Structural and Mutagenic Analysis of the RM Controller Protein C.Esp1396I

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Abstract

Bacterial restriction-modification (RM) systems are comprised of two complementary enzymatic activities that prevent the establishment of foreign DNA in a bacterial cell: DNA methylation and DNA restriction. These two activities are tightly regulated to prevent over-methylation or auto-restriction. Many Type II RM systems employ a controller (C) protein as a transcriptional regulator for the endonuclease gene (and in some cases, the methyltransferase gene also). All high-resolution structures of C-protein/DNA-protein complexes solved to date relate to C.Esp1396I, from which the interactions of specific amino acid residues with DNA bases and/or the phosphate backbone could be observed. Here we present both structural and DNA binding data for a series of mutations to the key DNA binding residues of C.Esp1396I. Our results indicate that mutations to the backbone binding residues (Y37, S52) had a lesser affect on DNA binding affinity than mutations to those residues that bind directly to the bases (T36, R46), and the contributions of each side chain to the binding energies are compared. High-resolution X-ray crystal structures of the mutant and native proteins showed that the fold of the proteins was unaffected by the mutations, but also revealed variation in the flexible loop conformations associated with DNA sequence recognition. Since the tyrosine residue Y37 contributes to DNA binding in the native complex, we have solved the structure of the Y37F mutant protein/DNA complex by X-ray crystallography to allow us to directly compare the structure of the DNA in the mutant and native complexes.

Introduction

Restriction-modification (RM) systems encode a restriction endonuclease (ENase) and a DNA methyltransferase (MTase). The DNA MTase protects the host DNA from cleavage by the associated restriction enzyme, whilst the ENase cleaves foreign DNA that attempts to enter the bacterial cell, before it has time to be protected by methylation [1,2]. Control mechanisms exist to ensure the correct temporal expression of RM genes, so that all recognition sites on the host DNA are methylated prior to exposure to the ENase.

The most widespread of these mechanisms employs a “controller” (C) protein encoded by a gene downstream of its own promoter, and usually co-transcribed with the endonuclease (R) gene as a single transcriptional unit [3–7]. The C-protein binds at various sites within the C/R promoter to regulate transcription of its own gene and the associated endonuclease gene [8]. ENase expression has been shown to be delayed with respect to the MTase when the C-protein is expressed in a new host leading to a regulatory feedback mechanism [9]. Transcription of the C-gene is itself dependent on the concentration of the protein it encodes, leading to a regulatory feedback circuit [10].

In the C.Esp1396I system, and other related systems, the operator sequence at the C/R promoter has two operator sites (denoted O₁ and O₂) that are distal and proximal, respectively, to the transcription unit comprised of the C and R genes [11,12]. The high-affinity O₁ site binds a C-protein dimer and recruits the sigma subunit of RNA polymerase to switch both the C and R genes on. As the C-protein concentration rapidly increases, the low-affinity O₂ site proximal to the gene then becomes occupied and the gene is down-regulated through displacement of bound RNA polymerase [12–14]. Unusually, in the RM system Esp1396I (Figure 1), the C-protein also represses the MTase (M) gene by binding to the promoter at the transcriptional start site, denoted O₄₆ where the C/R genes and the M gene are transcribed convergently from different promoters [15].

Analysis of C-protein binding sites in a wide variety of RM systems suggested a repeating quasi-symmetrical “consensus” sequence consisting of two sets of inverted repeats or “C-boxes” upstream of the C/R genes [6,8,12,16]. The first published structure of a controller protein bound to DNA was that of C.Esp1396I bound as a tetramer, i.e. with two dimers bound convergently on the 35 bp operator sequence (O₁+O₂) of the C/R promoter to form the “repression complex” [11]. The structure revealed the mechanism of the switch from activation to repression
of the C and R genes. In the crystal structure of the complex (PDB code: 3CLC), two dimers are bound to the DNA, each centred on the pseudo-dyad located between the central A and T bases in the TATA sequence within each operator site, and interacting across the major groove at the centre of the DNA.

