

Assembly Mutants of the Motor Subunit of the Type IC Restriction-Modification Enzyme EcoR124I


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Summary

We attempted mutagenesis of the motor subunit (HsdR), of the Type IC Restriction-Modification enzyme EcoR124I, in order to determine the possibility of altering the subunit in its ability to correctly assemble into a functional R₂-complex (a bidirectional motor). The overall aim was to produce a molecular motor capable of unidirectional translocation of DNA.

However, isolation of the appropriate mutants provided to be impossible.

However, during the course of the Project we have been able to show that the motor assembles in a random manner in terms of ultimate direction of translocation of DNA, but that by suitable location of the DNA binding site, with regard to the surface attached end of the DNA, we can induce effective unidirectional motion.

	<p style="text-align: center;">Mol Switch IST-2001-38036 A Molecular Magnetic Switch that links the Biological and Silicon Worlds</p>
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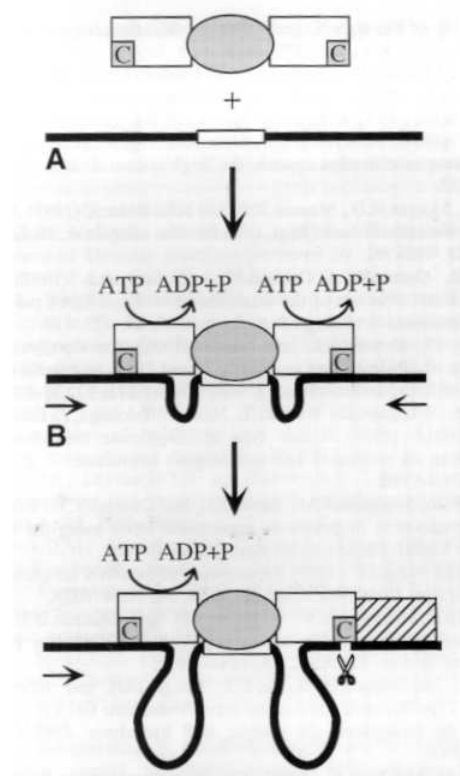
Background

Type I Restriction-Modification (R-M) enzymes are the most complex of the many R-M systems known (for recent reviews see Murray, 2000). They are multisubunit, multifunctional enzymes composed of three separate subunits (HsdR – the restriction subunit, HsdM – the methylation subunit, and HsdS – the DNA binding subunit). The active endonuclease (ENase) is composed of all three subunits in a ratio 2:2:1 (HsdR₂:HsdM₂:HsdS₁, or R₂-complex). The R₂-complex also functions as a DNA methyltransferase (MTase), an ATPase, and as a DNA pulling molecular motor.

HsdM and HsdS alone are sufficient to assemble an independent MTase with a stoichiometry of M₂S₁ (Taylor et al., 1992). The R-M enzyme EcoR124I can be assembled *in vitro* from the core methylase (MTase) enzyme by addition of the motor subunit HsdR (Janscák et al., 1998). However, the purified EcoR124I ENase exists as an equilibrium mixture of two species - R₂M₂S₁ and R₁M₂S₁ of which only the former is an active ENase (Janscák et al., 1996; Janscák et al., 1998). The R₂-complex is relatively weak, dissociating into free HsdR subunit and the restriction-deficient R₁-complex intermediate, under concentrations expected *in vivo* (Janscák et al., 1998).

Unlike other restriction endonucleases, Type I R-M enzymes cut distal to the DNA recognition site to which they bind. DNA cleavage can occur many thousands of basepairs from the recognition site, using a process of DNA movement known as DNA translocation (Yuan et al., 1980). This ENase-based motor activity is driven by ATP hydrolysis (Endlich and Linn, 1985), but unlike other DNA-based motors (e.g. DNA polymerase) it does not involve a linear tracking motion along the DNA; instead the motor remains bound at the recognition site and ‘pulls’ the adjacent DNA toward the bound enzyme (Figure 1). This motor activity presents the enzyme with an interesting topological problem at the initiation of translocation – how to ‘grasp’ adjacent DNA and produce the first loop of DNA.

Figure 1 DNA Translocation



The complex molecular motor function makes these enzymes particularly interesting. The restriction subunit (HsdR) is responsible for this motor activity and contains a series of conserved amino acid motifs (DEAD box motifs), which are associated with helicase-like activities (Gorbalenya and Koonin, 1991) including a Walker-type ATP binding site. They belong to a large superfamily (SF-II) of helicase-like enzymes (Flaus and Owen-Hughes, 2001) that also include type III R-M enzymes, chromatin remodelling factors and a few chimeric enzymes. It has been suggested that chromatin remodelling factors also make use of DNA translocation, in a similar mode as Type I restriction enzymes, which stresses the significance of a detailed analysis of the translocation process of the SF-II superfamily.

