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Purification of the C1 repressor of bacteriophage P1 by fast protein liquid chromatography

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ABSTRACT

A fast protein liquid chromatographic method is described for the purification of the C1 repressor of bacteriophage P1 and its truncated form C1*. By using one crude extract, both repressor proteins were purified in parallel to homogeneity and were shown to interact specifically with P1 operator DNA *in vitro*. The method involves an affinity chromatographic step on heparin-Sepharose, followed by a combination of ion-exchange chromatography on Q Sepharose and S Sepharose. The availability of a homogeneous preparation of the phage repressor is a prerequisite for studies on its structure–function relationship.

INTRODUCTION

Proteins that regulate gene expression generally bind to specific DNA sequences. These regulatory proteins can control gene expression negatively or positively. Many of these regulatory proteins were reported to be repressor proteins, which act by binding to specific operator DNA sequences. Usually, an operator is overlapping with a promoter, and therefore an operator-bound repressor is preventing the RNA polymerase from binding to this promoter.

We are interested in the molecular mechanisms regulating gene expression in bacteriophage P1. As temperate phage, P1 is able either to (i) lyse an infected bacterial cell after forming new phage particles and thus release many progeny phages or (ii) lysogenize its host by maintaining the injected phage chromosome as a prophage in the cell. At various stages in its life cycle the phage has to switch on or off different sets of genes. A key element in this circuit of phage P1 gene control is the C1 repressor as a negative regulator of phage gene expression. As long as this repressor is active, the phage is maintained as a stable extrachromosomal low-copy plasmid in the cell. C1 turns off the lytic phage genes by binding to several specific operators, which are scattered over the P1 chromosome. Analysis of the C1 protein sequence did not reveal any homology to the classical helix-turn-helix motif [1] or to any other of the known DNA-binding structures. A review on phage P1 was published by Yarmolinsky and Sternberg [2].

By using conventional chromatographic methods for the purification of the C1 repressor and of mutant C1 repressor proteins [3,4], the repressor activity was resolved into two overlapping peaks. Two polypeptides, called C1 and C1*, were found to be active as P1 operator-binding proteins *in vitro*. The polypeptide C1* (268 amino acids; relative molecular mass, M_r = 30 900) has been shown to be a truncated form of C1 (283 amino acids, M_r = 32 500 [3,5]. The C1 repressor protein is positively charged (net charge of +4 at pH 7.0, pI = 9.1) and exists as a monomer in solution [6].

On the basis of the previously published purification procedure [3], this paper describes the application of fast protein liquid chromatography (FPLC) to the isolation of the P1 C1 and the C1* repressor proteins. By using one crude cellular extract, both

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repressor proteins were purified in parallel. During the purification, the repressor proteins were monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by a DNA mobility shift assay that measured selective binding to P1 operator DNA.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma (Deisenhofen, Germany). Heparin-Sepharose CL-6B gel and the prepacked fast protein liquid chromatographic columns Q Sepharose High Performance 16/100, S Sepharose High Performance 16/100, Mono S HR 5/5 and Fast Desalting Column HR 10/10 were obtained from Pharmacia (Freiburg, Germany). The DNA (1000 base pair ladder) and protein molecular mass markers were obtained from Bethesda Research Labs. (Eggenstein, Germany) and Bio-Rad Labs. (Munich, Germany).

Preparation of the crude extract

The method used for the preparation of extracts and the isolation of C1 and C1* repressor proteins is based on previously described procedures [3,4]. For the overproduction of the C1 repressor the recombinant plasmid pMV2w [4] was used. Here, the *c*1 repressor gene is under the control of the *tac* promoter, which is inducible by isopropyl β-D-thiogalactoside (IPTG). Unless noted otherwise, all steps were performed on ice and dialysis was performed against the appropriate buffer.