Subsequent high resolution crystallographic studies of the complex with the \( O_L \) and \( O_M \) operators [17,18] showed the sequence-specific contacts to the bases within the recognition site ("direct readout"), as well as the non-specific interactions with the severely bent phosphodiester backbone ("indirect readout"). We also investigated the affinities of the protein for its three natural promoters, \( O_M \), \( O_K \) and \( O_L \) in order to understand the structural and mechanistic basis of differential DNA sequence recognition in this system [18].

Here, we are interested in dissecting the contribution to DNA binding of key amino acid side chains involved in sequence specific interactions with the bases (T36, R46), and those involved in interactions with the DNA backbone (Y37, S52). By individually mutating these residues, and assessing DNA binding affinity by SPR, we analyse the contribution of the mutated side chains to DNA binding. To confirm that these mutations have not affected the overall fold of the protein, we have determined their structures by X-ray crystallography, and these structures show further conformational details of the flexible loop region involved in DNA sequence recognition. We also crystallized one of the mutant proteins bound to DNA and have determined the structure of this DNA-protein complex, to compare the conformation of the DNA in the native and mutant complexes.

**Results**

**SPR Analysis of DNA Binding**

To evaluate the effects of the individual mutations on binding DNA, we used Surface Plasmon resonance (SPR) to follow binding to the \( O_M \) operator DNA sequence (see Figure S1). Since it was not possible in all cases to obtain accurate on- and off-rates, we determined the binding affinity of the mutants (and wild type)
proteins from the SPR signal (RU) at equilibrium, using a range of protein concentrations (Figure 2A). The location of the mutated residues with respect to the bound DNA is shown in Figure 2B. The range of protein concentrations used for the SPR experiments varied from 20–200 nM total protein, corresponding to 0.2 nM to 17 nM dimer (see Methods section) and all except T36A were shown to interact with the Om operator site within this concentration range. In further experiments, the maximum total protein concentration was increased to 1000 nM (corresponding to 210 nM dimer) but still no interactions were observed with those mutants (data not shown). The wild type protein, as expected, showed the highest binding affinity (\(K_D = 0.5\) nM). Of the mutant proteins, Y37F showed the strongest binding to the Om site, followed by S52A and Y37A. The mutant protein R46A bound with a much lower affinity (\(\sim 16\) nM), and T36A did not show any measurable binding under these conditions (Figure 2 and Table 1).

The \(K_D\) for the R46A mutant was \(~30\) fold greater than that of the wild type, showing a much weaker interaction with the Om operator site, and consistent with a key DNA-binding role for this arginine side chain. An even more important role is indicated for T36, since the DNA binding ability of the T36A mutant was completely abolished, as measured by SPR. The \(K_D\) for the S52A interaction with the Om operator was 5 fold higher than the wild type, confirming the importance of this hydrogen bond interaction.

### Table 1. Equilibrium SPR analysis of the C.Esp1396I mutant proteins binding to the Om operator.

| Protein | \(K_D\) (nM) | Error (nM) | \(\text{Chi}^2\) | \(\Delta G\) (kJ mol\(^{-1}\)) |
|---------|-------------|------------|----------------|---------------------|
| Wild type | 0.51        | \(\pm 0.04\) | 1.90          | \(-53.0\)          |
| Y37F    | 1.80        | \(\pm 0.26\) | 7.20          | \(-49.9\)          |
| S52A    | 2.60        | \(\pm 0.45\) | 4.80          | \(-49.0\)          |
| Y37A    | 5.50        | \(\pm 1.70\) | 0.16          | \(-47.1\)          |
| R46A    | 15.8        | \(\pm 4.70\) | 0.18          | \(-44.5\)          |
| T36A    | N/D         | N/D        | N/D           | N/D                 |

Analysis of the SPR data was performed using the 1:1 affinity model from the BiaEval software. The dimerisation constant of C.Esp1396I was fixed at 1.6 \(\mu\)M to enable estimation of the dimer concentration from the total protein concentration. N/D = not detectable.

