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Control of A Multisubunit DNA Motor by a Thermoresponsive Polymer Switch

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The recognition and modification of DNA by biomacromolecules are a central part of cellular function, protection, and reproduction.[1−3] The control of DNA modification by these proteins is of fundamental and technological interest, offering insights into biochemical reaction mechanisms and providing means by which biological information might be stored or processed synthetically.[4] Natural DNA recognition and modification pathways are regulated in elaborate ways or rely on energy sources (e.g., ATP) that are difficult to place under external control, particularly in biological media. Here, we report the first example wherein a multisubunit and multifunctional DNA restriction−modification (R−M) enzyme has been regulated by a thermoresponsive polymer "switch" conjugated at a subunit distal to the DNA recognition and restriction sites. In this polymer−enzyme hybrid, the changes in one of three different operations of the R−M enzyme, i.e., DNA methylation, follow the temperature-mediated phase transitions of the polymer.

We based our studies on a hybrid EcoR124I R−M enzyme. Natural type I R−M systems provide bacteria with protection against DNA-based bacteriophage,[5] via a complex restriction activity that cuts phage DNA at random locations, which can be more than 20 kbp from the enzyme's recognition sequence. Fully functional EcoR124I endonuclease (REase) comprises three subunits (HsdR,M,S) in a stoichiometric ratio of R_2M_2S.[6−8] The HsdS subunit is responsible for DNA sequence recognition specificity; HsdM carries out DNA methylation while HsdR, along with the core M_2S (methylase or MTase), is required for DNA cleavage (restriction). The EcoR124I holoenzyme is in equilibrium with a subassembly complex R_1M_2S that exhibits methylase activity and also translocates DNA without restriction.[7] In vivo, the REase binds specifically to DNA, reads the methylation status of the recognition site (GAAnnnnnnRTCG[9]), and either methylates hemimethylated DNA (produced in vivo following replication of host DNA) or switches to REase mode and moves the rest of the nucleic acid through this bound complex. Random cleavage occurs when this ATP-dependent translocation is blocked (e.g., through collision with another translocating enzyme) or via topological effects on single-site circular DNA.[10] The DNA pulling function is a unique feature of this protein (most proteins traverse along DNA rather than pull it) and is the basis on which nanoscale molecular motor devices can be developed.[11]

To regulate the activity of the enzyme complex—the first stage in regulation of molecular motor function—we chose the thermoresponsive polymer poly(*N*-isopropylacrylamide) (PNIPAm), which as a pure homopolymer undergoes a phase transition from a chain-extended soluble state to a chain-collapsed and insoluble state at ∼32 °C (the lower critical solution temperature or LCST). This property has been used to effect temperature control of ligand binding in polymer−protein conjugates[12−17] while related polymers have been shown to exhibit photochemical switching of single unit enzyme activity.[18]

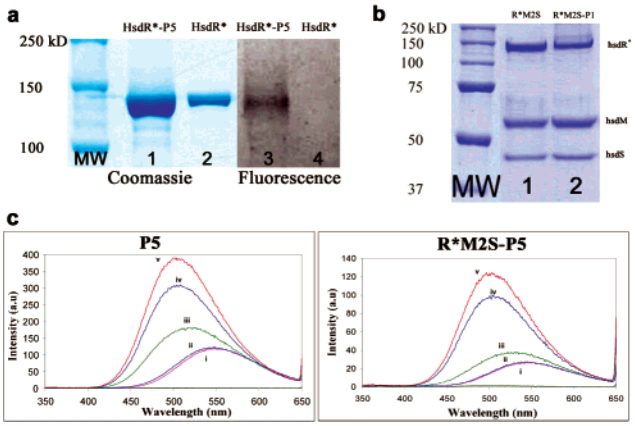

Figure 1. SDS PAGE. (a) Coomassie detection: lane 1, P5-HsdR*; lane 2, HsdR*. Fluorescence detection: lane 3, P5-HsdR*; lane 4, unlabeled HsdR*. (b) Coomassie stain detection of R^*M_2S enzyme (lane 1) and polymer−enzyme complex R^*M_2S-P1 (lane 2). (c) Variable temperature fluorescence emission spectra of P5 and R^*M_2S-P5 at (i) 20, (ii) 25, (iii) 30, (iv) 35, and (v) 40 °C.

The challenge in this study was to regulate the function of a large, multiunit enzyme without blocking the subunit self-assembly sites or the primary DNA sequence recognition site. Initial conjugation of PNIPAm polymers to the M subunits in the enzyme complexes irreversibly blocked enzyme activity, presumably because of the proximity of the polymer chains to the methylation site.[19] We reasoned therefore that enzyme activity might be controlled most effectively by attaching the polymer chain(s) distal to the M subunit DNA methylating sites, employing PNIPAm of varying chain lengths to alter the steric demands in the vicinity of the enzyme complex. PNIPAm polymers P1−P3 were accordingly prepared with a range of molecular masses (14−30 kDa), phase transition temperatures (33−38 °C), and maleimide functionality for attachment to the protein subunits. We also prepared fluorescent-tagged PNIPAm copolymers P4 and P5 (M_w 58 and 15 kDa, respectively; LCST, 27−30 °C) with maleimide termini and prepared a specific hybrid of HsdR (HsdR*) to contain an accessible cysteine residue close to the N terminus of the R subunit. These polymers were coupled under mild conditions (pH 7.4, phosphate buffer) to the cysteine thiol; single site conjugation to the HsdR* subunit and the thermal response of the attached polymers were confirmed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and fluorescence spectroscopy (Figure 1).

