A Molecular Motor that Links the Biological and Silicon Worlds

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ABSTRACT

This project was funded by the European Community as part of the Future Emerging Technologies (FET-OPEN) initiative and represents an exciting and innovative approach to the use of biological systems in bionanotechnology.

We describe the initial stages in the development of a nanoactuator that is based on a molecular motor, which translocates DNA. The nanoactuator involves the movement of a magnetic bead, attached to the DNA, past a sensor/detector that is able to operate a silicon-based electronic device. Since the motor is biological in origin, using the biological 'fuel' ATP to drive the motor and, therefore, translocate the DNA, the nanoactuator links the biological and silicon worlds.

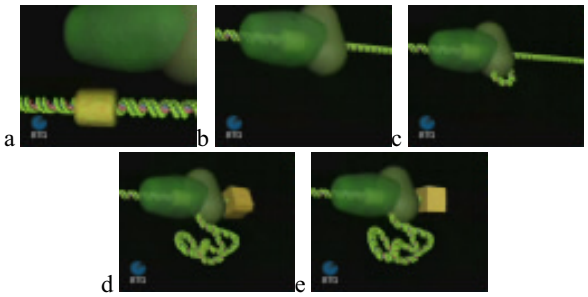
Keywords: Type I restriction-modification enzyme, endonuclease, DNA translocation, magnetic tweezers, atomic Force Microscopy (AFM)

1 INTRODUCTION

Type I Restriction-Modification (R-M) enzymes are the most complex of the many R-M systems known [for recent reviews see 1]. They are multisubunit, multifunctional enzymes composed of three separate subunits (HsdR – the restriction/motor 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 ($HsdR_2$:$HsdM_2$:$HsdS_1$, or R_2-complex). The R_2-complex also functions as a DNA methyltransferase (MTase), an ATPase, and as a DNA pulling molecular motor [2, 3].

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 [2]. This ENase-based motor activity is driven by ATP hydrolysis [3], 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). HsdM and HsdS alone are sufficient to assemble an independent MTase with a stoichiometry of M_2S_1 [4]. The R-M enzyme EcoR124I can be assembled *in vitro* from the core MTase by addition of the motor subunit HsdR [5]. However, the purified EcoR124I ENase exists as an equilibrium mixture of two species - $R_2M_2S_1$ and $R_1M_2S_1$ of which only the former is able to cleave DNA [5, 6]; although the R_1-complex is an ATPase and is able to translocate DNA [5, 7]. The R_2-complex is relatively weak, dissociating into free HsdR subunit and the restriction-deficient R_1-complex intermediate, under concentrations expected *in vivo* [5]. Therefore, to produce a more useful molecular motor, we engineered a special hybrid motor subunit (which we call HsdR(prrI)) that we were able to show was unique in forming exclusively a R_1-complex even when a vast excess of HsdR is mixed with MTase.

Figure 1 Motor activity of type I R M Enzyme



(a) The yellow block represents the DNA-binding (recognition) site of the enzyme, which is represented by the green object approaching from the top of the screen and about to dock onto the recognition sequence. (b) The motor is bound to the DNA at the recognition site and begins to attach to adjacent DNA sequences. (c) The motor begins to translocate the adjacent DNA sequences through the motor/DNA complex, which remains tightly bound to the recognition sequence. (d) Translocation produces an expanding loop of positively supercoiled DNA. The motor follows the helical thread of the DNA resulting in spinning of the DNA end (illustrated by the rotation of the yellow cube). (e) When translocation reaches the end of the linear DNA it stops, resets and then the process begins again.

The complex molecular motor function makes these enzymes particularly interesting. The restriction subunit (HsdR) contains a series of conserved amino acid motifs (DEAD box motifs), which are associated with helicase-like activities [8] including a Walker-type ATP binding site. They belong to a large superfamily (SF-II) of helicase-like enzymes [9] that also include type III R-M enzymes, chromatin remodeling factors and a few chimeric

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enzymes. It has been suggested that chromatin-remodeling 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.

