Positive co-operative interaction between the subunits of *CeqI* restriction endonuclease

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CeqI restriction endonuclease, an isoschizomer of *EcoRV*, forms complexes of 12–20 subunits under physiological conditions, in the absence of DNA. These molecules partially dissociate in the presence of DNA sequences recognized by *CeqI* or in the presence of non-ionic detergents. In solutions containing high concentrations of salts (e.g. 1 M-NaCl), the enzyme dissociates into subunits, concomitantly losing its activity. According to our experiments, it is the tetrameric form of the enzyme that binds the DNA and represents the catalytically active molecule. Analysis of the enzyme kinetics revealed a positive co-operative interaction between the subunits of the enzyme. Computer-assisted analysis of these data yielded a Hill coefficient of approx. 1.35, suggesting two binding sites per tetrameric enzyme molecule, two subunits per palindromic recognition site.

INTRODUCTION

CeqI restriction enzyme, an isoschizomer of *EcoRV*, is purified from *Corynebacterium equi* [1]. *EcoRV* exists as a dimer under physiological conditions [2], and the molecular mass of native *CeqI* is approx. 400–600 kDa as determined by gel-filtration and centrifugation experiments. Since the molecular mass of the subunits is determined to be 32 ± 2 kDa (Z. Izsvák, Z. Jobbágy & E. Duda, unpublished work), this value suggests the existence of 12–20 subunits in the oligomeric enzyme molecules.

CeqI is not the only restriction enzyme in which the subunits display a tendency to aggregate. Johannssen *et al.* [3] reported a similar observation on *BglII*, where 20 monomers form an enzyme complex of molecular mass approx. 1200 kDa. Widely used restriction enzymes, such as *EcoRI* [4], *BamHI* [5] and *Bst1503* [6], show somewhat similar properties also. In their native forms they exist as a mixture of dimers and tetramers. According to Modrich & Zabel [4], the tetrameric form of *EcoRI* constitutes a large fraction (80 %) of this restriction endonuclease within the cell.

It is still not clear what the role of these complexes could be (if they have a role at all). In most cases a moderate increase in the ionic strength eliminates the high-molecular-mass forms. Since *CeqI* restriction endonuclease exhibits maximal activity in a relatively high-ionic-strength environment, it is not unexpected that high salt concentrations are required for the dissociation of its subunits.

In this paper we demonstrate that the tetrameric *CeqI* enzyme is the catalytically active form. This is quite unusual, since most restriction enzymes are either monomers cleaving non-palindromic sequences or composed of two identical subunits that carry out a co-ordinated action on palindromic recognition sequences. Our experiment suggests another unusual property of *CeqI*, the positive co-operation between the subunits of the tetrameric enzyme binding to two recognition sequences.

MATERIALS AND METHODS

DNA

All the plasmids we used were purified in our laboratory according to standard procedures [7]. The plasmid pPC9 is a derivative of pUC19. A GATATC-containing decamer oligonucleotide was cloned into the polylinker of pUC19 at the *SmaI* site. Ethidium bromide dot quantification was used to estimate the concentration of fragments in solution. Ethidium bromide

fluorescence of droplets of the solutions of known DNA concentrations were compared with that of the measured solutions, as described in [8].

Enzymes

Corynebacterium equi cells were disrupted by using a French press, and *CeqI* was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation (50–70 % saturation), followed by DEAE- and aminopentyl-Sepharose chromatography. No other nuclease, phosphatase, DNA methylase or ligase activity was detected in this preparation. Final purification was achieved by chromatography on a Mono Q f.p.l.c. column (Pharmacia). *CeqI* constituted more than 85 % of the protein after these purification steps as assessed by SDS/PAGE. Hydrophobic interaction chromatography and non-ionic detergents (used in the preparation of homogeneous *CeqI*) were not used during the purification. Protein concentration was determined spectrophotometrically at 280 nm or by the dye-binding method.

All other restriction enzymes, the Klenow fragment of *Escherichia coli* DNA polymerase and T4 DNA ligase were purified in our Institute. Buffer A contained 10 mM-potassium phosphate, pH 7.4, 100 mM-NaCl, 10 mM-2-mercaptoethanol and 0.1 mM-EDTA.

