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A mutational analysis of DNA mimicry by ocr, the gene 0.3 antirestriction protein of bacteriophage T7

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ABSTRACT

The ocr protein of bacteriophage T7 is a structural and electrostatic mimic of approximately 24 base pairs of double-stranded B-form DNA. As such, it inhibits all Type I restriction and modification (R/M) enzymes by blocking their DNA binding grooves and inactivates them. This allows the infection of the bacterial cell by T7 to proceed unhindered by the action of the R/M defence system. We have mutated aspartate and glutamate residues on the surface of ocr to investigate their contribution to the tight binding between the EcoKI Type I R/M enzyme and ocr. Contrary to expectations, all of the single and double site mutations of ocr constructed were active as anti-R/M proteins *in vivo* and *in vitro* indicating that the mimicry of DNA by ocr is very resistant to change.

The ocr protein encoded by gene 0.3 of bacteriophage T7 [1,2] is the best characterised example of an antirestriction protein and of a structural mimic of DNA [3,4]. Phage- or plasmid-encoded antirestriction proteins alleviate the effects of host restriction endonucleases during invasion of the host by the phage or plasmid DNA [5,6]. Ocr has the same shape and charge distribution as a bent, double-stranded DNA molecule approximately 24 base pairs in length [7,8]. This molecular mimicry accounts for the ability of ocr to inhibit virtually irreversibly, all known Type I restriction and modification (R/M) enzymes found in most eubacteria and archaea [8–12]. Ocr binds to and completely occupies the DNA-binding site on the enzyme and prevents the enzyme from acting as a restriction endonuclease on the phage genome as it enters the bacterium. Ocr thereby greatly assists the spread of the phage infection in the bacterial population. The mimicry by ocr of the general shape and charge of DNA rather than any specific base pair sequence means that ocr acts against any of the vast variety of Type I R/M enzymes, each of which recognises a different base pair sequence [13].

The structure of ocr is an elongated, banana-shaped dimer decorated on its surface with a surfeit of aspartate and glutamate residues which can be superimposed upon the equivalent phosphate groups on the DNA molecule [8]. In addition to mimicking the charge distribution, ocr mimics the bend of approximately 50 in the DNA helical axis which is induced upon DNA binding to the R/M enzyme [8]. The introduction of the bend in DNA by the R/M enzyme is energetically costly and this cost is "saved" when the R/M binds to ocr as ocr is already "pre-bent" [14]. This would account for the ~50-fold tighter binding of the enzyme to ocr than to DNA.

We have explored the effect of replacing aspartate and glutamate residues in the ocr molecule on the interaction between this DNA mimic and Type I R/M systems *in vivo* and *in vitro* in an attempt to assess the importance of electrostatic mimicry of the DNA. Our selection of Asp and Glu residues was guided by the overlay of the structure of ocr on duplex DNA, Fig. 1. The residues chosen mimic the phosphate backbone of one strand of the duplex DNA recognised by the R/M enzyme. We found that the activity of ocr and its binding affinity for the archetypal purified Type I R/M enzyme, EcoKI, were extremely robust to mutations removing one or two acidic side chains from each monomer of ocr.

Materials and methods

Escherichia coli JM109 was purchased from Promega (Madison, WI). *E. coli* NM1261 (r^-m^-, no R/M system) and *E. coli* NM1049 (r^+m^+, EcoKI Type IA R/M system) were a kind gift of Professor Noreen Murray (School of Biology, University of Edinburgh, UK). The expression strain *E. coli* BL21(DE3)pLysS was purchased from Invitrogen (Groningen, The Netherlands). All cell growth was conducted

