Purification and Characterization of *Ceq*I Restriction Endonuclease

Zsuzsa Izsvák, Zsolt Jobbágy, and Ernő Duda

Institute of Biochemistry, MTA Biological Research Center, Pf. 521, Szeged, H-6701 Hungary

Z. Naturforsch. **47c**, 830–834 (1992); received May 26/August 17, 1992

Restriction Enzyme, Endonuclease, Purification, *Corynebacterium*, Multimeric Enzyme

*Ceq*I, a type II restriction endonuclease, an isoschizomer of *Eco*RV was purified to apparent homogeneity by a combination of salt precipitation, ion exchange, dye affinity and hydrophobic interaction chromatographies. The crude enzyme was present in the form of large aggregates that could be pelleted by high speed centrifugation. The enzyme was not associated with cellular membranes, though non-ionic detergents lowered the apparent size of the aggregates. The purified enzyme also showed a tendency to form large molecular mass (66–600 kDa) complexes under physiological conditions, in the absence of cleavable DNA. The enzyme formed smaller complexes in the presence of DNA and non-ionic detergents and dissociated into subunits (and undergoes reversible loss of activity) in the presence of high concentrations of salts. According to SDS gel electrophoresis and sedimentation analysis the molecular mass of the monomer 32 ± 2 kDa. The enzyme had a rather broad pH optimum, extending into the alkaline range and lost specificity and activity in buffers below pH 6.

Introduction

We have found a new restriction endonuclease, *Ceq*I, in *Corynebacterium equii*, that recognizes the sequence GATATC and cleaves it in the middle, producing blunt ended fragments [1]. *Ceq*I shows quite unusual properties for a restriction endonuclease. It sediments as a large complex by ultracentrifugation, Biogel columns do not separate the enzyme from DNA.

In this paper we describe the purification of *Ceq*I to homogeneity and some of the biochemical properties of the enzyme. Beside its unusual properties the pure enzyme might be of interest since its isoschizomer, *Eco*RV, is one of the best studied restriction enzymes [2–5]. The two enzyme producing organisms are not related and the proteins exhibit very different structural properties, yet they recognize the same sequence, cleave it similarly and show similar loss of specificity under certain conditions [6–8]. It would be therefore interesting to compare these proteins at the molecular level.

Materials and Methods

Cells and enzyme purification

Corynebacterium equii cells were grown in L broth or on the surface of LB agar plates at 37 °C for 7 days and stored at 4 °C for two additional days. The cells were washed with buffer A (10 mM potassium phosphate, pH 7.5, 10 mM β-mercaptoethanol and 10 mM EDTA), centrifuged (3000 × *g*, 30 min) and kept frozen until used, at −20 °C.

Proteins were analyzed on 10% SDS polyacrylamide gels as described by Laemmli [9]. The gels were visualized by Coomassie Brilliant Blue or silver staining [10]. Protein content was quantitated by the dye binding method [11] or by measuring the absorbance of the solution at 280 nm (using bovine serum albumin as a standard protein).

DNA, oligonucleotides

Bacteriophage lambda DNA, pBR 322, its restriction fragments and most restriction enzymes were produced and purified in our institute according to standard procedures. The oligonucleotide GGGATATCCC was a kind gift of Dr. A. Pingoud, Hannover, Germany, other oligonucleotides were synthesized in our institute by L. Szilák. DNA, DNA fragments and oligomers were end-labelled with the use of T4 polynucleotide kinase, purchased from Boehringer, Mannheim, Germany.

Determination of enzyme activity

*Ceq*I activity was determined in buffer R (50 mM tris(hydroxymethyl)aminomethane, Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM magnesium chloride, 1 mM dithiothreitol) in a total volume of 50 µl for 1 h at 37 °C, either with 1 µg DNA or synthetic oligonucleotides. DNA fragments were separated

Reprint requests to E. Duda.

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/92/1100–0830 $ 01.30/0

in agarose gels using a home-made apparatus and Tris-borate buffer (90 mM Tris-borate, pH 8.0, 2 mM EDTA).

The DNAs used in these experiments were pBR322, the 377 bp long *Eco*RI-*Bam*HI fragment of pBR322 and a 20 bp oligonucleotide containing the GATATC sequence.