The *Escherichia coli* strain HB101 harbouring plasmid pMV2w was grown at 37°C in 1.2 l TY medium [1.0% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.5% (w/v) NaCl]. To induce repressor synhesis, IPTG was added to a final concentration of 2 m*M* at a cell density of about $2 \cdot 10^8$–$3 \cdot 10^8$ cells/ml. Incubation was continued at 37°C for 2 h and finally the cells were harvested by centrifugation [6000 *g* (Sorvall GS3 rotor), 10 min, 4°C]. The resulting pellets (13.7 g of wet cell paste) were resuspended in 2.5 m*M* EDTA (pH 8.0), 200 m*M* NaCl and 20 m*M* spermidine (5 ml/g of wet cell paste). The addition of one volume of lysis buffer containing 25 m*M* Tris–HCl (pH 8.0), 120 m*M* NaCl, 3.5% (w/v) sucrose, 0.2% (w/v) Brij 58, 1.25 m*M* EDTA (pH 8.0), 10 m*M* spermidine and 0.5 mg/ml of lysozyme led to bacterial lysis. After incubation for 30 min at 0°C the samples were warmed to 30°C and stirred at the same temperature for 5 min to complete lysis. The suspension was immediately cooled again, adjusted to 1 *M* NaCl and centrifuged [105 000 *g* (Beckman 45Ti rotor), 60 min, 4°C]. The supernatant (105 ml) was diluted 1:1 with buffer A [20 m*M* Tris–HCl (pH 7.6), 0.1 m*M* EDTA (pH 7.5), 50 m*M* NaCl, 1 m*M* dithiothreitol (DTT) and 10% glycerol]. An ammonium sulphate precipitation (60% saturation = 390 mg/ml) was followed by centrifugation [105 000 *g* (Beckman 45Ti rotor), 60 min, 4°C]. The pellets were dissolved in buffer A (11 ml) and subsequently dialysed against buffer A, resulting in a final volume of 13.5 ml with a protein concentration of 21.6 mg/ml (fraction I).

Fast protein liquid chromatography

An automated FPLC system (Pharmacia) was used, consisting of a controller (LCC-500 Plus), two high-precision pumps (P-500), solvent mixer, prefilter, a UV monitor (UV-M), one MV-7 and four MV-8 multi-position valves, a recorder (REC-482) and a fraction collector (FRAC 200). For the FPLC application of samples of up to 2 ml, loops of various sizes were used. A 50-ml Superloop was used for sample volumes larger than 2 ml.

Heparin-Sepharose CL-6B gel was laboratory-packed in an XK 16/20 glass column with an attached RK 16/26 packing reservoir [7]. The column was packed, washed and equilibrated under FPLC low-pressure conditions (operating pressure ≤ 0.5 MPa). The calculated column volume was 22.0 ml. The prepacked FPLC columns were handled as recommended by Pharmacia. High Performance (HP) 16/100 columns were equilibrated and developed under FPLC low-pressure conditions (operating pressure range 0–0.5 MPa). During the purification procedure the temperature of the columns and of the collected fractions was kept constant at 2°C by using a MultiTemp II refrigerated thermostatic circulator unit (Pharmacia) as an external cooling system. All buffers were degassed and filtered through 0.22-μm membrane filters (Sartorius, Göttingen, Germany) prior to use. The protein absorbance was monitored at 280 nm.

C1 repressor assay

The *in vitro* interaction of C1 repressor with P1 Op86 operator DNA in buffer E [20 m*M* Tris–HCl (pH 7.6), 0.1 m*M* EDTA, 1 m*M* DTT, 50 m*M* NaCl, 10% glycerol, 100 μg/ml bovine serum albumin (BSA)] was studied by a DNA mobility shift assay [8,9] as modified by Velleman *et al.* [5].

Protein analysis

SDS-PAGE of proteins was performed on 15% slab gels as described [10]. Proteins were stained with Coomassie Brillant Blue R250 (Sigma). Protein molecular mass markers were myosin (200 000), phosphorylase *b* (97 400), BSA (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (21 500) and β-lactoglobulin (18 400). The protein concentrations were determined by the method of Miller [11] using BSA as a protein standard.

RESULTS AND DISCUSSION

Heparin-Sepharose chromatography

Affinity chromatography is commonly used as one of the final steps in protein purification, but it has been shown that chromatography on heparin-Sepharose is a very effective first step in the purification of C1 repressor [3]. Heparin is a linear glycosamine–glycan composed of mostly sulphated 1,4-linked glycosamine and glucuronic acid residues, covalently coupled to Sepharose CL-6B.

