Inhibiting the dimeric restriction endonuclease *Eco*RI using interfacial helical peptides

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Background: Many enzymes are active only in a dimeric form, including a variety of type II restriction endonucleases. Disruption of subunit interactions is therefore a potential method for multimeric enzyme inhibition. *Eco*RI is a homodimeric restriction endonuclease, the dimeric interface of which consists of a four-helix bundle. We set out to design helical peptides to interact with this interface and block dimer formation, thus rendering *Eco*RI inactive.

Results: Here we describe two synthetic, helical peptides based on the interfacial region of *Eco*RI. Both peptides inhibit the enzyme, but the peptide derived from the α4 helix of *Eco*RI had both a higher helical content and better efficacy than a variant peptide, α4(Leu), that has three Ile→Leu mutations (IC_{50} values of 27 μM and 90 μM, and helical contents of 29% and 10%, respectively). Size-exclusion chromatography confirmed that the α4 peptide disrupted dimerization of *Eco*RI, and circular dichroism indicated that *Eco*RI remained folded upon binding to α4. Inhibition with α4 and α4(Leu) was shown to be specific for *Eco*RI, as the dimeric restriction enzyme *Pvu*II was not affected by the peptides.

Conclusions: Interfacial peptide inhibitors of the dimeric *Eco*RI were obtained that both inhibit dimerization and endonuclease activity. The peptide sequence with a preference for a helical conformation was a more effective inhibitor, presumably because the more preorganized state enhanced interactions with the helical interface of *Eco*RI. The specific nature of this endonuclease–peptide interaction was also confirmed. The potential of this strategy for inhibiting other enzyme classes is currently being addressed.

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Introduction

Many enzymes that have multimeric subunits function only when in an associated form. Extensive work has shown that enzyme active sites might be targeted by small molecules, thereby limiting access of the substrate to the catalytic site. An alternative method that we have developed is to target the interfacial area of multimeric enzymes rather than focusing on the active site. Here, vulnerable areas can often be found to disrupt the enzyme interface, and, through dissociation of the subunits, inhibition of the enzyme might be achieved.

Dissociative inhibition has been applied to the enzymes ribonucleotide reductase [1–3] and HIV-1 protease [4–7]. In both cases peptide sequences corresponding to terminal, interfacial regions of the enzymes were used as starting points for the design of dissociative inhibitors. Here, a new dimeric interface from the widely used restriction endonuclease *Eco*RI was targeted for inhibition. Inhibition of *Eco*RI has been observed by disrupting the protein–DNA interactions using intercalators [8], or by the addition of triple-helix-forming oligonucleotides [9]. As the two active-site regions of dimeric *Eco*RI are approximately 18 Å apart, it would be difficult to design a small molecule that could span both sites. The design of agents that target the dimerization interface of *Eco*RI, therefore, might be a more feasible approach to inhibition in this case.

The overall fold of *Eco*RI consists of eight α helices and eleven β strands, two helices of which, α4 and α6, make up the majority of interfacial contacts, and assemble as a four-helix-bundle dimerization interface (Figure 1) [10]. The shorter of these helices, α6, consists of only seven residues, and was judged to be too small to form a stable helix in solution. The longer of the helices, α4, is involved in many of the interfacial contacts, and was therefore used as the basis of our design. On examining the α4 sequence it appeared that the peptide might not favor a helical conformation because of the presence of the three isoleucine residues [11]. A mutated sequence of α4, α4(Leu), in which three isoleucine residues were replaced with three leucine residues was therefore also synthesized (Figure 2).

Figure 1

Crystal structure of dimeric *Eco*RI showing each monomer in green and white and the interfacial helix (α4) in red [10].

Results and discussion

Peptide conformation and aggregation

Circular dichroism (CD) spectra for α4 and α4(Leu) were evaluated to determine whether the peptides had helical characteristics [12] (Table 1). In aqueous phosphate buffer, the α4 peptide produced a spectrum that is consistent with a helical conformation, having minima at 208 nm and 222 nm, with a helical content of 29%. Contrary to expectation, the helical content for α4(Leu) was only 10% at comparable concentrations. The sequence designed on the basis of the greater helical propensity of leucine than isoleucine did not enhance the stability of the α helix in solution. It was predicted, therefore, that the natural sequence, α4, would interact more effectively with the interface of *Eco*RI.