Results

Enzyme purification

Cells (40 g) were suspended and disrupted in 3 volumes of buffer A by sonication or by shearing in a French press. Cell debris was removed by careful centrifugation at low speed (5000 × *g*, 30 min) to avoid losses of enzyme activity. The enzyme was present in the crude extract in the form of a purple complex of proteins which sedimented at 5-300 S.

The intensely colored supernatant was mixed with 0.1 volumes of a 10% streptomycin solution and agitated at 0 °C for 30 min to precipitate nucleic acids and ribosomes. After centrifugation (Beckman JA-20 rotor, 5000 × *g*, 20 min) the supernatant was mixed with solid ammonium sulfate to achieve 70% saturation and stirred on ice (pH of 7.4 was maintained by addition of 2 M tris(hydroxymethyl)aminomethane (Tris) base). The precipitate was collected by centrifugation (50,000 × *g*, 20 min) and resuspended in buffer A that contained 2 M ammonium sulfate. The proteins were loaded onto a phenyl-Sepharose column (2 × 43 cm), previously equilibrated with the same buffer. A decreasing gradient of ammonium sulfate in buffer A was applied. The colored material eluted with approx. 500 mM ammonium sulfate. The gradient was stopped here and the endonuclease was eluted after this peak with buffer A.

Enzyme activity was assayed and active fractions were dialyzed and loaded onto an aminopentyl(or aminohexyl-)-Sepharose column (1.5 × 8 cm) equilibrated with buffer A. 0–1 M KCl gradient in buffer A was used to elute the enzyme. The peak of activity was detected between 350–400 mM KCl.

A Mono-Q FPLC column was used for further purification. After load and washing, 57 ml of a linear gradient of 0–0.7 M KCl in buffer B was used (0.5 ml/sec, 1 MPa). The catalytically active fractions were dialyzed against buffer A, filtered through a phosphocellulose column and final purification was achieved by chromatography on Blue-Sepharose. The enzyme was eluted with a 0–200 mM KCl gradient in buffer A. The active fractions were dialyzed against buffer A containing 50% glycerol and stored at −20 °C without freezing. Table I summarizes data of the enzyme purification and Fig. 1A shows the electrophoretogram of the purified *Ceq*I.

A simplified protocol was used for the production of partially pure enzyme preparations for recombinant DNA experiments. The protocol included streptomycin and ammonium sulfate precipitations as above, DEAE column chromatography or MonoQ FPLC in the absence of detergents. The enzyme was approx. 70–80% pure in these preparations and exhibited high specific activities and a remarkable stability even at room temperature.

Preparations exposed to detergents or phenyl-Sepharose chromatography (essential steps to purify the enzyme to homogeneity) became less active and stable. This explains the fact that homoge-

Table I. Summary of the purification procedure. The amount of the enzyme that catalyzes the complete digestion of 1 μg DNA/h in 50 μl of buffer R at 37 °C is defined as one unit of the enzyme.

Step	Sample	Total protein [mg]	Total activity [1000 U]	Specific activity [U/mg]	Purification	Recovery [%]
1.	Crude extract	7050	189	26.8	1	100
2.	Streptomycin-sulfate	5600	240	42.85	1.6	127
3.	Ammonium-sulfate	2780	178.5	64.2	2.4	94.4
4.	Phenyl-Sepharose	123	34.8	282.9	10.6	18.4
5.	Aminohexyl-Sepharose	10.5	32.0	3047	113.7	16.9
6.	MonoQ	0.55	18.0	32727	1221.0	9.5
7.	Phosphocellulose + Blue-Sepharose	0.15	8.05	53667	2002.5	4.26

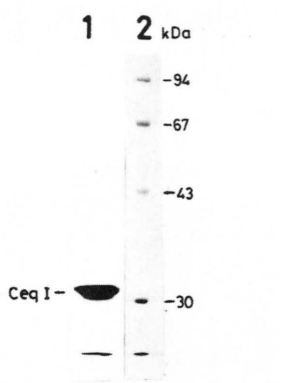

Fig. 1. Electrophoretic analysis of the pure *Ceq*I enzyme and the determination of the molecular mass of the subunits of *Ceq*I according to the electrophoretic mobility. Lane 1: *Ceq*I enzyme, purified as in Table I (5 μg); lane 2: mol. mass marker proteins. The proteins were electrophoresed in an SDS polyacrylamide denaturing gel, according to Laemmli [9].

neous preparations of *Ceq*I display a rather low specific activity (Table I), compared to other type II restriction enzymes.

