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Abbreviations

HMWS-high molecular weight species, PCP-precut primary site, res-residue(s), PNK-polynucleotide kinase, PAGE-polyacrylamide gel electrophoresis, OAc-Acetate, AUC-analytical ultracentrifugation, ATP-adenosine triphosphate, dsDNA-double-stranded DNA, ssDNA-single-stranded DNA, wtSgrAI-wild-type SgrAI, DTT-dithiothreitol, APS-ammonium persulfate, DNA-deoxyribonucleic acid, MW-molecular weight

Abstract

An 8-bp recognition sequence, secondary activity, and allosteric activity for the primary sequence make SgrAI a rather peculiar endonuclease. Recent evidence suggests that SgrAI exists as a dimer in solution in the absence of DNA and is able to bind to one duplex of DNA. However, the molecule mechanisms of secondary site and allosteric activity are poorly understood. Through a series of kinetic and binding assays we have reason to believe that the rate of DNA cleavage by SgrAI is significantly accelerated through the formation of HMWS (high molecular weight species), an aggregate of SgrAI dimers bound to DNA. Native gel electrophoresis suggests that increasing concentrations of PCP (precut primary site), in the presence of excess SgrAI enzyme, increases formation of the HMWS. Furthermore, our kinetic assays show acceleration of DNA cleavage by the addition of PCP in the presence of excess SgrAI. As such, it appears that the HMWS is an activated form of the enzyme. Crystal structures show that the tetramer has an N-terminal region which is domain swapped with another dimer to form a tetramer, which may be a building block of the HMWS. In order to further test this hypothesis, we measured HMWS formation and the stimulation of DNA cleavage by mutated enzymes, P27G/W SgrAI. Both mutants enzymes were designed to disrupt the domain swapped tetramer. Native gel electrophoresis suggests that P27W/G SgrAI enzymes do not form HMWS to the same extent as wild-type SgrAI (wtSgrAI). Additionally, P27W/G SgrAI do not exhibit the same acceleration of DNA cleavage as wtSgrAI, which leads us to believe that formation of the tetramer is necessary for formation of the HMWS. It also further supports the hypothesis that the active conformation of the enzyme can be found in the HMWS. This work is supported by Howard Hughes Medical Institute and NIH, General Medical Sciences.

Introduction

SgrAI is a unique type II restriction endonuclease native to *Streptomyces griseus*.

Restriction endonucleases play an important role in prokaryotes protecting the host genome from potential invasion of foreign DNA [1]. Endonucleases are often part of a system that protects the host cell from foreign DNA. The host DNA is often differentiated from foreign DNA by methylation by native DNA methylases. Methylation of host DNA significantly decreases endonuclease activity for the host DNA [2]. Unmethylated DNA from an invading species is usually susceptible to cleavage by host restriction endonucleases, thus protecting the host genome. Though methylation can be used to distinguish between host and invading DNA, there are also cases in which restriction endonucleases are specific to sequences that do not exist in the host genome. Furthermore, endonucleases such as NciI can cleave symmetrically methylated, hemimethylated, or unmethylated DNA [3].

Before a crystal structure for SgrAI was available, there were crystal structures available for other type II restriction endonucleases such as EcoRI, EcoRV, NgoMIV. Type II restriction endonucleases often bind and recognize palindromic DNA sequences as dimers (reviewed in [1]). Positioning of the endonuclease dimers allows for the two catalytic domains to be positioned such that cleaved DNA can have blunt or sticky-ends. Type II restriction endonucleases typically cut sequences of 4-8 bp in length [4]. Rare restriction enzymes that are capable of cleaving sequences of up to 8 bp in length have also been identified with crystal structures available for NotI [5] and SfiI [6].

SgrAI is a rather promiscuous restriction endonuclease that can recognize and cleave primary and secondary sites. The primary site consists of an 8 bp sequence CR|CCGGYG, where | denotes the site of cleavage. Secondary site activity, involving cleavage of CR|CCGGY(A,C,T)

and CR|CCGGGG, is much slower. However, the efficiency of cleavage for secondary sites is greatly increased with high concentrations of SgrAI and primary site. Interestingly enough, SgrAI, shows allosteric properties which are quite unique in comparison to other restriction endonucleases. Not only does a pre-cut 40-mer, (i.e. PCP), stimulate cleavage of the secondary site, but it also accelerates the cleavage of the primary site. Interestingly, the uncut primary sequence can also increase cleavage rates (reviewed in [7]). X-ray crystallographic data provides additional insight into the activation mechanism responsible for these unique biochemical properties.