Determination of enzyme activity

To detect slight changes in the activity of the enzyme we used the following assay: end-labelled DNAs containing one *CeqI*-recognition site in the middle of the sequence were cleaved by the enzyme to achieve approx. 50 % digestion. In this case the separating gel resolved two bands of almost the same intensity [undigested (U) and digested (D)]. Any change in enzyme activity results in a different proportion of the two bands. We determined the value of $D/(D+U)$ by both liquid-scintillation counting of the radioactivity found in the gel slices and scanning the films of the autoradiograms (or ethidium bromide-stained gels). The DNAs used in this type of experiment were either the 377 bp-long *EcoRI*–*BamHI* fragment of pBR322 or a 20 bp-long oligonucleotide containing the GATATC sequence, both end-labelled with ^{32}P .

Mobility-shift electrophoresis

A 61 bp *EcoRI*–*HindIII* fragment of pPC9 was used for mobility-shift electrophoresis as described by Fried & Crothers [9], after end-labelling by the Klenow fragment of the *E. coli* DNA polymerase. The reaction mixture contained

10 ng (0.27 pmol) of end-labelled fragment of pPC9, 1 g of poly(dI-dC), 200 g of BSA, 5 % glycerol, 100 mM-NaCl, 1 mM-dithiothreitol, 50 mM-Tris/HCl (pH 7.8), 1 mM-EDTA and 0.003–3.0 pmol of crude or partially purified *CeqI* in a final volume of 20 μ l. The samples were incubated for 20 min at room temperature before loading.

When purified enzyme preparations were used, 1.3–0.0065 pmol of *CeqI* and 5 ng (0.13 pmol) of fragment were incubated for 20 min at room temperature, then glycerol and Bromophenol Blue were added and the samples were loaded on 4.7 % polyacrylamide gels in low-ionic-strength buffer [10] at room temperature. $MgCl_2$ (10 μ l; 1 mM) was added to one reaction mixture instead of EDTA just before the loading of the sample.

Kinetic analysis

PvuII-linearized pBR322 was the substrate DNA in the digestion reactions. The substrate concentration varied between 0.125 and 10 nM. The reaction mixture contained 0.06 pmol of purified *CeqI*, 100 mM-NaCl, 50 mM-Tris/HCl (pH 7.5), 10 mM- $MgCl_2$ and 1 mM dithiothreitol in a final volume of 20 μ l. The digestion was stopped by adding 0.1 vol. of stop solution (0.1 % Bromophenol Blue/0.2 M-EDTA/50 % glycerol) to the samples, which were run in 1 % agarose gels. Negatives and/or autoradiograms were scanned with a Beckman DUV-8 spectrophotometer. Similar methods have been reported earlier [10,11]. Initial velocity versus substrate concentration plots were fitted and analysed by using the computer program COSY [12], a gift from Dr. M. Eberhard (Biozentrum, University of Basel, Basel, Switzerland).

RESULTS

Multimeric forms

Molecular-sieve chromatography (Fig. 1) and sedimentation-analysis experiments (not shown) suggest that *CeqI* is present in a high-molecular-mass form under physiological conditions, in the absence of DNA (Fig. 1, stippled bars). On the basis of these data we calculated that the native enzyme consists of 12–20 monomers. The monomer size was determined to be 32 ± 2 kDa by SDS/PAGE (Z. Izsvák, unpublished work).

In the presence of non-ionic detergents (e.g. Triton X-100; Fig. 1, open bars) or cleavable DNA, the molecular mass of the enzyme decreases to a value roughly equivalent to the sum of four subunits. High concentrations of salts, e.g. 1 M-NaCl, dissociate the enzyme complexes completely, converting them into monomers (Fig. 1, closed bars). Whereas the activity of the enzyme is not, or only marginally, affected by non-ionic detergents, the presence of 1 M-salt totally abolishes catalytic activity. The inhibition is completely reversible; after removal of the salt, the enzyme regains its activity simultaneously with its tetrameric form.

Any enzyme fraction of the molecular-sieve chromatography can be converted into the other forms by selecting appropriate conditions.