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Figure 3. Coordination of the sulphate ion required in the crystal structure. Top panel shows the asymmetric unit of the C.Esp1396I high resolution wild type X-ray crystal structure (4I6R) in orange with a symmetry related dimer in light orange. The sulphate ion is shown in pink. The zoom shows atoms hydrogen bonding directly to the sulphate including water molecules (dark blue spheres). Hydrogen bonds are shown as grey dashed lines.
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Figure 4. Conformation of the flexible loop of C.Esp1396I. (A): The three observed loop conformations observed in crystal structures of the native protein. The two alternative C.Esp1396I loop positions in 3G5G are shown in light blue (conformation I) and light green (conformation II) and the high resolution wild type loop position from 4I6R is shown in orange (III). (B): The side chains of residues S45, R46 and N47 are displayed as sticks.
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in stabilising the DNA-protein complex. The Y37A and Y37F mutants had $K_D$s that were 11 and 3.5 fold higher than the wild type respectively. The three-fold difference between these two mutants indicates that both the phenyl ring and the hydroxyl group of the tyrosine are important for interacting with the DNA.

**Structural Analysis of Native and Mutant Proteins**

In order to confirm that the structures of the mutant proteins closely resembled the native protein, and that the amino acid substitution had not introduced any inadvertent structural changes, we crystallized each mutant protein and solved their structures by molecular replacement. Since the resolution of the diffraction data from each mutant protein (1.5–2.0 Å) was significantly better than the previously determined native structure (2.7 Å), we also crystallized the native protein and obtained crystals that diffracted to much higher resolution (1.4 Å) than the published structure, to permit a more accurate comparison.

Overall the new triclinic wild type and mutant protein structures closely resemble the original protein structure [19,20]. The root mean square deviations (RMSD) between the main chain atoms of each of the structures presented here and the previous structure (3G5G) were <1 Å in all cases. One obvious feature of the higher resolution structures was the presence of several water molecules on the surface of the proteins. The side chain positions could also be placed with a higher degree of confidence. Because the mutants crystallized in multiple space groups and unit cells, the crystal contacts were mostly unique to each structure. One key crystallisation contact in the majority of the structures relied upon an $SO_4$ ion contacting symmetry related chains. The B-factors across the mutant structures are all fairly consistent with the C-terminus having the highest B-factors.

