2, lane 7).

3.4. *Ca^{2+}-binding properties of P100*

The properties of the interaction between P100 and Ca^{2+} were studied measuring the binding of $^{45}Ca^{2+}$ to purified protein adsorbed onto membrane filters as described in section 2. Fig.3A shows the data on a Scatchard plot. The results indicate a dissociation constant of 50 μM and the existence of approx. 12 high-affinity Ca^{2+}-binding sites per molecule of P100, values comparable to those reported for other unrelated Ca^{2+}-binding proteins [13].

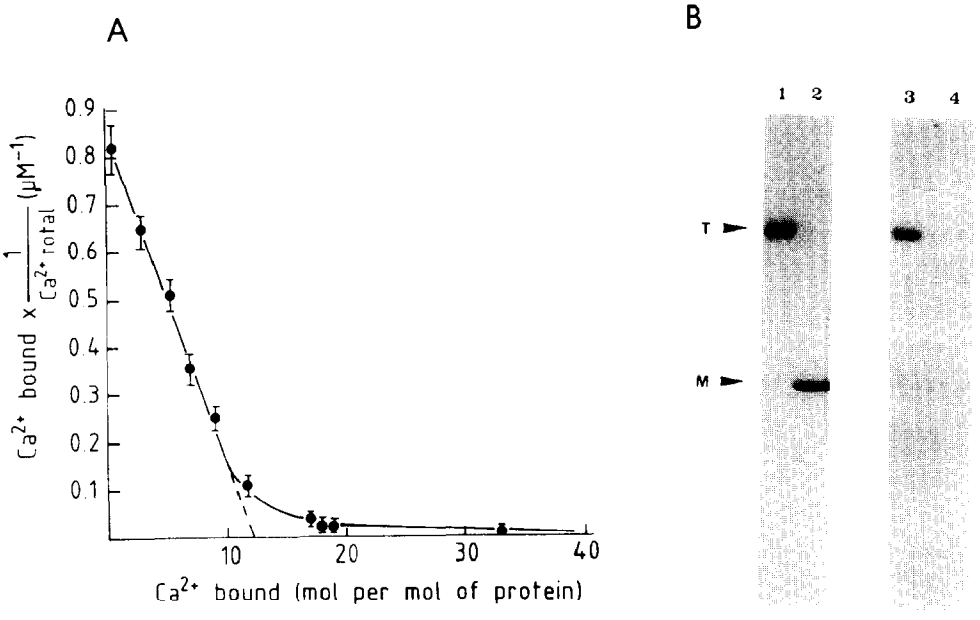

Fig.3. Binding of $^{45}Ca^{2+}$ to purified P100. (A) Samples of purified P100 were incubated in the presence of increasing concentrations of $^{45}CaCl_2$, and further processed as described in section 2. Data are displayed on a Scatchard plot. (B) Aliquots (50 μg protein) of purified P100 were incubated for 15 min in boiling 10% SDS, in the presence or absence of 1 mM EDTA, subjected to SDS-PAGE, transferred onto nitrocellulose paper sheets, and further processed as described in the text to detect binding of Ca^{2+} to the blotted proteins. Lanes: 1,2, Coomassie blue-stained gel of EDTA untreated and treated P100, respectively. Lanes: 3,4, autoradiography of EDTA untreated and treated P100, respectively. T,M, position of P100 in the trimeric and monomeric forms, respectively.

A second point of interest was to determine whether the presence of EDTA during incubation of P100 in boiling 10% SDS would affect the ability of the protein to bind Ca^{2+}. To study this aspect, samples of P100 in 10 mM Tris-HCl buffer, pH 7.8, were boiled (30 min) in 10% SDS in the presence or absence of 1 mM EDTA and subjected to SDS-PAGE. The ability of the protein in the monomeric (EDTA treated) and oligomeric (EDTA untreated) forms to bind $^{45}Ca^{2+}$ was assayed as described in section 2. The results shown in fig.3B indicate that only the oligomeric form retained the ability to bind $^{45}Ca^{2+}$, suggesting that removal of Ca^{2+} made the Ca^{2+}-binding sites of P100 susceptible to irreversible denaturation by hot SDS.

Acknowledgements: The technical assistance of J. de la Rosa is greatly acknowledged. M.L.M.F. holds a fellowship from the Fondo de Investigaciones Sanitarias. This work has been supported by grants PR84-0213-C02-01 and BT9/85 from the CAICYT, and an institutional grant from Fondo de Investigaciones Sanitarias.

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