Reaction optima of CeqI

The enzyme displayed a fairly broad pH optimum and exhibited its highest activity in relatively high salt buffers (Fig. 2A and B). Concentrated salt solutions (*e.g.* 300 mM KCl) caused reversible inhibition of the enzyme activity. Low salt buffers, especially at pH values below 6 destabilized the protein. The enzyme worked in the presence of both Mg^{2+} and Mn^{2+} ions, though the latter gave rise to *Ceq*I* (star) activity [8]. Similar loss of specificity was induced by the presence of organic solvents, *e.g.* dimethyl sulfoxide (DMSO) and glycerol, but the enzyme became unstable and almost inactive in these solutions [8]. The enzyme activity showed a maximum between 37–42 °C as the protein became denatured above 42 °C (Fig. 2C).

Molecular mass and molecular forms

On the basis of its electrophoretic mobility in SDS polyacrylamide gels, the molecular mass of the subunit of *Ceq*I was estimated to be 32 ± 2 kDa. The native enzyme was present as large aggregates in crude extracts. The possibility that the enzyme was associated with membranes or other cellular structures seemed quite likely, since the presence of Triton X-100 (or Nonidet P-40 a non-ionic surfactant) changed both the sedimentation profile of the enzyme activity and the elution pattern of the crude enzyme on DEAE cellulose columns.

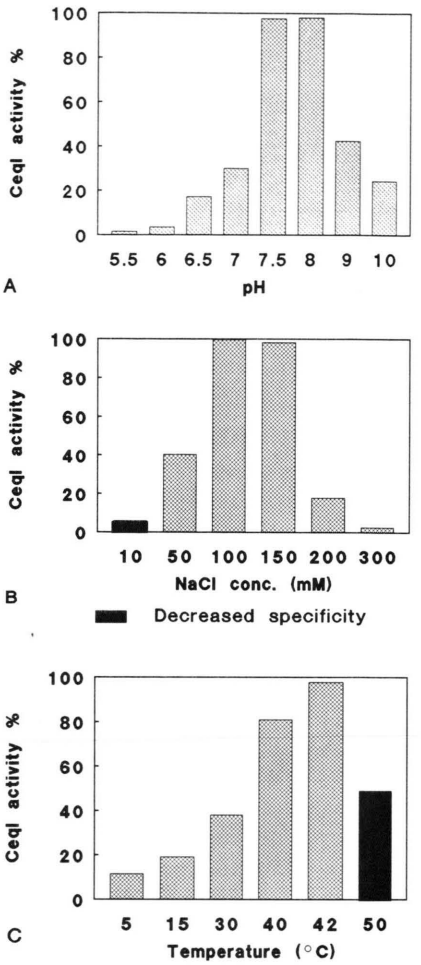

Fig. 2. pH, salt and temperature dependence of *Ceq*I endonuclease.
A: pH dependence. 50 mM Na-acetate buffer was used between pH 5–6, 50 mM Tris-HCl between pH 7–9 and 50 mM NaOH-glycine between pH 9–10.5 in the presence of 10 mM magnesium chloride, 100 mM NaCl and 1 mM DTT.
B: The effect of ionic strength. Indicated concentrations of NaCl were present in a buffer of 10 mM potassium hydrogen phosphate pH 7.4, 10 mM magnesium chloride and 1 mM DTT. Decreased specificity refers to *Ceq*I star activity.
C: Enzyme activity was measured at different temperatures in (optimal) buffer R. The enzyme denatured over 42 °C.

When crude extracts were loaded onto DE 52 columns, the KCl gradient eluted two enzyme peaks, between 165–200 mM and around 1 M KCl. If proteins present in the 1 M eluted, dialyzed fraction were loaded again onto DEAE column all *Ceq*I activity eluted at 165–200 mM (data not shown). Addition of 0.1% Triton X-100 to the crude extract eliminated the second peak, eluting *Ceq*I quantitatively with 200 mM KCl.

Further experiments excluded the possibility that the enzyme was associated with membranes. *Ceq*I is very stable in crude form and tolerates the presence of organic solvents. Precipitation and repeated washings of the protein preparation with acetone and chloroform–methanol, known to dissolve membrane lipids, did not change the sedimentational and chromatographic profiles. Experiments to incorporate the protein into the membranes of artificial lipid vesicles (liposomes) have also been unsuccessful.