**The native protein.** The high-resolution (1.4 Å) triclinic wild type structure presented here differs significantly in crystal contacts when compared to the previously described wild type structure 3G5G [19]. In terms of crystal contacts, Y29 does not stack with a symmetry related Y29 residue in the manner observed in several C.Esp1396I crystal structures. Instead the majority of the contacts are direct hydrogen bonds between side chains. A large number of crystal contacts occur through the C-terminal tail of C.Esp1396I and symmetry related chains. This gives that previously flexible region a high degree of stability, allowing the complete C-terminus up to the terminal carboxyl oxygen atoms to be modelled. The other primary intermolecular interface is centred on one of the $SO_4$ ions. The $SO_4$ ion essentially bridges the gap between symmetry related chains as in this region no direct protein-protein interactions occur (Figure 3). Of these intermolecular contacts, only R35 of the recognition helix is involved in DNA interactions in the protein-DNA complex. We see no difference in protein...
| Protein | Wild Type | T36A | Y37A | Y37F | R46A | R46A | S52A | Y37A-19OaM |
|---------|-----------|------|------|------|------|------|------|-------------|
| PDB code | 4I6R | 4I6T | 4IA8 | 4I6U | 4F8D | 4FBI | 4FN3 | 4IVZ |
| Space Group | P1 | P6 | P1 | P1 | P2,2,2 | C2 | P3 | C2 |
| Cell Dimensions | a, b, c (Å) | 34.76, 34.78, 41.86 | 65.45, 65.45, 72.29 | 34.26, 34.28, 41.12 | 48.61, 81.85, 135.08 | 46.40, 51.00, 74.60 | 65.33, 65.33, 71.38 | 46.59, 51.32, 74.38 | 47.50, 146.70, 47.80 |
| α, β, γ (°) | 110.47, 105.60, 96.00 | 90, 90, 120 | 104.44, 108.92, 97.42 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Solvent content (%) | 37.2 | 40.1 | 37.2 | 37.5 | 36.1 | 36.3 | 36.5 | 45.0 |
| Monomers in ASU | 2 | 2 | 2 | 6 | 2 | 4 | 2 | 4 (+4 DNA) |
| Data Collection: | | | | | | | | |
| Beamline | DLS-I04-1 | DLS-I04-1 | DLS-I04-1 | DLS-I04-1 | DLS-I04-1 | DLS-I04-1 | DLS-I04-1 | DLS-I04-1 |
| Wavelength (Å) | 0.92 | 0.92 | 0.92 | 0.92 | 0.98 | 0.92 | 0.92 | 0.98 |
| Resolution (Å) | 1.4 | 2.0 | 1.9 | 2.0 | 1.5 | 1.5 | 1.8 | 3.1 |
| No. measured reflections | 112030 | 130244 | 44469 | 527601 | 81886 | 128533 | 96678 | 41888 |
| No. unique reflections | 29258 | 12474 | 11313 | 38881 | 27204 | 53279 | 30869 | 11810 |
| Completeness | 83.5 (83.8) | 99.6 (100) | 79.4 (82.6) | 99.8 (100) | 97.8 (94.9) | 95.1 (97.0) | 95.2 (94.3) | 99.5 (99.6) |
| CC1/2 | 0.97 | 0.93 | 0.97 | 0.97 | 0.97 | 0.97 | 0.97 | 0.97 |
| Multiplicity | 3.8 (3.8) | 10.4 (10.7) | 3.9 (4) | 136 (137) | 3.2 (3.2) | 2.4 (2.4) | 3.1 (3.2) | 3.8 (3.8) |
| Rmerge | 4.3 (31) | 41.8 (238.9) | 110 (103) | 80 (40.8) | 4.0 (40.0) | 3.4 (24.6) | 6.5 (39) | 9.8 (55.4) |
| CC1/2 | 0.998 (0.881) | 0.932 (0.549) | 0.994 (0.705) | 0.999 (0.963) | N/D | 0.999 (0.881) | 0.998 (0.895) | 0.996 (0.701) |
| Wilson B (Å²) | 10.64 | 18.92 | 21.54 | 16.87 | 23.01 | 14.61 | 19.34 | 58.46 |

Values in parenthesis are for the highest resolution shell. The CC1/2 value was not determined for the monoclinic R46A structure.

Rmerge = \frac{\sum_i |\sum_j (I_{ij} - \langle I_{ij} \rangle)|}{\sum_i \sum_j I_{ij}},

CC1/2 = \sqrt{\frac{\sum \langle I_{ij} \rangle}{\sum \langle I_{ij} \rangle} - \langle I_{ij} \rangle^2},

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In the Y37A, S52A and monoclinic R46A structures, the SO₄ based interaction was observed in 3G5G and 4I6R are shown in the same colours as in Figure 5 and are translucent. (A) T36A loop positions shown in green. (B) Y37A loop position shown in dark red. (C) Loop positions in both R46A structures shown in red (monoclinic space group) and purple (trigonal space group). (D) Loop position of S52A shown in light green. (E) Loop positions in Y37F (blue).