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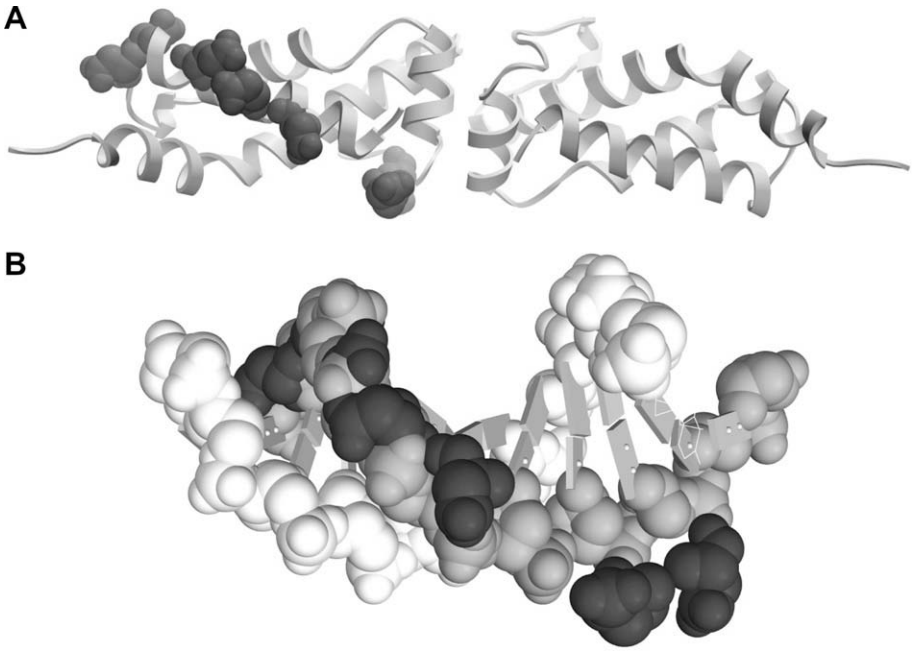

Fig. 1. (A) A ribbon diagram of the ocr dimer showing amino acids 5–110 in each subunit. The C-termini are at the extreme left and right and the N-termini are located at the interface between the two subunits. The amino acids changed by site-directed mutagenesis are shown as space-filling spheres in the left hand subunit. These charged residues run around the surface of the ocr protein. The ocr monomer has pdb code 1s7z. (B) A DNA duplex is shown with the base pairs as slabs and the phosphate backbone as space-filling spheres in light grey and grey. The atoms of the amino acids in ocr targeted for mutagenesis are shown as very dark grey space-filling spheres. It can be seen that they overlap well with the phosphate backbone of the grey strand of DNA [8].

at 37 °C unless stated otherwise. *In vivo* restriction assays using virulent unmodified bacteriophage lambda, $\lambda_{v.o}$, or modified lambda $\lambda_{v.k}$ were performed as described previously [15]. Plasmids pAR3786 and pAR3790 encoding for C-terminal deletions of the last 7 or 17 amino acids of wild-type ocr were a kind gift from Dr. Alan Rosenberg and Professor William Studier (Brookhaven National Laboratory, USA).

Single or double codon mutagenesis was achieved using the QuikChange II Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) following the manufacturers guidelines. All constructs were sequenced to ensure no mistakes had been introduced during amplification. Edman sequencing of purified ocr [16] indicated that the N-terminal methionine was subject to post-translational cleavage. Therefore for clarity, mutated amino acids in the present work are numbered by taking this into account (i.e. the ocr sequence starts AMSNM).

To prepare the mutant versions of ocr, we adapted the procedure used to purify wild-type ocr [17]. *E. coli* BL21(DE3)pLysS was transformed with the appropriate construct. Cells were grown at 37 °C in 2 L conical flasks containing 1 L LB broth supplemented with 34 μg/ml chloramphenicol and 50 μg/ml of carbenicillin and shaking at ~230 rpm. Once the optical density at 600 nm reached ~0.5, heterologous gene expression was induced by the addition of IPTG (final concentration of 1 mM) and growth continued for a further 2.5 h before harvesting the cells by centrifugation at 8000*g* for 10 min at 4 °C. Cell pellets were stored at −20 °C until required. The cells were resuspended in ice-cold buffer A (20 mM Tris–HCl, 300 mM NH_4Cl, pH 8.0) in the presence of a protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were then broken on ice by sonication using a Soniprep 150 Sonicator (Sanyo, Tokyo, Japan) fitted with a 9-mm probe (1 min/g of cell paste). The cell debris was then removed by centrifugation (20,000*g* for 1 h at 4 °C). The supernatant was loaded onto a 20 × 1.6 cm diameter DEAE-Sepharose fast flow ion-exchange column (GE Healthcare, Piscataway, NJ), which had been pre-equilibrated in buffer A, at a flow rate of 48 ml/h. The column was extensively washed with buffer A and then a 500 ml gradient from 0.3 to 1.0 M NH_4Cl in buffer A was run at 24 ml/h. Fractions containing ocr, identified by SDS–PAGE, were subsequently pooled. UV-spectroscopy indicated that the sample was contaminated with nucleic acid. The nucleic acid was removed in the following manner. The crude ocr preparation was precipitated by the addition of 1.2 volumes of 10% trichloroacetic acid (TCA) and incubated on ice for ~10 min. The precipitate was collected by centrifugation at 20,000*g* for 20 min at 4 °C and the pellet was resuspended in 95% ethanol with gentle mixing for ~10 min. After centrifugation (20,000*g* for 20 min at 4 °C) the supernatant, which contained the ocr protein, was transferred to a clean tube. This cycle of TCA precipitation followed by resuspension in 95% ethanol was then repeated a further two times except the final precipitate was resuspended in 20 mM Tris–HCl, pH 8.0, instead of 95% ethanol. The sample was then dialysed against ~4 L 20 mM Tris–HCl, pH 8.0, for 16 h at 4 °C and concentrated by centrifugation using a Vivaspin concentrator (10,000 MWCO; VivaScience AG, Hannover, Germany). Finally, an equal volume of glycerol was added to the sample, which was then stored at −20 °C until required. The M.EcoKI methyltransferase was purified as previously described [18,19].