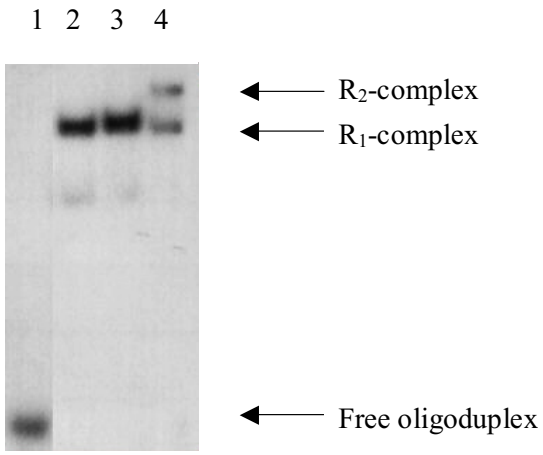
2 RESULTS

The use of the EcoR124I R-M enzyme as a molecular motor has been compounded by the existence of an equilibrium mixture between R_1- and R_2-complexes during purification of the restriction endonuclease [5, 6]. Therefore, we have produced a hybrid endonuclease that, as shown below, has a drastically increased dissociation constant for the R_2-complex, resulting in the production of R_1-complex only.

2.1 Production of a stable R_1-complex

The HsdR subunit of EcoprrI differs from the HsdR subunit of EcoR124I only in the N-terminal region (Figure 2). The bulk of these differences are located within the first 150 residues. To engineer a system for overproduction of the HsdR(prrI) subunit we utilized the plasmid pACR124, which over-produces the HsdR(R124) subunit [10] and constructed a hybrid gene consisting of 600 bp from the 5'-end of the *hsdR* gene of EcoprrI fused to the 3'-end of the *hsdR* gene of EcoR124I. To prepare this hybrid gene we produced a 600 bp PCR fragment of *hsdR*(prrI), produced by cleavage at the unique PstI site (which overlaps amino acid 200, and ligated this to the remaining *hsdR* gene of EcoR124I also cleaved at the unique PstI site.

Figure 2 Gel retardation using the hybrid ENase.



10nM oligoduplex carrying a single s_{R124} recognition site was incubated with 200nM MTase. HsdR(prrI) was added at the following concentrations and the assembled products analyzed on a native 6% PAGE. ***Lane 1****, free oligoduplex;* ***lanes 2-3****, MTase, oligoduplex + 3200nM and 4000nM HsdR(prrI) respectively;* ***lane 4****, EcoR124I ENase produced from a 5:1 molar ratio of HsdR(R124):MTase, showing the location of R_1- and R_2-complexes.*

2.2 Structural analysis of motor subunit

The HsdR subunit of a type I R-M system is a large protein >100kDa, which means that to date there is no structural information for the protein (no crystals have been produced and it is too large for NMR). Therefore, an ideal approach to gain some information is to look for specific domains that might be isolated separately for NMR analysis. Several domains have been identified for the closely related EcoKI R-M system [11] using limited proteolysis to probe the structure. We have initiated a variety of structural studies of the HsdR(prrI) subunit including a similar analysis using limited proteolysis. We have shown that there is a similar number of proteolytic products from limited proteolysis of HsdR(R124I) as observed with EcoKI and these are very similar in HsdR(prrI). This suggests that the N-terminal region, used to make HsdR(prrI) may represent a domain in all HsdR subunits.