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Figure 6. Conformation of the flexible loop of C.Esp1396I in the mutant crystal structures. The three wild type loop conformations observed in 3G5G and 4I6R are shown in the same colours as in Figure 5 and are translucent. (A) T36A loop positions shown in green. (B) Y37A loop position shown in dark red. (C) Loop positions in both R46A structures shown in red (monoclinic space group) and purple (trigonal space group). (D) Loop position of S52A shown in light green. (E) Loop positions in Y37F (blue).
differences either in the local binding regions in terms of helix or side chain positions, or in the overall fold. Comparing the native and mutant (Y37F) protein-DNA complex structures, the tyrosine and the phenylalanine were equally buried and inaccessible to solvent and in both cases the phenyl ring stacks against the deoxyribose ring in the DNA.

From the Y37F-19OΔ co-crystal structure, it is clear that the only major alteration to the binding interface is the loss of the hydroxyl-phosphate hydrogen bonds, of which there is one per protein subunit. The ΔG values were calculated from the equilibrium binding constants for the wild type, Y37F and Y37A proteins binding to DNA to be \(-53.0\), \(-49.9\) and \(-47.1\) kJ mol\(^{-1}\).
respectively (Table 1). The ΔG for the native C-protein-DNA of −55.0 kJ mol⁻¹ falls within the range of other HTH DNA binding protein-DNA interactions including SinR (−36.7 kJ mol⁻¹) [21], the C-protein C.AhdI (−45.7 kJ mol⁻¹) (12) and the Cro repressor (−64.4 kJ mol⁻¹) [22].

The ΔG difference (ΔΔG) due to loss of a side chain in the mutant proteins can be estimated. For the hydroxyl group only, the ΔΔG between the wild type and Y37F mutant was found to be −3.1 kJ mol⁻¹ (−1.55 kJ mol⁻¹ per hydrogen bond). The ΔΔG for the phenyl group alone (comparing the values for Y37F and Y37A), is −2.8 kJ mol⁻¹ (−1.4 kJ mol⁻¹ per monomer). Comparison of these two values indicates that the ring stacking and hydrogen bond interaction with the DNA make similar energetic contributions. Together, these interactions of Y37 with the DNA backbone contribute 5.9 kJ mol⁻¹ per dimer. Comparing ΔGs for S52A and WT, we obtain ΔΔG of 4.0 kJ mol⁻¹, comparable with that for the Y37F mutant (3.1 kJ mol⁻¹), and indeed in the crystal structure of the protein-DNA complex, both of these residues form a hydrogen bond to the phosphodiester backbone of the DNA. The ΔΔG for R46A is significantly larger (8.5 kJ mol⁻¹), and that for the T36A mutation must be larger still, since we could not measure any DNA binding activity of this mutant protein. Both R46 and T36 of C.Esp1396I are seen in crystal structures of DNA-protein complexes to interact directly with the base pairs involved in sequence recognition [18], specifically with the G and C bases, respectively, of the C-box recognition sequence GTC. Further hydrogen bonds from these amino acid residues to the thymine base may be mediated by water molecules.

The high-resolution structural studies of the mutants revealed the dimerisation interface of the proteins in great detail. Comparative analysis was then performed between these structures, previously published structures and the newly determined high-resolution (1.4 Å) wild type structure. It was observed that the
| Protein     | Wild Type | Y37A | Y37F | R46A (P2₁) | S52A |
|-------------|-----------|------|------|------------|------|
| Refinement Parameters: | | | | | |
| R<sub>work</sub>/R<sub>free</sub> (Å<sup>2</sup>) | 14.9/18.2 | 18.4/24.5 | 21.3/27.7 | 21.7/21.7 | 15.4/19.6 |
| No. atoms/B-factors (Å<sup>2</sup>) | 1390/14.3 | 1376/28 | 1373/28 | 3797/23 | 1384/17 |
| R.m.s. deviations from standard: | | | | | |
| Bond lengths (Å) | 0.030 | 0.019 | 0.018 | 0.018 | 0.033 |
| Bond angles (°) | 2.20 | 1.88 | 1.86 | 1.86 | 2.01 |
| Ramachandran statistics (% of residues) | | | | | |
| Favored | 100 | 96.7 | 99.6 | 99.8 | 99.7 |
| Allowed | 0 | 0.2 | 0 | 0 | 0 |
| Outliers | 0 | 0 | 0 | 0 | 0.3 |

*Refinement statistics from the C. Esp1396I and mutant free protein crystals.