Mobility-shift analysis

To answer the obvious question, how many subunits form the active enzyme, we used mobility-shift gel-electrophoretic analysis. Type-II restriction endonucleases cleave the DNA (at their recognition sites) only in the presence of Mg^{2+} (some enzymes, including *CeqI* also in the presence of Mn^{2+}). The presence of DNA–endonuclease complexes can be detected by mobility-shift electrophoresis in the absence of these bivalent cations. Fig. 2 shows DNA–protein complexes formed between *CeqI* and the 61 bp-long DNA fragment, containing one recognition site.

Fig. 1. Different molecular forms of *CeqI* and the effect of high salt concentrations and non-ionic detergents on the complexity of *CeqI* restriction endonuclease

Purified *CeqI* was subjected to gel filtration on Sephacryl-300 in buffer A + 0.1 % Triton X-100 (□), buffer A + 1 M-KCl (■) and buffer A (▨). Protein markers used for calibration were ferritin (F, 440 kDa), catalase (C, 232 kDa) and BSA (B, 67 kDa) indicated by arrows. Enzyme activity was determined after the removal of the salt and detergents.

Fig. 2. Mobility-shift analysis of a partially purified *Corynebacterium equi* extract

The reaction conditions were as described in the Materials and methods section. Some 1.35 pmol of labelled 61 bp-long pPC9 (*EcoRI*–*HindIII*) fragment was added to each sample. Lanes 1–3 contained 0, 1.3 and 0.65 pmol respectively of crude enzyme (molarity was calculated as monomers); lanes 4 and 5 contained 0.13 and 0.065 pmol of partially purified *CeqI* enzyme in the reaction mixture. The two retarded bands (a and b) were calculated to be approx. 130 and 75 kDa according to their relative mobility [13], corresponding to the tetramer and presumably the methylase. Lane 6 contained 3 pmol of purified enzyme and 1 mM- Mg^{2+} instead of EDTA. Band a corresponds to the protein–DNA complex, two additional bands, d and e, are the cleavage products of the 61 bp-long fragment (35 and 26 bp). Band c represents the unbound fragments.

Fig. 3. Effect of Mg^{2+} ions on the mobility-shift pattern of *CeqI* enzyme

The experiment was carried out as described in the Materials and methods section. The binding mixtures contained 2.7 pmol of labelled 61 bp-long fragment and decreasing amounts of purified *CeqI*. Lanes 1–9 contained 0, 3, 1.3, 0.6, 0.3, 0.06, 0.03, 0.01, 0.0065 and 0.03 pmol of purified *CeqI* respectively. Mg^{2+} (10 μ l; 10 mM) was added to slot 10 when the electrophoresis started. The filled arrow indicates the 130 kDa protein–61 bp-long DNA complex. The empty arrow shows a gap in the radioactive DNA smear at the same position in samples (8–) 9–10.

Fig. 4. Lineweaver–Burk and Hill plots of *CeqI* enzyme kinetics

Enzyme activity was measured as described in the Materials and methods section. The indicated amounts of *PvuII*-linearized pBR322 plasmid were used as substrate for the digestions. Reactions were followed for 15 min. During this reaction time only partial digestion was achieved. Agarose gels were run, photographed under u.v. light and exposed to X-ray films. The negatives and autoradiograms were scanned with a Beckman DUV-8 densitometer. The radioactivity in the bands was counted in a liquid-scintillation counter. The Lineweaver–Burk plot of the enzyme (a) indicated positive co-operativity of the subunits, the Hill plot (b) suggests two binding sites per enzyme complex.

Mobility-shift experiments, carried out in low-ionic-strength buffer systems, suboptimal for the activity of the enzyme, revealed the presence of several complexes formed between the enzyme and the DNA probe (results not shown). The relative electrophoretic mobilities of the retarded bands (relative to the free

DNA) were measured and these values were plotted against the values of $\log M_r$ for the putative DNA–enzyme complexes [13]. The values that fitted in a straight line suggested one dimer of subunits per complex being the smallest and two dimers per complex being the most abundant of all bands. Large complexes, containing up to 12 dimers, were slightly visible (results not shown). The increase in ionic strength in the gels eliminated most of the bands, except the increasingly dominant one corresponding to a 130 kDa tetrameric form (Fig. 2).