A crude cellular extract was prepared from an *Escherichia coli* strain which overexpresses the *c*1 repressor structural gene from the *tac* promoter. Proteins of the crude extract were concentrated by salting-out with ammonium sulphate at a concentration of 60% saturation (fraction I) and finally loaded on to a laboratory-packed heparin-Sepharose column (50-ml Superloop, flow-rate 0.5 ml/min), equilibrated with buffer A. Unbound proteins were washed from the column, at a flow-rate not exceeding that used for loading, until the absorbance of the flow-through dropped to the absorbance of buffer A. Under these conditions, all repressor material is bound to the matrix. The repressor protein was eluted with a flow-rate of 2.5 ml/min using an NaCl gradient in buffer A (Fig. 1). The SDS-PAGE analysis of protein extracts from uninduced and induced cells, fraction I, the flow-through and fractions 23–30 eluted from heparin-Sepharose is shown in Fig. 2. The preparation of fraction I (Fig. 2, lane 3) is a very critical step in the purification of the C1 repressor (see purified C1 re-

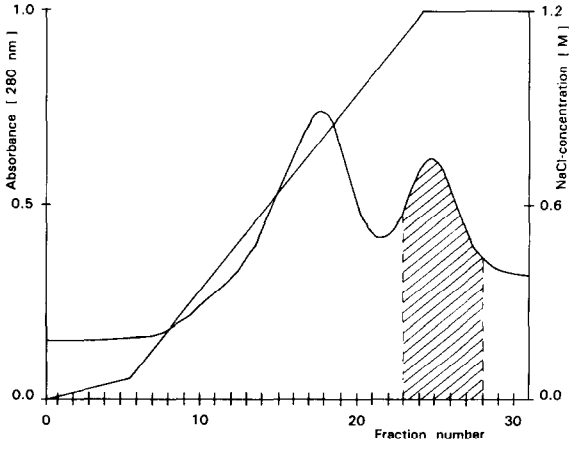

Fig. 1. Elution profile of C1 repressor on heparin-Sepharose. Fraction I (13.5 ml, 21.6 mg/ml) was applied to a heparin-Sepharose column (22 ml), pre-equilibrated and washed with buffer A. Bound proteins were eluted using a gradient: 0% buffer B (buffer A containing 1.2 *M* NaCl) in 5 ml, 0–5% buffer B in 40 ml, 5–100% buffer B in 150 ml, 100% buffer B in 30 ml. Fractions of 8 ml were collected at a flow-rate of 2.5 ml/min. The fractions containing the repressor protein (hatched area) were pooled and dialysed against buffer C (fraction II).

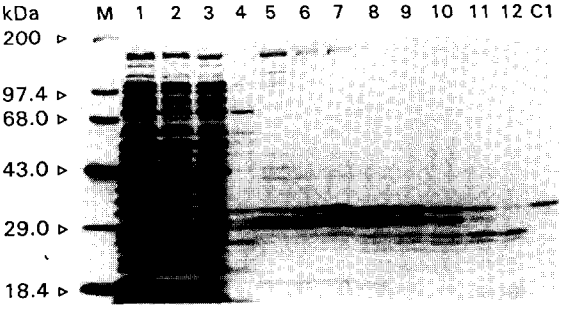

Fig. 2. SDS-PAGE of protein fractions eluted from heparin-Sepharose on a 15% gel. Lanes: M = protein molecular mass markers [in kilodaltons (kDa) as indicated] in descending order (8 μg each): myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin; 1 and 2 = crude cellular extracts (15 μl each) of uninduced (lane 1) and induced cells (lane 2); 3 = fraction I (5 μl); 4 = flow-through heparin-Sepharose (70 μl); 5–12 = fractions 23–30 eluted from heparin-Sepharose (20 μl each); C1 = purified C1 repressor protein (1 μg).

pressor as marker in the last lane of Fig. 2 on the right). The truncation of C1 (upper band at *ca.* 32 500) to C1* (lower band at *ca.* 31 000) is drastically increased in this step, possibly owing to the activity of proteases in fraction I (compare lane 2 with lane 3). Therefore, the portion of C1* in the pooled fractions 24–28 eluted from heparin-Sepharose (Fig. 2, lane 5–10) is very high. The pooled fractions were dialysed against buffer C [20 m*M* Tris–acetate (pH 6.5), 0.1 m*M* EDTA (pH 7.5), 1 m*M* DTT, 15% glycerol] and applied to ion-exchange chromatography (fraction II).