References:
1. Engh & Huber 1991 [33].
2. MolProbity [34].

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dimerisation interfaces of the mutants were identical to the wild type protein, complete with the same hydrogen bonding networks and hydrophobic interactions.

As well as confirming that the mutant proteins were folded correctly, the structural studies revealed a more detailed structural picture of C.Esp1396I. Previously a flexible loop in C.Esp1396I had been identified from the wild type structure, where two alternative conformations were observed [19]. The wild type structure of C.Esp1396I presented in this study revealed further details including an additional, third conformation of the flexible loop region that was subsequently observed in several of the mutant protein structures. Between the 16 published and 20 newly determined structures of the C.Esp1396I monomer (from nine free protein X-ray crystal structures containing multiple monomers) and across seven different crystal forms, only these three loop conformations were observed. The T36A mutant protein crystal structure showed both the major conformation (I) and the novel conformation observed in this study (III), indicating that both could exist simultaneously in different subunits of the protein dimer. Flexibility in this loop is very important for DNA sequence recognition, as it has been observed in the nucleoprotein structures to adopt different conformations depending on which operator site the protein is bound to. Thus the side chains of DNA binding residues within the loop are presented in different orientations and so accommodate the different DNA sequences in the operator sites, albeit with very different affinities as required for finely tuned operation of the genetic switch control mechanism.

Methods

Mutagenesis of C.Esp1396I to create the R46A and T36A mutant protein constructs was carried out as previously described [11]. All other mutant protein construct genes were synthesised by GenScript (USA). C.Esp1396I native and mutant constructs were purified as described previously [11]. In brief, all protein constructs were over-expressed in E. coli strain BL21 (DE3) pLysS with an N-terminal hexahistidine sequence for nickel affinity chromatography. After removal of the hexahistidine tag using the serine protease thrombin, size exclusion chromatography was used to further purify the C.Esp1396I proteins.

For SPR experiments the purified C.Esp1396I native and mutant proteins were dialysed into SPR running buffer (130 mM NaCl, 10 mM HEPES pH 7.4 and 0.05% (v/v) Tween-20). A 5’ biotinylated single stranded DNA oligonucleotide comprising the entire O M operator site and its complementary sequence were synthesised by ATDBio (Southampton, UK) for X-ray crystal structures containing multiple monomers) and across seven different crystal forms, only these three loop conformations were observed. The T36A mutant protein crystal structure showed both the major conformation (I) and the novel conformation observed in this study (III), indicating that both could exist simultaneously in different subunits of the protein dimer. Flexibility in this loop is very important for DNA sequence recognition, as it has been observed in the nucleoprotein structures to adopt different conformations depending on which operator site the protein is bound to. Thus the side chains of DNA binding residues within the loop are presented in different orientations and so accommodate the different DNA sequences in the operator sites, albeit with very different affinities as required for finely tuned operation of the genetic switch control mechanism.

Supporting Information

Figure S1 Representative SPR data. Sensorgrams for the wild type and mutant constructs of C.Esp1396I binding to the O_M operator site (200 nM total protein).

Table S1 Crystallisation conditions.

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Author Contributions

Conceived and designed the experiments: GGK JEM RNAM. Performed the experiments: RNAM JEM. Analyzed the data: RNAM JEM GGK. Contributed reagents/materials/analysis tools: RNAM. Contributed to the writing of the manuscript: GGK RNAM JEM.

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