DNA cleavage is accomplished by protein sequences within the N-terminal domain of HsdR. These sequence motifs are known as Motif X and mutations at key residues within this motif have been shown to remove DNA cleavage activity of the closely related enzyme EcoKI (Davies *et al.*, 1998).

Aim of Deliverable

To use random mutagenesis of the 5'-end of the *hsdR* gene to identify amino acids involved in subunit assembly with an aim to produce only R₁-complex capable of unidirectional translocation.

Tasks scheduled:

1. PCR-based mutagenesis of the *hsdR* gene of EcoR124I at amino acids at the N-terminus of the protein that may be involved in protein-protein interactions.
2. Assay cleavage capability of such mutants.
3. Analysis subunit assembly using gel-shift technology to identify mutations that shift the equilibrium between R₂- and R₁-complex formation.

Methodology Used:

Site directed mutagenesis. The Quick-Change procedure (Stratagene) was employed for site-directed mutagenesis of the wt *hsdR* gene present on pGEX-2T plasmid. Listed below are the top strand sequences of the oligonucleotides used for mutagenesis with this kit, the following changed (mutated) nucleotide (underlined) were used:

E141A, 5'GATTATCCAGCAGTTTGCACAGGCGGGTTCTCACG 3';

T280P, 5'GTACCAGATTGCCGCCCCGAGCGCATTCTGTG 3';

D341A, 5'GTGGTCGACAGGAAAGCCCTCGATTACCAGACC 3';

D372A, 5' GCAGGCCTTAAACGAAATCTGGCTAAGGACGATAACAAAATTATCG 3'

D374A, 5' GGCCTTAAACGAAATCTGGATAAGGCCGATAACAAAATTATCGTC 3'

D375A, 5' CGAAATCTGGATAAGGACGCTAACAAAATTATCGTCACTAC 3';

E610A, 5' CAATGGACAGCAGTGCTAAAGCGTTTCTGGACGCTGCAATTC 3';

D613A, 5' GTGCTAAAGAGTTTCTGGCCGCTGCAATTCGTG 3';

E618A, 5' CTGGACGCTGCAATTCGTGCATATAACAGCCATTTTAAAAC 3';

D630A, 5' CTAACCTTAGCACCGCAGTAACGGTTTTTCAGAAC 3';

D640A, 5' CAGAACTACTATCGTGCTTTAGCCCAGCGGG 3';

Phenotypic analysis:

The restriction phenotype was determined using a classical complementation analysis, based on both the positive and negative (trans-dominant test) complementation. Combination of these tests allow us distinguish between cleavage mutations and subunit assembly mutations.

For screening of large collection of potential mutants the spot tests were used as described in Colson et al. (1965a). Those clones, where mutations were confirmed by sequencing of the *hsdR* gene, were further quantitatively analysed for precise estimations of restriction as described in Hubacek and Glover (1970).

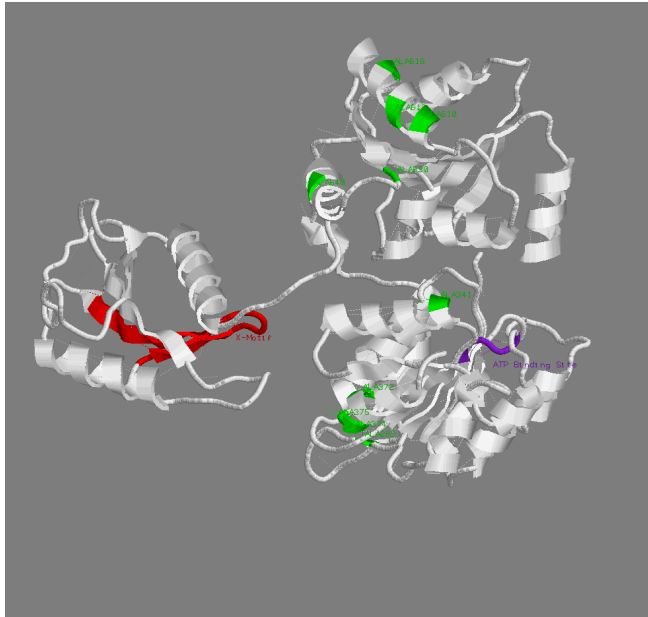
Results achieved:

Following a meeting between Ports and IMIC during Year 2, at which a structural model of the HsdR subunit was discussed (Janusz Bujnicki, Pers. Comm.), a combination of site-directed mutagenesis and random mutagenesis was outlined for regions of the *hsdR* gene thought to be involved in protein-protein interactions (based on predictions from the structural model). In between the endonuclease domain and the C-terminal domain is a region



containing seven DEAD-box amino acid motifs. These motifs probably fold into two domains to form a cleft through which the DNA would pass (Davies *et al.*, 1999). Therefore, in the light of this predictive structural model for the motor subunit (Blundell *et al.*, 2004), the strategy for mutagenesis of the *hsdR* gene, with the aim of disrupting R₂-complex formation, was modified.