Polymer phase transitions in the polymer−enzyme conjugates were further characterized by UV and dynamic light scattering (DLS). Observed hydrodynamic radii were higher for polymer−enzyme conjugates below LCST (78−99 nm) than above LCST (55−66 nm), indicative of the lower diffusion coefficients of the conjugates prior to collapse of the covalently attached polymer

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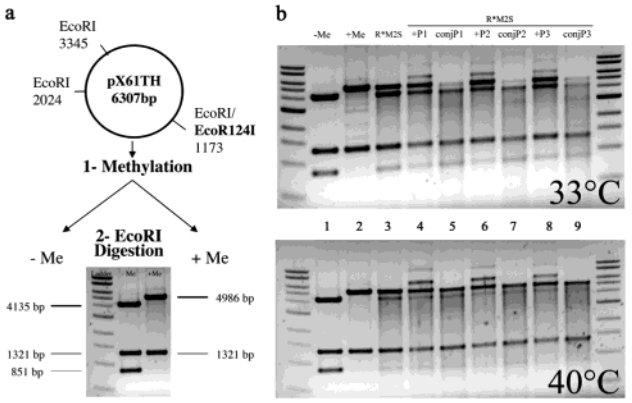

Figure 2. (a) Example of restriction patterns of plasmid pX61TH with and without methylation. (b) Agarose gel electrophoresis showing the EcoRI restriction pattern of pX61TH following methylation by enzyme complexes and polymer−enzyme complex conjugates at temperatures below polymer LCST (upper panel) and above LCST (lower panel).

chains. The enzyme complexes and the polymer−enzyme conjugates were able to bind DNA containing the sequence GAAnnnnnn-RTCG but did not bind DNA without this sequence. No temperature dependence was observed in these assays, establishing that the primary DNA recognition site was unaffected by polymer conjugation and phase transition. Also, the ATPase activity was independent of polymer conjugation and LCST. However, the MTase function of the RM_2S complex was notably affected by conjugation of thermoresponsive polymers P1−P3. Methylation in vitro at adenine residues within the specific EcoR124I recognition sequence of DNA (which included the EcoRI restriction site) in the test pX61TH plasmid[4] allowed us to determine whether the polymer conjugated enzyme complex could be switched "on" and "off" by polymer phase transitions. Incubation of the plasmid with the conjugate followed by DNA digestion with EcoRI resulted in the presence of two bands in the gel, whereas the absence of methylation at this specific site yielded three bands. Marked differences in DNA methylation (Figure 2) occurred dependent on whether the assays were carried out below or above polymer LCST. Nonconjugated EcoR124I was used in all of these assays to control and compensate for the effect of the temperature difference on the enzyme activity.

Mixtures of polymers and enzyme complexes (equimolar in RM_2S content) displayed similar activity as compared to the enzyme complex alone, excluding the nonspecific effect of the unconjugated polymer. Quantification of bands indicated that RM_2S and RM_2S mixed with polymers P1−P3 caused 50% methylation of DNA when the experiment was carried out at 33 °C, i.e., below the polymer LCST (Figure 2b; lanes 3, 4, 6, and 8, upper panel), whereas RM_2S covalently bound to polymers P1−P3 yielded only 10% methylation (Figure 2b; lanes 5, 7, and 9, upper panel) at the same temperature.

By contrast, the enzyme complex alone, or mixed with the polymers, caused 80% methylation of pX61TH at 40 °C (Figure 2b; lanes 3, 4, 6, and 8, lower panel). At this temperature, RM_2S covalently bound to polymers P1−P3 produced 90−100% methylation of the plasmid (Figure 2b; lanes 5, 7, and 9, lower panel). To verify that the switch was due to the coil−globule transition of the pendant polymer, fluorescent PNIPAm copolymer P5, with a reduced LCST (27−29 °C) as a result of dansyl monomer content, was conjugated to the R subunit. Although the polymer phase transition at 27 °C was verified by fluorescence, no change in DLS behavior or DNA methylation was observed over the 33−40 °C range as the attached polymer was in its collapsed state at both of the latter temperatures. We also attached a low molecular mass fluorescent PNIPAm polymer (M_w = 7 kDa; LCST, 33−34 °C), which we reasoned, on the basis of preliminary molecular modeling calculations, would be too small to exert an influence on the methylation site when attached to the R subunit. This polymer enzyme complex displayed the LCST transition over the 33−40 °C range but did not affect the pattern of methylation. The LCST transitions of the polymer−enzyme conjugates could be reversed and repeated over multiple cycles, and the MTase function of the enzyme was retained over at least five DNA methylation assays at 33 and 40 °C.

Overall, the data show that responsive polymers can switch the activity of multiunit enzyme complexes even where the point of attachment of the polymer chains is distant from the enzyme active sites. Because the methyltransferase activity required DNA recognition by the enzyme, the changes in DNA methylation via the polymer phase transition—in a conjugate that did not change DNA recognition behavior with temperature—indicate that the polymer may have a role in controlling the switch of the enzyme complex to its DNA translocating function. Experiments to evaluate this hypothesis—in effect control of a DNA translocating "motor"—are under way in our laboratories. The transfer of information from one subunit of the enzyme complex to another via the polymer switch is a first step toward the use of these chimeric systems as molecular devices.

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Supporting Information Available: Details of synthetic procedures, structures, and properties of polymer−protein conjugates and assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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