The faint band (of approx. 75 kDa) probably represented the *CeqI* methylase, because it could only be detected in crude extracts, not in purified preparations.

In the presence of Mg^{2+} , the above described bands disappeared and the cleavage products of the 61 bp DNA were visible below the free DNA front (Fig. 2; lane 6). When low concentrations of Mg^{2+} diffused into the gel (Fig. 3), a gap formed in the radioactive DNA smear at the presumed place of the tetrameric-enzyme–DNA-fragment complex in slots 6–10. (The X-ray film was slightly overexposed.)

Summarizing these results, it seems very likely that the DNA-binding form of *CeqI* is the tetrameric enzyme not the aggregated high-molecular form. The fact that the substrate DNA has a dissociating effect on the large protein complex could play an important physiological role.

Kinetic analysis

Several lines of evidence suggested a possible co-operativity between the subunits of the enzyme. Although the enzyme was more active in the presence of Mg^{2+} ions (6 mM) than in the presence of Mn^{2+} ions (6 mM), the addition of Mg^{2+} ions to the Mn^{2+} -activated enzyme decreased its catalytic activity. The activity reached a minimum at equimolar concentrations. The activity of the isoschizomer, *EcoRV*, increased with increasing Mg^{2+} concentrations, displaying a maximum (between 3 and 10 mM) regardless of the presence of Mn^{2+} ions.

Addition of short oligonucleotides containing the recognition sequence (GATATC) to the reaction mixtures increased the specific activity of *CeqI* enzyme. On the other hand, oligonucleotides with similar sequences (five or six homologues) had an inhibitory effect. In parallel experiments, the specific activity of *EcoRV* was not influenced at all by the addition of either oligomers (Z. Jobbágy and Z. Izsvák, unpublished work).

We used kinetic analysis of the enzyme-activity data to demonstrate the co-operation of the subunits. *PvuII*-linearized pBR322, which contains one recognition site, was used as substrate, as detailed in the Materials and methods section. As shown in Fig. 4, the Lineweaver–Burk plot of the results unambiguously differed from the ones obeying Michaelis–Menten kinetics and indicated positive co-operation of subunits.

We applied the computer program COSY [12] to the analysis of our kinetic data. The program calculated a Hill coefficient of approx. 1.35, which suggested two substrate-binding sites [14]. These results and data of the mobility-shift analysis suggested a native conformation of the enzyme with four subunits capable of binding to and cleaving two double-stranded DNA molecules in a co-ordinated action.

DISCUSSION

Experiments on *CeqI* restriction endonuclease, an isoschizomer of *EcoRV*, revealed a complex structure of the enzyme. In extracts, the enzyme is present as a large complex of 12–20 subunits which dissociate in the presence of non-ionic detergents or high concentrations of salts. We assume that the high-molecular-mass form represents the enzyme form found in the intact cells. The presence of substrate DNA facilitates a con-

formational change, a dissociation of the multimeric form into active tetrameric enzyme molecules.

Subunits of the active enzyme exhibit some degree of co-operation. Previous experiments showed that addition of short oligonucleotides containing the recognition site of the enzyme increased its activity towards DNA substrates (Z. Izsvák & Z. Jobbágy, unpublished work), whereas sequences that differed by one nucleotide from the authentic recognition site [15] interfered with the activity of the enzyme.

Gel-shift assays prove the DNA-binding property of the tetrameric form and the same conformation seems to be responsible for the catalytic activity of the enzyme. Kinetic analysis of the enzyme-activity data suggests a positive co-operative interaction between the four subunits. The tetrameric enzyme consisting of two dimers each binding one double-stranded DNA is not far from the model of type-II restriction endonucleases. What seems to be new is the co-ordinated action of the two dimers on two DNA molecules. Theoretically the positive co-operativity of the restriction enzyme molecules and the presence of high-molecular-mass complexes as reservoirs of (inactive) subunits could be an advantage for the host cells. As the complexes dissociate in the presence of substrate, stored enzyme molecules can be immediately mobilized in the case of foreign DNA 'invasion'. Since many restriction enzymes are ready to form higher-molecular-mass complexes and very few results are available about the kinetics of restriction enzymes, it could turn out that the behaviour of *CeqI* is not so exceptional after all.

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