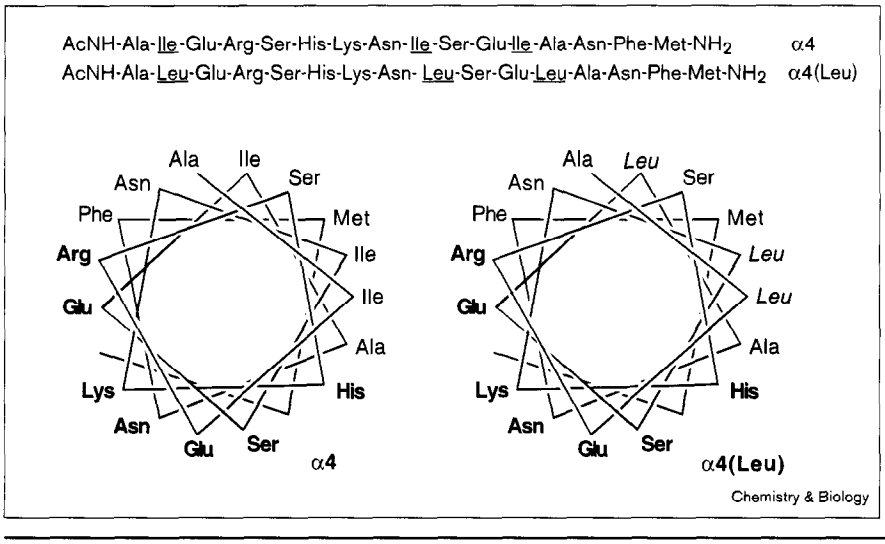
CD spectra of the peptides were also determined with varying concentrations of trifluoroethanol (TFE) to determine if enhanced helical contents were possible. Peptide α4, although more helical in aqueous buffer than the peptide α4(Leu), actually showed less of an increase in helicity with added TFE than α4(Leu); α4 and α4(Leu) reached maximum helical contents of 59% and 83% in 50% TFE, respectively. The presence of leucine residues in α4(Leu) does, therefore, contribute to the overall stability of a helical conformation under forcing conditions.

Size-exclusion chromatography was performed to determine if aggregation of the peptides could account for their differences in helicity. Both α4 and α4(Leu) had apparent molecular weights that corresponded to aggregation states of 1.1 and 1.2, respectively, even when loaded on the column at concentrations as high as 2 mM. These results indicate that the peptides are monomeric in aqueous solution, and would be available in this state to interact with *Eco*RI.

Inhibition of *Eco*RI by synthetic α-helical peptides

An assay based on *Eco*RI DNA cleavage was developed to evaluate inhibition of *Eco*RI by the interfacial peptides. In this assay, the peptide and *Eco*RI were incubated, and the mixture was evaluated for DNA-cleavage ability. The DNA substrate used was pUC19 plasmid that had been linearized with the restriction enzyme *Alw*NI. *Eco*RI cleavage of the linearized DNA should produce two DNA fragments containing 1865 and 821 basepairs as observed using polyacrylamide gel electrophoresis (PAGE). If *Eco*RI was inhibited, however, disappearance of the two bands would be observed with

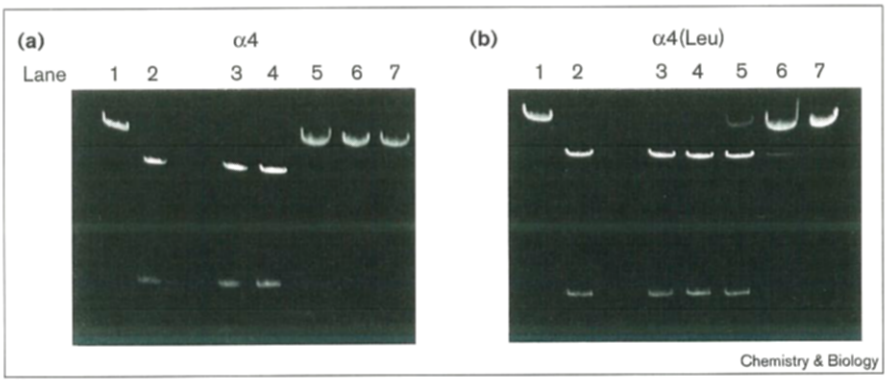
Figure 2



Sequences and helical wheel diagrams of α4 and α4(Leu). Mutated residues are underlined.