Figure 2 Structural model of the HsdR subunit of EcoR124I



Predictive structural model for the motor subunit (Blundell *et al.*, 2004). The amino acids possibly associated with protein-protein interaction between HsdR and MTase are in green. Nuclease motif is shown in red.

From this model the amino acids possibly associated with protein-protein interaction between HsdR and Mtase were predicted and subjected to site-directed mutagenesis instead of original planned N-terminal region (Figures 2 and 3). Mutagenesis was performed on plasmid pGEX-2T harbouring the wt *hsdR* gene.

The amino acid residues (Figure 3) were individually substituted mostly by neutral alanine. The basic procedure utilizes a supercoiled double-stranded DNA, vector with an insert of interest (*hsdR* gene) and two synthetic oligonucleotide primers containing the desired mutation. This method is performed using PfuTurbo DNA polymerase, which replicates both plasmid strands with high fidelity without displacing the mutant oligonucleotide primers.

The putative mutants were screened for restriction deficiency. The restriction

phenotype of mutant endonucleases was determined by testing the ability of cells expressing these mutants to restrict the growth of bacteriophage lambda. For this assay the plasmid DNAs after mutagenesis were transformed into both *E. coli* r⁺m⁺ (expressing the wt REase) and *E. coli* r⁻m⁺ (expressing the wt MTase) cells. Efficiency of plating (e.o.p.) of lambda phage (e.o.p.; ratio of the phage titre on tested host to the titre on non-restricting host) on these strains was determined. The wt HsdR subunit introduced into r⁻m⁺ cells should cause r⁺m⁺ phenotype (positive complementation) and not affects the wt phenotype in r⁺m⁺ cells. The latter assay is called negative complementation or trans-dominant test and serves as a positive evidence of a restriction-deficient phenotype (Hubáček *et al.*, 1989). The trans-dominant effect of the cleavage mutant HsdR subunit on the wt HsdR produced by plasmid is reflected in a 100-1000-fold reduction in the level of EcoR124I restriction and the phenotype r⁻m⁺ also prove the ability of mutant HsdR to bind with MTase (Davies *et al.*, 1998). The combination of these two tests allowed us to distinguish between cleavage mutants (the above case) and assembly mutants which should not be able both negative and positive complementation.

For screening of large collection of potential random mutants the spot tests were used as described in (Colson *et al.*, 1965b). Those clones where mutations were confirmed by sequencing of *hsdR* gene were further quantitatively analysed for precise estimations of restriction as described in (Hubacek and Glover, 1970). Since none of substitutions altered the wt restriction phenotype in r⁺m⁺ host (no negative complementation) as expected for assembly mutants, the quantitative tests were performed in JM109(DE3) harbouring the MTase plasmid (pACYC184 with hsdM+hsdSR124II) together with pGEX-2T carrying individual substitutions (Table 1).

Figure 3 Summary of amino acid residue substitutions in HsdR motor subunit

MTHQTHTTIAESNNFIVLDKYIKAEPTGDSYQSESDLERELIQDLRNQGYE	
FISVKSQSAMLANVREQLQNLNGVVFNDSEWRRFTEQYLDNPSDGILDK	
TRKIHIDYICDFIFDDERLENIYLIDKKNLMRNKVQIIQQF	E141A
<u>DVTILVNGLPVQI</u> <u>ELK</u> KRGVAIREAFNQIHRYSKESFNSENLSFKYLQLF	D151A, E165A, D, H, E167A
VISNGTDTRYFANTTKRKDNSFDFTMNWAKSDNTLIKDLKDFATATCFQK	
HTLLNVLVNYSVFDSSQTLVMPYQIAA	T280P
SGGYIWHHTTGSKTLTSTFKAARLATELDFIDKVVFFVDRKDLDYQTMKE	D341A
YQRFSPDSVNGSENTAGLKRNL	D372A, D374A, D375A
NQQVVFIFDECHRSQFGEAQKNLKKKFKRYYQFGFTGTFIPFENALGSET	
TASVFGRELHSYVITDAIRDEKVLKFKVDYNDVRPQFKSLETETDEKKLS	
AAENQQAFLHPMRIQEITQYILNFRQKTHRTFPGSKGFNAMLAVSSVDA	
AKAYYATFKRLQEEAANKSATYKPLRIATIFSFAANEEQNAIGEISDETFD	
TSAMDSSAK	E610A, D613A, E618A, D630A, D640A
<u>EFLDAAIR</u> <u>EYNSHF</u> <u>KTNF</u> <u>STDS</u> <u>NGFQNYRDLAQRVKNQDI</u>	
DLLIVVGMFLTGFDAPTLNLTFLVDKNLRYHGLMQAFSRTNRIYDATKTFG	
NIVTFRDLERSTIDAITLFGDKNTKNVVLEKSYTEYMEGFTDAATGEAKR	
GFMTVVSELEQRFDPPTSIESEKEKKDFVKLFGEYLRAENILQNYDEFATL	
KALQQIDLSDPVAVEKFKAEHYVDDEKFAELQTIKRLPADRKIQDYRSAYN	
DIRDWRREKEAEKKEKSTTDWDDVVFVDLLKSQEIINLDYILGLIFEHN	
RQNKGGEMIEEVKRLIRSSLGNRAGEGLVDFIQQTNLDDLDPKASIIIDA	
FFTF	A957V
QREQQREAEALIKEENLNEDAARKYIRTSLKREYATENGTELNETL	
PKLSPLNPQYKTKKQAVFRKSSRLLRSLKA	