Isothermal titration calorimetry (ITC) was carried out using a VP-ITC instrument (Microcal, Northampton, MA). The stocks of ocr (wild-type or mutated versions) and M.EcoKI were buffer exchanged into 20 mM Tris–HCl pH 8.0, 6 mM $MgCl_2$, 7 mM 2-mercaptoethanol using a PD-10 gel filtration column (GE Healthcare). The concentration of the protein solution was adjusted either by dilution into the same buffer or by concentration using a Vivaspin concentrator (10,000 MWCO for ocr, 30,000 MWCO for M.EcoKI; VivaScience AG). *S*-Adenosyl-L-methionine (New England Biolabs, Ipswich, MA, USA) was then added to a final concentration of 100 μM. All solutions were thoroughly degassed prior to use. Typically, ocr at a concentration of 30 μM was titrated into a M.EcoKI solution at a concentration of 3 μM in the VP-ITC cell (1.4 ml active volume). All titrations were carried out at 25 °C. The heat of dilution was obtained by injecting ocr into buffer or

buffer into buffer and these values were subtracted from the ITC titration data. The calorimetric data were converted into differential binding curves by integration of the resultant peaks. The data were fitted with a single-site binding model using the Microcal LLC Origin software package.

Competition between DNA and the wild-type or mutated ocr for binding to M.EcoKI was determined using the fluorescence anisotropy assay described previously [9]. Assays were performed in 20 mM Tris–HCl pH 8.0, 6 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 100 μM *S*-adenosyl-L-methionine with the concentration of M.EcoKI varying between 0.25 and 500 nM. The 21 base pair DNA duplex containing the M.EcoKI target sequence and end-labelled with hexachlorofluorescein was present at 2 nM [20]. The ocr dimer, if present, was at a concentration of 50 nM. The anisotropy data were fitted to an equilibrium binding model using Dynafit (Biokin Ltd., Watertown, MA, USA) as described previously [9].

Results

The results of all our in phage infection assays, ITC measurements and fluorescence anisotropy assays are given in Table 1.

Once the mutations had been verified by sequencing of the entire gene on both strands, we tested whether the mutated ocr protein was active *in vivo*. These experiments were conducted at low levels of heterologous gene expression in the absence of induction by IPTG. *E. coli* cells with (NM1049) or without (NM1261) the chromosomal EcoKI R/M system were transformed with either the expression vector (a control) or the vector containing the wild-type ocr gene or the mutated ocr genes. Each of these transformed strains was then challenged with phage lambda $\lambda_{v.o}$ or $\lambda_{v.k}$. As expected, cells transformed with the vector alone showed a strong reduction (six orders of magnitude) in the efficiency of plating of $\lambda_{v.o}$ compared to $\lambda_{v.k}$. Cells transformed with the plasmid expressing the wild-type ocr showed essentially identical numbers of plaques with both $\lambda_{v.o}$ and $\lambda_{v.k}$ showing that ocr had knocked out the R/M system. Assaying the mutated forms of ocr showed that all of the mutants were fully active and indistinguishable within the limits of the errors associated with this sort of assay. There was no significant difference between single amino acid substitutions, double substitutions or the C-terminal deletions of 7 or 17 amino acids.