Figure 3

Inhibition of *Eco*RI cleavage. **(a)** Lane 1, linearized pUC19; lane 2, linearized pUC19 cleaved with *Eco*RI; lane 3, same as lane 2 with 16 μM α4; lane 4, same as lane 2 with 21 μM α4; lane 5, same as lane 2 with 32 μM α4; lane 6, same as lane 2 with 43 μM α4; lane 7, same as lane 2 with 65 μM α4. **(b)** Lane 1, linearized pUC19; lane 2, linearized pUC19 cleaved with *Eco*RI; lane 3, same as lane 2 with 22 μM α4(Leu); lane 4, same as lane 2 with 45 μM α4(Leu); lane 5, same as lane 2 with 90 μM α4(Leu); lane 6, same as lane 2 with 135 μM α4(Leu); lane 7, same as lane 2 with 203 μM α4(Leu).



the concomitant reappearance of the starting 2686 base-pair DNA.

Varying concentrations of each peptide were pre-incubated with *Eco*RI (4 nM) for three hours at 37°C. This mixture was added to linearized pUC19 DNA and equilibrated for another three hours to allow maximum cleavage. (One to three hour incubation times between the peptide inhibitors and EcoRI yielded the same amount of inhibition.) Analysis of the cleavage reactions using PAGE demonstrated that the α4 peptide inhibited *Eco*RI activity with an IC_{50} value of 27 μM, with complete inhibition at 32 μM peptide (Figure 3a). Significantly higher concentrations of α4(Leu) were required to achieve similar inhibition with an IC_{50} of 90 μM (Figure 3b). These data correlate well with the observed CD data in that the more helical the peptide the better the inhibition, and, presumably, the better the interaction with an *Eco*RI monomer.

Size-exclusion chromatography was employed to evaluate whether or not the observed inhibition with the interfacial peptides is a result of the disruption of protein–protein interactions in the *Eco*RI dimer (Figure 4). An apparent molecular weight of 62,000 was obtained for *Eco*RI (MW 31,000), which corresponds to an aggregation state of 2.0 as anticipated for the dimeric enzyme. Addition of α4 (83 μM) to *Eco*RI (4 μM) produced a dramatic shift in the elution pattern of *Eco*RI; an apparent molecular weight of 24,000 was obtained, indicating that the peptide disrupted the interactions between the two subunits of *Eco*RI. These data, therefore, confirm that α4 inhibits the dimerization of *Eco*RI, ultimately leading to inhibition of DNA cleavage.

Table 1

Circular dichroism data.

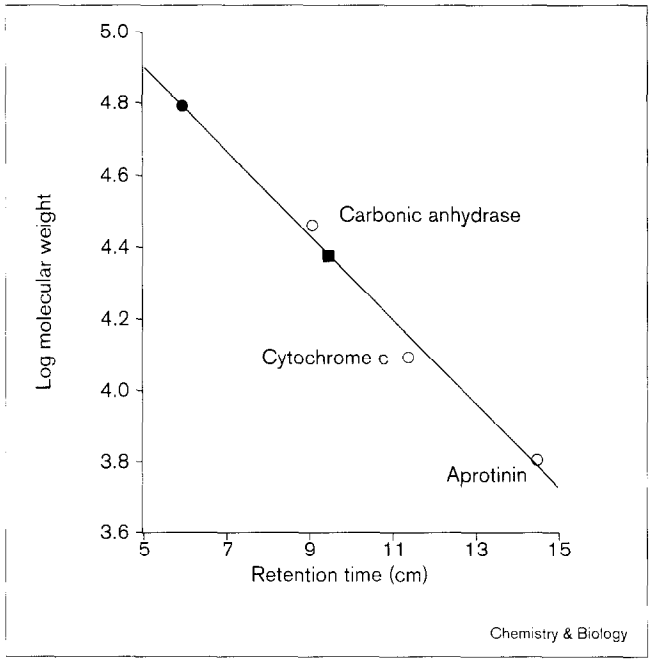
Peptide	θ_{208nm} (deg)	θ_{222nm} (deg)
α4	−11,221	−8,689
α4(Leu)	−7,377	−3,093