Ion-exchange chromatography

For further purification of heparin-Sepharose fractions containing a mixture of C1 and C1* repressor and other proteins, we chose a combination of anion- and cation-exchange chromatography. Under the experimental conditions, C1 and C1* repressor were bound to the cation-exchange column.

Collected fractions from the affinity chromatography on heparin-Sepharose were passed through a Q Sepharose HP column (50-ml Superloop, flow-rate 0.5 ml/min), equilibrated with buffer C. The flow-through of this anion-exchange column was loaded directly on to an S Sepharose HP column coupled in series. The columns were washed free from unbound protein using buffer C and were subsequently decoupled. The bound proteins were eluted from the cation-exchange column with a flow-rate of 1.5 ml/min using an NaCl gradient in buffer C (Fig. 3). The overlapping peaks of the C1* and the C1 repressor proteins resulting from the heparin-Sepharose step were separated on this cation-exchange column. Two well defined peaks were obtained, the first peak containing only C1* (Fig. 4, lanes 2–4, corresponding to fractions 18–20 in Fig. 3) and the second peak mainly containing C1 repressor (Fig. 4, lanes 5 and 6, corresponding to fractions 21 and 22 in Fig. 3, hatched area).

For further purification and concentration of C1, fraction 22 (Fig. 4, lane 6) was desalted on a Fast Desalting HR 10/10 column by using buffer C at a flow-rate of 3 ml/min and subsequently rechromatographed on an analytical cation-exchange column (Mono S). The C1 repressor was eluted from the column with an NaCl gradient in buffer C as shown in Fig. 5. In contrast to the C1 repressor, which was eluted from the Mono S column at ≥ 0.6 *M* NaCl (hatched area in Fig. 5), the C1* repressor was elut-

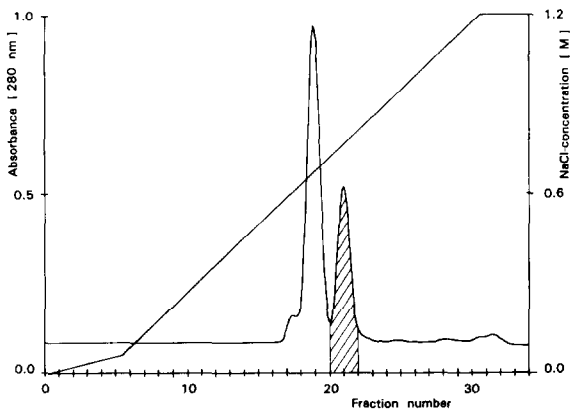

Fig. 3. Elution profile of C1 and C1* repressor proteins on S Sepharose. Fraction II (42 ml, 3.3 mg/ml) was applied to a Q Sepharose column (20 ml) coupled in series with an S Sepharose column (20 ml), each pre-equilibrated and washed with buffer C. The C1 and C1* proteins were only bound to S Sepharose and eluted from this column with a gradient: 0% buffer D (buffer C containing 1.2 *M* NaCl) in 5 ml, 0–5% buffer D in 40 ml, 5–100% buffer D in 200 ml, 100% buffer D in 30 ml. Fractions of 8 ml were collected at a flow-rate of 1.5 ml/min. The fractions containing the C1 repressor protein (fraction 21 and 22, hatched area) or the C1* repressor (fractions 18–20) were separately desalted on a Fast Desalting column and concentrated on Mono S.