Figure 3 Motif X the site required for DNA cleavage

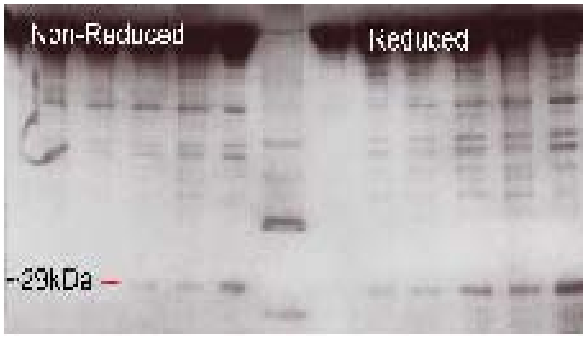
EcoKI (IA)	AD**Y**VLFV--**G**LKPIAVV**EA**K	332
EcoAI (IB)	AD**I**VLYHKP**G**I-PLAVI**EA**K	78
EcoR124I (IC)	Y**D**VTILVN-**G**L-PLVQI**EL**K	167

This region includes the region responsible for DNA cleavage (motif X – Figure 3) that is another important structural feature of these proteins [11]. We have used protein-structure prediction algorithms to analyze the possible structure for this motif. Figure 4 shows the predicted structure.

Figure 4 Computer Generated Prediction (X-Motif)

Predicted X-motif structure generated from superposed structural predictions of many aligned homologous sequences deposited on the protein sequence databank (*http://protinfo.compbio.washington.edu/*)

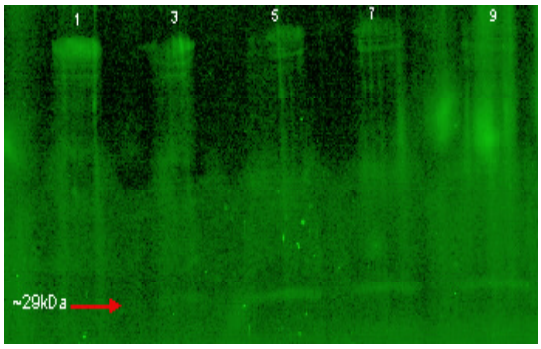
Figure 5 Limited Proteolysis of HsdRPrrI



The above gel (13.5% SDS-PAGE) shows a limited proteolytic of the HsdR(prrI) subunit, using a modified trypsin protease. The proteolysis was performed on both the Non-Reduced and Reduced form of the subunit, respectively. The fragment of interest resides at ~29kDa and increases with intensity as proteolysis proceeds. This region has been mapped to the HsdR(prrI) sequence, suggesting a putative correlation with the N-terminal region of the subunit. This fragment will now N-terminal sequenced.

The hybrid HsdR(prrI) subunit was found to have an available cysteine at position three (as determined by chemical means and using cross-linking to a variety of ligands and polymers). This provides us with an ideal route for identification of the N-terminal proteolytic fragment (Figure 6), surface attachment of the motor, as well as a mechanism to study translocation through FRET. Since it has been previously shown that the R_1-complex is a unidirectional motor [7], it was important to show that this was also true for the hybrid R_1-complex.

Figure 6 HsdR N-Terminal Fluorescence Labeling



The above gel (13.5% SDS-PAGE) shows a limited proteolytic cleavage of the HsdR(prrI) subunit, carried as for Figure 5 and fluorescently labeled with Alexa-633 maleimide probe. Lanes 1,3,5,7,9 show a time-course trypsin digest (1, 5, 30, 60, & 90min, respectively). The fragment of interest resides at ~29kDa and increases with intensity the longer the proteolysis is allowed to continue.

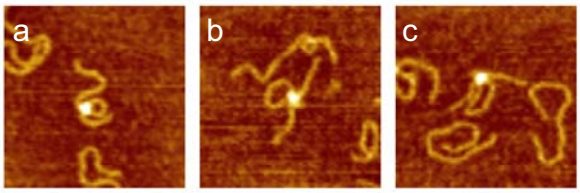
We have initiated studies of the translocation process by measuring FRET between DNA-bound labeled motor protein and end-labeled DNA using Alexa 633 maleimide fluor (Molecular Probes) on the motor protein and Black-Hole Quencher-2 (BHQ-2) fluor on the DNA. A set of four individual 30-mer ssDNA oligonucleotide primers have been synthesized, of which two possess BHQ-2 (A_{max} 580nm) attached to the 5'-ends of two of the 30-mer's.