The position of all substitutions either in the nuclease motif (red) or in helicase domains (green) is demonstrated. DEAD boxes are underline. The blue region was predicted for the random mutagenesis using PCR-based mis-incorporation

Four substitutions expressed the restriction function very low but comparable with the wt gene. Protein with substitutions E141A, T280P, D372A expressing restriction deficient phenotype were purified and analysed *in vitro* for subunit assembly, using gel retardation technology as previously described (Janscak *et al.*, 1998). Unfortunately none of the mutants produced an interesting effect on the stability of the R₂-complex.

Substitutions D341A, D374A, E610A, and D640A expressed also restriction deficient phenotype in screening test, but sequence analysis revealed a secondary mutation in the *hsdR* gene. With regards to this fact and to general difficulties with the plasmid pGEX-2T, when even plasmid with wt *hsdR* gene expresses very low restriction activity *in vivo* (Table 1), further mutagenic work with this plasmid was stopped. We suggest to prepare in future these substitutions on plasmid pACR124, which was successfully used for mutagenesis of the nuclease motif and consequently for purification of the cleavage mutant HsdR subunits.

Following a request for a specific mutation through the TUDelft group (via Ports) we have produced both the A957V substitution in the C-terminal region of HsdR subunit and double mutation D151A + A957V and confirmed these mutations by DNA sequencing.

While the substitution A957V retains restriction proficient phenotype stabilising the R2 complex (Makovets *et al.* 2004), our results show that the double mutant is restriction deficient *in vivo* and *in vitro* due to the presence of D151A substitution in the endonuclease motif.

Table 1 **Complementation analysis of restriction phenotype in r^-m^+ cells**

Plasmid with substitution	Specificity of phage λ	e.o.p. ^a	Restriction phenotype
E141A	λ .R124I	0.7	r-
	λ .R124II	1.0	
T280P	λ .R124I	1.0	r-
	λ .R124II	1.0	
D372A	λ .R124I	0.1	r-
	λ .R124II	1.0	
D375A	λ .R124I	0.06	r±
	λ .R124II	1.0	
D613A	λ .R124I	0.06	r±
	λ .R124II	1.0	
E618A	λ .R124I	0.02	r±
	λ .R124II	0.9	
D630A	λ .R124I	0.01	r±
	λ .R124II	0.8	
wt	λ .R124I	0.2	r±
	λ .R124II	0.5	
MtasA	λ .R124I	1.0	r-
	λ .R124II	1.0	
JM109(DE3)	λ .R124I	1.0	r-
	λ .R124II	1.0	

^aThe numbers represent the efficiency of plating (e.o.p.) of lambda phage (e.o.p.; ratio of the phage titre on tested host to the titre on non-restricting host). Two colonies of individual substitution were tested for restriction of phage λ .R124I. λ .R124II is for specificity control only.

Conclusion:

It has not been possible to identify a region or amino acid substitution in the HsdR subunit that clearly affects subunit assembly *in vitro*.

However, Motif X mutations, produced during D1.1 and D1.2 all behaved as predicted and provide cleavage-deficient, translocation-proficient motor subunits for use in the Mol Switch Device.

In the light of new fact that translocation occurs in both directions even with the R_1 -complex, because the enzyme can switch directions before it starts to translocate again (Seidel *et al.*, 2005), both the HsdR motor and the HsdS DNA recognition subunits should be taken into consideration for mutagenic analysis with the aim to produce stable R_1 -complex capable of unidirectional translocation.

Since the HsdS is the only odd subunit in the enzyme complex, the opportunity exists to use HsdS assembly mutants for asymmetric assembly with cleavage mutant of the HsdR motor subunit.

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