CD was used to determine whether *Eco*RI remained folded in the presence of α4. The helical content of *Eco*RI (4 μM) was calculated to be 32% using CD. Addition of α4 at a concentration that was sufficient to inhibit dimerization (83 μM) had little effect on the helical content of *Eco*RI (35%).

Specificity of α-helical peptides for *Eco*RI

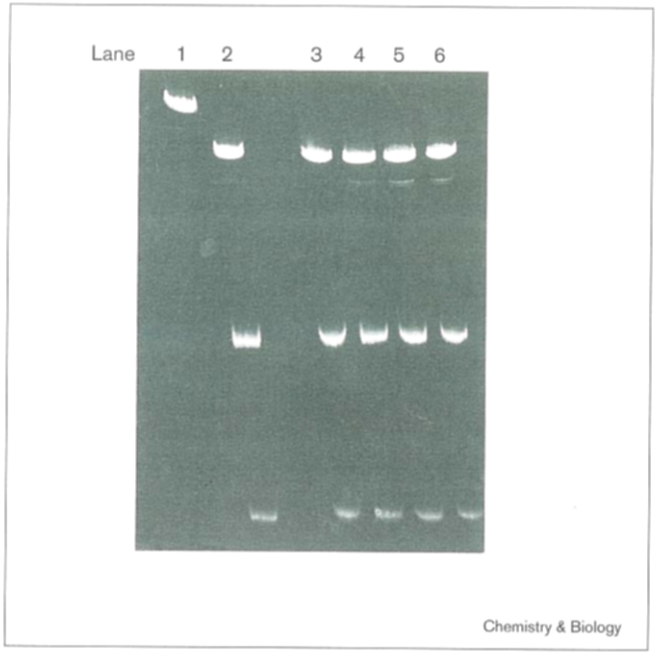
To evaluate the specificity of the interaction between α4 or α4(Leu) and *Eco*RI, the peptides were also assayed for inhibition with another restriction endonuclease, *Pvu*II.

Figure 4



Size-exclusion chromatography of *Eco*RI with (■) and without (●) α4.

Figure 5



Inhibition of *Pvu*II. Lane 1, linearized pUC19; lane 2, linearized pUC19 cleaved with *Pvu*II.; lane 3, same as lane 2 with 7 μM α4; lane 4, same as lane 2 with 14 μM α4; lane 5, same as lane 2 with 28 μM α4; lane 6, same as lane 2 with 56 μM α4.

*Pvu*II is structurally quite dissimilar to the overall fold of *Eco*RI [13]. Instead of a dimerization interface consisting of a four-helix bundle as found in *Eco*RI, the dimeric interface of *Pvu*II contains only two overlapping amphiphilic helices, one from each monomer. It was not anticipated, therefore, that our designed peptides would interact with the interfacial regions of Pvu II in a specific manner because they were derived from the four-helix-bundle interfacial region of *Eco*RI.

*Alw*NI-linearized pUC19 was used to evaluate the effect of the interfacial peptides on *Pvu*II. Three DNA fragments, 1775, 589 and 322 basepairs in length, would result from cleaving linearized pUC19 by *Pvu*II. Incubation of Pvu II with α4 or α4(Leu), followed by addition of the linearized DNA, resulted in no inhibition of cleavage in both cases (Figure 5). Even up to a peptide concentration of 56 μM, all the DNA was in the cleaved state. The finding that α4 and α4(Leu) did not inhibit the DNA-cleaving ability of dimeric *Pvu*II, but could completely block DNA cleavage by dimeric *Eco*RI, supports the assertion that a specific interaction occurs between *Eco*RI and these interfacial peptides.