We obtained a successful FRET assay for the ENase complex when conjugated with Alexa-633 and BHQ2 (data not shown). However, further studies are underway to extend this work.

2.3 DNA translocation by hybrid R_1-complex

As a final check whether the hybrid HsdR(prrI)-MTase(R124I) R-M enzyme functions normally, its DNA translocation ability was tested. Figure 7 shows AFM images of typical hybrid R_1-complexes after incubation with 1 mM ATP and 724 bp DNA with a single recognition site. As anticipated, following the mechanism described in Figure 1, a DNA loop was formed by the hybrid R_1-complex. Only single loops were observed, consistent with the formation of R_1- rather than R_2-complexes. The size of the loops was comparable to those formed by wild-type enzymes at similar incubation times and under similar conditions (data not shown). With longer incubation times the average length of the loop increased. Both the presence of loops and the increase in size of such loops indicate regular translocation activity.

Figure 7 AFM images of DNA translocation



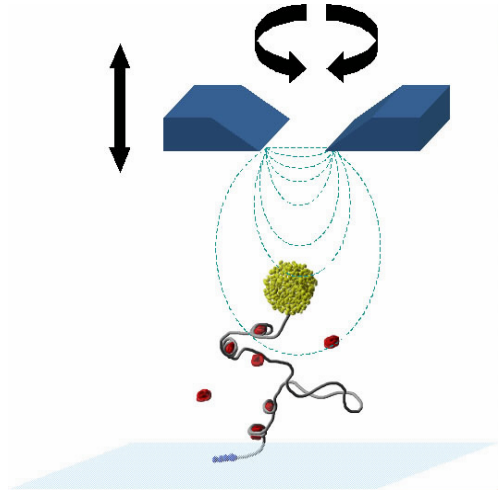
Typical images of translocation activity by HsdR(prrI)-MTase(R124I) after incubation for a) 10s, b) 30s, and c) 60s. in the presence of ATP and 724 bp DNA fragments, containing a single recognition site at 175 bp. At the position of the protein, which shows up as a white globular feature, one small DNA loop is observed that increases in size with longer incubation times. Scan range 250 nm, z range 3 nm.

2.4 Magnetic Tweezer studies

The study of these motor enzymes is encouraged by their unusual property of behaving as simple actuators – they move one part of the DNA relative to another section of the DNA and do not require immobilization on a surface to reproduce measurable motion (although the DNA needs to be surface attached for an observable displacement of a

bead). This suggests a simple mechanism for detecting DNA movement through the use of a magnetic bead.

Figure 8 A simple Magnetic Tweezer setup



The simplest device for measuring such movement is a magnetic tweezer device (Figure 8). Magnetic tweezers allow real time monitoring of protein DNA interactions without surface interference and with femtonewton sensitivity. In addition, these systems can measure DNA displacements as low as 10nm as well as being able to produce negative, or positive, supercoils into the DNA, one turn at a time, through manipulation (spinning) of the magnetic bead [12, 13].

We have begun to measure the rate of translocation for the EcoR124I R-M enzyme in a single molecule situation and we expect this project to yield an abundance of interesting data over the next two years.

3 DISCUSSION

The EcoR124I R-M enzyme represents a very useful molecular motor that can be used as a nanoactuator. We have produced a derivative of the wild-type enzyme motor subunit, which only assembles as a R_1-complex that is also a unidirectional molecular motor, which can be used as a nanoactuator. We have initiated structural studies of this subunit and fluorescence analysis of DNA translocation. Single molecule studies have shown the motor is fully functional and we have initiated translocation studies using a magnetic tweezer setup.

Therefore, this motor has the potential to be a nanoactuator that will link the biological and silicon world through detection of the moving magnetic bead. Such a device could be silicon based with a variety of possible in built sensors for detecting the moving magnet from a simple magnetic force microscope cantilever etched into the device, to a Hall-type sensor built into the silicon.

4 ACKOWLEDGEMENTS

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