Gel filtration and sedimentation analysis of both crude and purified preparations of the enzyme indicated that the tendency to form aggregates is the endogenous property of the enzyme. The sedimentation velocity of *Ceq*I was estimated through linear 5–20% and 5–30% (w/v) sucrose gradients in solutions of high and low ionic strengths (Fig. 3 B and 3 C). Analysis of these data gave an apparent M_r of 32 ± 2 kDa in the presence of 1 M KCl. When physiological concentrations of salts (150–200 mM) were present in the gradients, *Ceq*I activity was measured in the fractions corresponding to more than 300 kDa (Fig. 3).

Gel filtration experiments supported these data, the large (>300 kDa) molecular forms seemed to be predominant under physiological conditions, while the majority of enzyme molecules were present as inactive monomers in 1 M NaCl (data not shown). The addition of DNA (or non-ionic detergents) prevented formation of large complexes or caused partial disaggregation of the oligomers (Fig. 4). They also stabilized the enzyme against heat denaturation (data not shown).

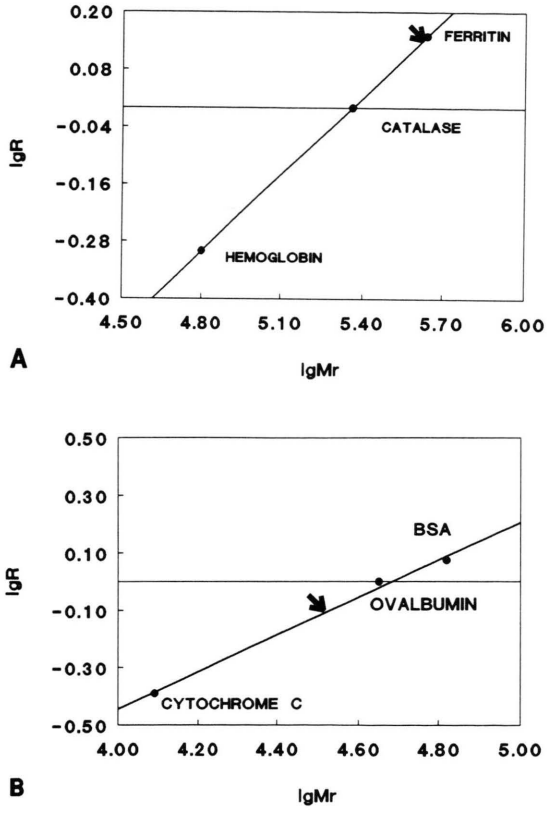

Fig. 3. Determination of the native molecular mass of *Ceq*I endonuclease by sucrose density gradient centrifugation.
A: Purified *Ceq*I and marker proteins were sedimented in a linear 5–20% (w/v) sucrose gradient in buffer B containing 1 M NaCl, as described in [15].
B: Purified *Ceq*I and marker proteins were sedimented in a linear 5–30% (w/v) sucrose gradient in buffer A. The arrows indicate the position of *Ceq*I. The distances travelled by each protein from the meniscus are expressed as a ratio (R), relative to that of ovalbumin (BSA = bovine serum albumin).

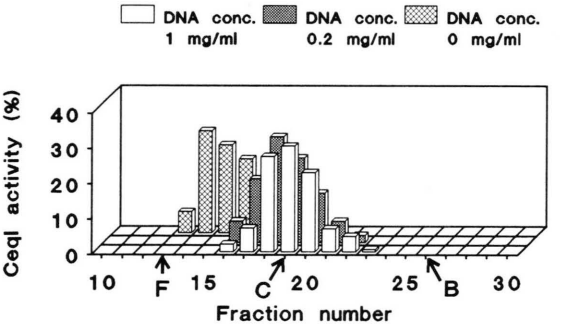

Fig. 4. Dissociation of the large enzyme complexes in the presence of DNA. 100 units (comp. Table I) of partially purified enzyme was used for gel filtration chromatography on a 8 × 400 mm Sephacryl S-300 column in buffer R. As indicated, none, 0.2 or 1 mg/ml sonicated chicken blood DNA (0.5–1 kb sized fragments) was present during the chromatography. F, C and B correspond to ferritin, catalase and bovine serum albumin, respectively. (The endogenous pigment of the extract eluted in fractions 13 and 14 in each cases.)