Significance

Like many other multimeric enzymes, *Eco*RI, a homodimeric restriction endonuclease, is only active in dimeric form. Disruption of the dimeric enzyme is therefore a strategy for enzyme inhibition. The results presented here indicate that inhibition of *Eco*RI with peptides corresponding to its dimerization interface is specific, and is due to a decreased concentration of the active, dimeric enzyme in solution. The restriction enzyme *Pvu*II is distinct from *Eco*RI in its dimerization interface and is not affected by the *Eco*RI interfacial peptides. *Eco*RI is the first enzyme in which subunit dissociation has been observed with peptide fragments internal to the protein sequence by employing a dissociative strategy. It is significant, therefore, that a 16 amino-acid peptide effectively disrupts the association of these large subunits to inhibit *Eco*RI activity. This work brings the prospect of designing low molecular weight, specific dimerization inhibitors of enzymes and receptors closer to a reality.

Materials and methods

Materials

Restriction enzymes and plasmids were purchased from New England Biolabs (Beverly, MA). Acrylamide was purchased from GibcoBRL (Gaithersburg, MD), and other electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Amino acids and solid-phase resin were purchased from Bachem Biosciences (King of Prussia, PA). Sephadex G-50 was purchased from Sigma (St. Louis, MO). All other chemicals and reagents were purchased from Aldrich (Milwaukee, WI) or Mallinckrodt (Paris, KY) unless otherwise specified.

Synthesis of peptides

The peptides were synthesized using a solid phase methodology on the Rink resin [14] using a fluorenylmethoxycarbonyl (Fmoc)-based strategy. The peptides were synthesized in a stepwise manner by the N-hydroxybenzotriazole method, and were purified by preparative reverse-phase high performance liquid chromatography (HPLC) on a C8 column. Peptides were characterized by fast-atom-bombardment mass spectrometry (glycerol/S-glycerol matrix), with an $M + H^+$ peak of 1901.8 (calc'd 1901.21) for both α4 and α4(Leu).

Size-exclusion chromatography

Size-exclusion studies were carried out with the peptides (83 μM) and *Eco*RI (4 μM) at 4°C using a 1.6 cm × 90 cm column of Sephadex G-50 and 50 mM phosphate buffer, pH 7.0. Concentrations of the peptide stock solutions were determined by amino acid analysis. The cluent was monitored by measuring the absorbance at 214 nm. A standard molecular weight curve was generated using bovine serum albumin, carbonic anhydrase, cytochrome *c* and aprotinin. Apparent molecular weights were determined by interpolation of the standards curve.

Circular dichroism spectroscopy

CD spectra were recorded on a Jasco J600 spectropolarimeter at 25°C, in 5 mM phosphate buffer, pH 7.0. The spectra were recorded using a 1 mm path cell scanned from 200 nm to 260 nm. The spectra were an average of three scans with a resolution of 0.2 nm and a scan speed of 20 nm/min^{-1}. Peptide concentrations were quantitated by quantitative amino acid analysis.

Activity assay of EcoRI

The enzyme *Eco*RI, 2.5 units in 1 μl, was pre-incubated with each peptide at the concentrations described in the text at 37°C for 3 h. To this was added 1 μg of pUC19 DNA that was linearized by 2 units of the restriction enzyme *Alw*NI. Enzyme for use in the control lane was also pre-incubated at 37°C for 3 h before addition of the linearized substrate. Each reaction was analyzed on an 8% acrylamide gel, and electrophoresed at 200V for 2–3 h. Plasmid DNA in acrylamide gels was stained with ethidium bromide and visualized at 312 nm.

Activity assays of Eco*RV and* Pvu*II*

The enzyme *Pvu*II, 2 units in 1 µl, was each pre-incubated with each peptide at the concentrations described in the text at 37°C for 3 h. To this was added 1 µg of plasmid DNA, pUC19, that was linearized by 2 units of the restriction enzyme *Alw*NI. Enzyme for use in the control lane was also pre-incubated at 37°C for 3 h before addition of the linearized substrate. Reactions of pUC19 plasmid were analyzed on an 8% acrylamide gel, and the gel was electrophoresed at 200V for 2–3 h. Plasmid DNA in acrylamide gels was stained with ethidium bromide and visualized at 312 nm.

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