The mutated proteins all overexpressed and purified in the same way as the wild-type ocr indicating that their structures were all likely to be similar to that of the wild-type protein as already suggested by the *in vivo* assay.

The purified proteins were then tested for binding to the methyltransferase core of EcoKI. The core protein, M.EcoKI, comprises one DNA specificity subunit and two DNA methylation subunits with a total molecular mass of 169 kDa. It has previously been shown that ocr binds extremely tightly to M.EcoKI (K_d ~50 pM) and can easily displace a DNA duplex from the enzyme [9,10,14]. We measured the interaction between the mutated ocr proteins and M.EcoKI using ITC as a direct method, and the fluorescence anisotropy competition assay as an indirect method.

The ITC data showed a highly exothermic interaction between ocr and M.EcoKI with a stoichiometry consistent with one ocr dimer per M.EcoKI. The enthalpy change on binding the mutated ocr varied by ±40% from the −85.8 kJ/mol observed for the interaction of the wild-type ocr with M.EcoKI. The substitutions D25C, E59C and D62C gave rise to the largest differences from the wild-type with D25A being less exothermic and the others more exothermic. It is interesting that removal of a charged residue can give both exothermic and endothermic effects. The ITC data indicate tight binding (K_d <10 nM), as evidenced from the sharp end point of the titration (data not shown), and it was not possible to obtain a reliable measure of the free energy change for the interaction from ITC.

To attempt to estimate whether the free energy change for binding was altered by the amino acid substitutions or the C-terminal deletions, we used a fluorescence anisotropy competition assay as previously described [9]. This assay is more appropriate than ITC in this K_d range. A control titration of a labelled DNA oligonucleotide duplex with M.EcoKI showed a typical binding behaviour with a K_d of 2.106 ± 0.350 nM similar to previous measurements [14,20]. A weaker non-specific binding event was observed at high concentrations of M.EcoKI with a K_d of 349 ± 73 nM. The addition of 50 nM ocr dimer to the solution containing the duplex reduces the proportion of M.EcoKI available to bind to the duplex so that more M.EcoKI has to be added to observe DNA binding. The binding of wild-type ocr to M.EcoKI is much stronger than the binding of DNA to M.EcoKI so no change in anisotropy is observed until al-

Table 1
A list of the mutant forms of the ocr protein created by mutagenesis and their behaviour *in vivo* and *in vitro*.

Protein	Eop[1] with unmodified phage $\lambda_{v.0}$	Eop with modified phage $\lambda_{v.k}$	Enthalpy[2] of binding of ocr to M.EcoKI (kJ/mol)	Stoichiometry[2] of binding, ocr:M.EcoKI	Dissociation constant (nM) for ocr binding to M.EcoKI determined by anisotropy
Plasmid vector alone	1.703×10^{-6}	0.973			
Wild-type ocr	0.792	1.493	−85.8 ± 1.3	0.87 ± 0.01	0.044 ± 0.040
D12N	0.912	1.039	−59.0 ± 1.3	0.94 ± 0.01	0.081 ± 0.044
E16A	2.504	2.985	−92.5 ± 1.3	0.82 ± 0.01	0.165 ± 0.150
E16Q			−72.0 ± 0.4	0.70 ± 0.00	0.129 ± 0.037
E20A	0.681	0.996	−70.7 ± 1.7	0.85 ± 0.01	0.102 ± 0.054
D25A			−59.9 ± 1.3	0.96 ± 0.01	0.208 ± 0.036
D25C	0.541	0.502	−102.6 ± 2.1	0.78 ± 0.01	
D26N	0.821	0.761	−91.2 ± 1.3	0.76 ± 0.01	0.158 ± 0.080
E59C	1.135	0.634	−107.6 ± 0.8	0.86 ± 0.00	
D62C	1.056	1.018	−122.6 ± 2.5	0.78 ± 0.01	
D12A, D26N	0.780	0.986	−72.0 ± 1.3	1.00 ± 0.01	0.144 ± 0.044
D12N, D26N	1.506	1.317	−72.0 ± 0.8	0.89 ± 0.01	0.101 ± 0.043
D12N, E87D	0.982	1.008	−63.6 ± 1.3	0.98 ± 0.01	0.063 ± 0.037
E16A, D25A	0.728	0.833	−85.8 ± 0.8	0.84 ± 0.01	0.051 ± 0.056
Ocr deletion, 109 amino acids long	0.603	1.395	−104.6 ± 2.5	0.74 ± 0.01	0.111 ± 0.030
Ocr deletion, 99 amino acids long	1.355	1.022	−93.8 ± 2.9	0.71 ± 0.01	0.098 ± 0.051