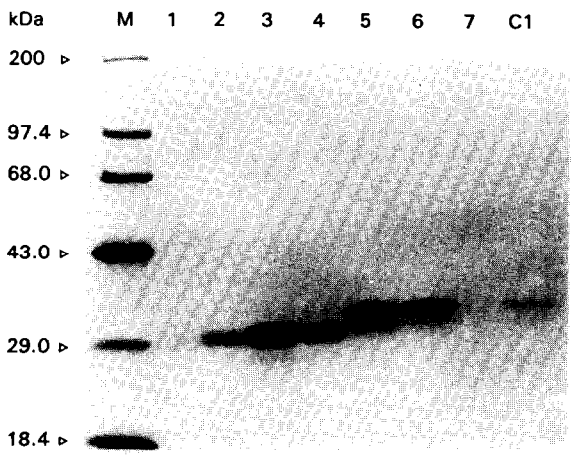

Fig. 4. SDS-PAGE of protein fractions eluted from S Sepharose on a 15% gel. M = protein molecular mass markers as described in the legend of Fig. 2; lanes 1–7 = fractions 17–23 from S Sepharose (20 μl each); C1 = purified C1 repressor protein (1 μg).

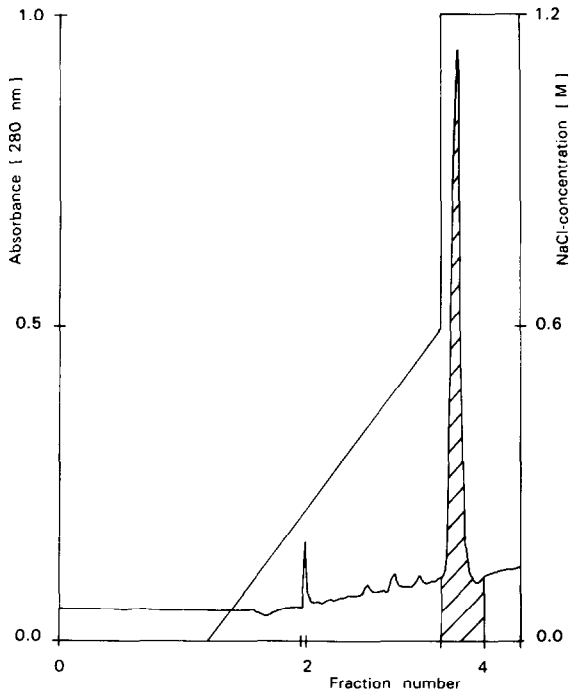

Fig. 5. Elution profile of C1 repressor on Mono S. The desalted fraction 22 obtained from S Sepharose chromatography (3 ml, 0.53 mg/ml) was loaded on to an analytical Mono S column (1 ml), pre-equilibrated and washed with buffer C. Elution was performed using a gradient: 0% buffer D in 10 ml, 0–50% buffer D in 15 ml and 100% buffer D in 5 ml. The flow-rate was 1 ml/min and the operating pressure range 0–3.5 MPa. Only peaks with a peak threshold of 3% full-scale were collected and the maximum fraction size was set to 8 ml. The hatched area indicates the fraction containing only the C1 repressor.

ed at ≥ 0.45 *M* NaCl (data not shown), corresponding to a very small peak in Fig. 5, which is due to contamination of the applied C1 repressor fraction 22 with C1*.

The Mono S column is the most effective step in separating the C1 from the C1* repressor. Desalting of the repressor-containing fraction and the final exchange of buffer were done by using a Fast Desalting HR 10/10 column, equilibrated with buffer F [20 m*M* Tris–HCl (pH 7.6), 0.1 m*M* EDTA, 1 m*M* DTT, 50 m*M* NaCl, 50% glycerol). The same purification and concentration procedure was applied to fraction 21 and the pooled fractions 18–20 obtained from S Sepharose chromatography (data not shown). The C1 and C1* fractions resulting from rechromatography of fraction 21 (Fig. 4, lane 5) on Mono S were pooled with the corresponding C1 and C1* fractions.

The results of the final purification step of C1 and C1* repressor are shown in Fig. 6. SDS-PAGE revealed highly purified C1 and C1* repressor proteins. In addition, the identity and purity of the repressor preparations were tested by the very sensitive Western blot analysis (data not shown). Aliquots of the purified proteins were kept at −70°C and were stable for at least 1 year.