Discussion

Several enzymes belonging to the type II restriction modification systems form active tetramers [12, 13] and Johannssen *et al.* described *Bgl*I as a 20-mer complex of 61 kDa monomers [14]. In this respect, *Ceq*I restriction endonuclease that forms large homooligomeric complexes under physiological conditions, in crude extracts as well as in purified form, is unusual but not unique. Molecular mass of the subunits is calculated to be 32 ± 2 kDa on the basis of denaturing gel electrophoresis, chromatographic and sedimentation analysis. Both electrostatic and hydrophobic forces participate in the formation of multimeric forms, since the high molecular mass forms disaggregate in concentrated salt solutions or in the presence of non-ionic detergents. High ionic strength solutions completely block the activity of the enzyme while non-ionic detergents do not inhibit the catalytic activity.

The behaviour of the protein during phenyl-Sepharose chromatography suggests the existence of hydrophobic surfaces. These are partially responsible for the multimerization and the binding of detergent molecules. Triton X-100 destabilizes the high molecular mass forms, though complete dissociation of the oligomers can not be achieved. Triton X-100 also protects the enzyme against heat denaturation.

The dynamic equilibrium between dimers and multimers under physiological conditions does not allow the exact measurement of the specific activities of different molecular forms, however drastic changes in the extent of aggregation – caused by the addition of 0.1% Triton X-100 – do not cause a significant change of the enzyme activity.

Enzyme preparations that are purified without detergents and hydrophobic interaction chromatography (these preparations always contained impurities) exhibit much higher specific activities and superior stability over preparations purified as described above. The role of other proteins in the formation and stability of the native enzyme forms is unlikely, but can not be excluded. The anomalous sedimentation and chromatographic behaviour of the enzyme suggests intimate interaction between the subunits (or dimers) of the enzyme. Though the association of the protein molecules seems reversible, it is conceivable that the capability of the enzyme to form superstructures that are present *in vivo* and in crude extracts is lost during the purification procedure.

Acknowledgements

We are very grateful to Drs. A. Kiss, M. Szekeres and J. Moitra for their helpful discussions and criticism, to Dr. A. Pingoud and L. Szilák for the oligonucleotides.

[1] E. Duda, Z. Izsvák, and A. Orosz, Nucl. Acids Res. **15,** 1534 (1987).
[2] A. D'Arcy, R. S. Brown, M. Zabeau, R. W. van Resandt, and F. K. Winkler, J. Biol. Chem. **260,** 1987–1990 (1985).
[3] L. Bougeleret, M. Schwarzstein, A. Tsugita, and M. Zabeau, Nucl. Acids Res. **12,** 3659–3676 (1984).
[4] G. V. Kholmina, B. A. Rebenitsh, Y. S. Koblov, A. A. Mironov, N. K. Yankovskii, Y. I. Kozlov, L. I. Glatman, A. F. Moroz, and V. G. Debabov, Dokl. Akad. Nauk. SSSR **253,** 495–497 (1980).
[5] C. A. Frederick, J. Grable, N. Melia, C. Samudzi, L. Jen-Jacobson, B. Wang, P. Greene, H. W. Boyer, and J. M. Rosenberg, Nature **309,** 327–331 (1984).
[6] S. E. Halford, B. M. Lovelady, and S. A. McCallum, Gene **41,** 173–181 (1986).
[7] S. E. Halford, B. M. Lovelady, and S. A. McCallum, Biochem. Soc. Trans. **14,** 260–262 (1986).
[8] Z. Izsvák and E. Duda, Biochem. J. **258,** 301–303 (1989).
[9] U. K. Laemmli, Nature **227,** 680–687 (1970).
[10] C. R. Merril, D. Goldman, S. A. Sedman, and M. H. Ebert, Science **211,** 1437–1438 (1981).
[11] A. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[12] L. A. Smith and J. G. Chirikijan, J. Biol. Chem. **254,** 1003–1006 (1979).
[13] B. Polisky, P. Green, D. E. Garfin, B. J. McCarthy, H. M. Goodman, and H. W. Boyer, Proc. Natl. Acad. Sci. U.S.A. **72,** 3310–3314 (1975).
[14] W. Johannssen, H. Schutte, F. Mayer, and H. S. Mayer, J. Mol. Biol. **143,** 707–726 (1979).
[15] R. G. Martin and B. N. Ames, J. Biol. Chem. **236,** 1372–1379 (1961).