[1] Eop, efficiency of plating from phage assays showing the ratio of plaques in NM1049–NM1261, with each of the two phages.
[2] The errors quoted are from the ITC analysis programme but we estimate that the real experimental errors are up to ±20% taking into account uncertainties in the determination of protein and DNA concentrations using UV absorption of around 5%.

most all of the ocr has been bound and excess M.EcoKI is added. The experiment involves three equilibria; two for binding of M.EcoKI to DNA and one for M.EcoKI binding to ocr. By keeping the binding affinities fixed in the analysis procedure for the DNA–EcoKI interactions, one can calculate the binding affinity of M.EcoKI for ocr. The accuracy of this method is not high because the affinity for ocr is so much greater than for DNA. Nevertheless, this methodology sets an upper limit for the dissociation constant, K_d. For all of the mutant proteins tested, tight binding between ocr and M.EcoKI was observed with little DNA binding being observed until the M.EcoKI had bound most of the available ocr. Given a K_d of 2.106 nM for the M.EcoKI–DNA interaction, we find that the mutant forms of ocr have K_d values ranging from roughly equal to the wild-type ocr to 4.7 times larger than wild-type ocr for binding to M.EcoKI. The mutant ocr proteins with substitutions of amino acids E16, D25 or D26 appear to have the weakest interactions with M.EcoKI although the magnitude of the effect is not large.

Discussion

Here, we have focussed our attention on a stretch of amino acids of ocr that mimic a single strand of the DNA recognised by the EcoKI R/M enzyme. Mutants were generated containing one or two amino acid changes of negatively-charged side chains to a neutral side chain per ocr monomer. These changes apparently do not perturb the ocr structure but we had postulated that they would seriously weaken the interaction with M.EcoKI due to electrostatic and structural mimicry of DNA by the ocr molecule. However, our results clearly show that this postulate is wrong as all of mutants were still active *in vivo* and bound strongly to M.EcoKI *in vitro*.

The binding to M.EcoKI is virtually unaffected by any of the amino acid substitutions, at least within the sensitivity limits of the ITC and fluorescence experiments. Only changes at E16, D25 or D26 appeared to slightly weaken binding but the effect was only marginally greater than the error in the measurements. The enthalpy of binding changed in an unpredictable manner with some mutants showing more exothermic binding and some less exothermic binding to M.EcoKI than the wild-type ocr. The enthalpy change varies by about a factor of two between −112.6 kJ/mol and −59.0 kJ/mol and the dissociation constant varies between 0.044 nM and 0.208 nM, a fourfold variation. This variation in K_d only amounts to free energy changes between −59.1 kJ/mol and −55.2 kJ/mol, a rather trivial variation. Given the small changes in the free energy of binding between all of our variants of ocr and M.EcoKI, it is apparent that changes in the enthalpy of binding are being largely compensated by changes in entropy.

There are three possible reasons for our observations: (i) the mutations are not in the ocr:M.EcoKI interface, (ii) the loss of a potential electrostatic interaction at the ocr:M.EcoKI interface is compensated by the proximity of other electrostatic interactions, or (iii) the ocr:M.EcoKI complex is based upon so many electrostatic interactions that it is largely unaffected by the removal of only a few interactions. Given that M.EcoKI is known to wrap around ocr with a resultant large interfacial area [10], the first reason is improbable hence we favour a combination of local (reason ii) and global effects (reason iii) as the explanation for our results. Thus, mutagenesis on a much larger scale than attempted in this paper is required to fully understand the DNA mimicry exhibited by ocr. Based on the three-dimensional structure of ocr, it should be possible to mutate multiple amino acid residues that correspond to entire regions of negative charge on the surface of the protein. In this way, we may be able to deduce regions of the protein that are particularly important for the interaction with M.EcoKI. This work is currently underway. The mimicry of DNA seems to be very robust from a structural viewpoint. However, it is also robust from an evolutionary perspective. Once a mimic has been evolved, it would appear to be resistant to incremental changes in its ability to function.

Acknowledgments

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