C1 repressor assay

This assay is based on the decreased mobility of protein–DNA complexes during electrophoresis compared with the mobility of free DNA [8,9]. C1 repressor has been shown to bind specifically to P1 Op86 operator DNA *in vitro* as revealed by DNAse I footprinting and by a DNA mobility shift [5].

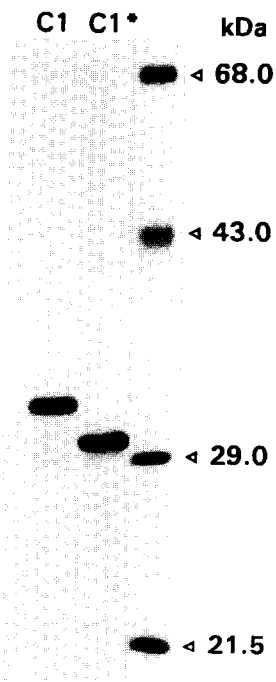

Fig. 6. SDS-PAGE of purified C1 and C1* repressor proteins eluted from Mono S on a 15% gel. The peak fractions from Mono S were desalted and the buffer was changed prior to electrophoresis. C1 and C1* = purified repressor proteins (2.5 μg each in buffer F); M = protein molecular mass markers (in kilodaltons as indicated) in descending order (3 μg each): bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor.

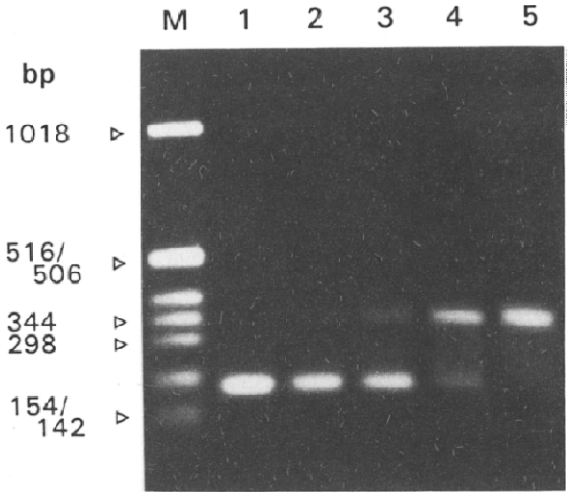

Fig. 7. C1 repressor assay. The purified C1 repressor protein was incubated with Op86 operator DNA for 15 min at 37°C in buffer E (total volume 25 μl) and subsequently subjected to analysis on a 1.5% agarose gel. Lanes: 1 = 198-base pair DNA fragment with P1 operator Op86 (1.2 pmol); 2–5 = increasing molar ratios of C1 repressor to constant amounts of operator DNA (1.2 pmol each lane); 2 = 0.6 pmol C1 (0.5:1); 3 = 1.2 pmol C1 (1:1); 4 = 2.4 pmol C1 (2:1); 5 = 4.8 pmol C1 (4:1); M = DNA molecular mass marker [in base pairs (bp) as indicated].

The P1 Op86 operator DNA was incubated with increasing amounts of FPLC-purified C1 repressor (Fig. 7). On binding of the purified C1 repressor, the operator DNA is gradually shifted to an upper position with decreased electrophoretic mobility (Fig. 7, lanes 2–5). At a molar ratio of four C1 repressor molecules per C1 repressor binding site (Fig. 7, lane 5) all operator DNA is shifted. Therefore, we conclude that the purified C1 repressor is active *in vitro*. When the experiment was repeated with C1* repressor, the DNA-binding activity was reduced at least twofold compared with the C1 repressor (data not shown).

CONCLUSIONS

This chromatographic procedure has been found to be well suited for the purification of C1 and C1* repressor proteins in parallel. Especially for the crystallization of the C1 repressor highly purified proteins are an essential requirement. Also, a reliable determination of biophysical data, such as binding or equilibrium constants, depends very much on homogeneous protein preparations. The use of the described procedure is not restricted to the isolation of the P1 C1 repressor but can also be used for the purification of other repressor proteins. We have already successfully purified the Bof co-repressor from bacteriophage P1 by a very similar procedure [12].

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