Recently, a crystal structure for SgrAI bound to an 8 bp target sequence was determined [7]. An SgrAI dimer binds to one duplex DNA molecule, along with two Ca^{2+} ions per active site. The SgrAI dimer bears remarkable similarity in structure to Cfr10I, Bse634I, and NgoMIV. Furthermore, SgrAI and NgoMIV target sequences share similarities, CRCCGGYG and GCCGGC respectively (reviewed in [7]). However, NgoMIV and Cfr10I are known to exist as tetramers in crystal structures [8, 9]. Data from analytical ultracentrifugation indicates that SgrAI forms a HMWS in the presence of 20 bp DNA [10]. At the same time, homology to NgoMIV implies that SgrAI might also exist as a tetramer. It has been proposed that the unusual biochemical properties of SgrAI derive from tetramerization of DNA bound SgrAI [10]. A tetrameric structure for SgrAI, recently solved by x-ray crystallography in the Horton Lab [11] reveals that domain swapping occurs between res 1-24. The domain swapped region, res 1-24, of one subunit interacts with a different subunit, in the opposite dimer (Figure 1). Therefore the peculiar allosteric modulation of DNA cleavage by SgrAI has been proposed to be due to the formation of this domain swapped tetramer, stabilized in part by domain swapping of residues 1-

24 of wtSgrAI, Through kinetic and binding assays we hope to determine the molecular mechanism of the allosteric modulation of DNA cleavage by SgrAI.

Data from AUC suggests that SgrAI binds to DNA as a dimer (reviewed in [10]). Once SgrAI binds the primary sequence, an SgrAI dimer can interact with another SgrAI dimer bound to DNA. Formation of SgrAI oligomers is followed by or induces a shift in conformation such that SgrAI has significantly higher DNA cleavage activity. Communication between dimers may take place through the domain swapped regions, res 1-24, as seen in the crystal structure [11]. The conformation of the enzyme in the domain swapped tetrameric form, and the unswapped dimeric form, is identical, with the exception of the swapped domain and the conformation of the linker connecting the swapped domain to the core of the enzyme [7, 11]. Proline 27 resides in this linker, and by mutating this proline to tryptophan or glycine we hoped to disrupt the domain swapping, and to test for disruption of HMWS formation. Since the activated form of SgrAI is proposed to reside in the HMWS, disruption of HMWS formation is predicted to disrupt the allosteric activity of SgrAI (Figure 2). By measuring HMWS formation and DNA cleavage rate constants of wild type and P27W/G SgrAI under stimulating and nonstimulating conditions, we will determine whether the mutation disrupts HMWS and/or the allosteric stimulation of DNA cleavage by SgrAI. If the HMWS formation is disrupted, then a role for the domain swapped tetramer in forming HMWS can be inferred. If both HMWS formation as well as the allosteric stimulation of DNA cleavage activity are disrupted, then a role for the HMWS as the activated species is further supported.

Type II restriction endonucleases are able to cut DNA in the presence of Mg^{2+} , Mn^{2+} or Co^{2+} [13]. However, in the presence of Ca^{2+} the restriction endonucleases are unable to cleave DNA, but remarkably retain the ability to bind target DNA sequences with enhanced affinity and

specificity [14]. Crystal structures show that the divalent cations bind in the active site of the enzyme, and the higher affinity of the endonuclease for DNA in the presence of the divalent cations derives from the screening of negatively charged side chains of the enzyme from the negatively charged phosphates of the DNA. The inability of the bound Ca^{2+} , which binds very close to the Mg^{2+} binding sites of the enzyme active site, to confer DNA cleavage activity to the enzyme is less well understood, but may be due to the larger size of Ca^{2+} relative to Mg^{2+} , causing mispositioning of active site groups [15]. With this information, we plan to investigate SgrAI binding by native gel electrophoresis. SgrAI binds to primary and secondary sequences with relatively high affinity. The K_D for primary and secondary sequences is within the nanomolar range [12]. Due to the extremely high affinity of type II restriction endonucleases, our assays will require a highly sensitive method for detection. Labelling substrate with radioactive ^{32}P - γ -ATP allows for high sensitivity in our assays when our DNA sequences are labeled with T4 phage polynucleotide kinase [16]. Studying the formation of HMWS as a function of PCP and primary or secondary sequence will provide details into ability of SgrAI to polymerize on DNA.

Kinetic activity of SgrAI can be measured via endpoint sample methods (Figure 3). If PCP increases the formation of HMWS and also accelerates the reaction rate constants for SgrAI it would suggest that the high activity conformation exists in the HMWS. In order to further support this hypothesis we can make mutations predicted to to disrupt HMWS through disrupting the domain swapping regions. Measurements of the binding affinity of the P27W and P27G mutant enzymes to the primary site DNA (18-1 and PCP) used in the assays have been performed and indicate that no significant effects on binding affinity occur as a result of the mutations[11]. As such, P27W and P27G are ideal mutations for our mutagenesis studies. The

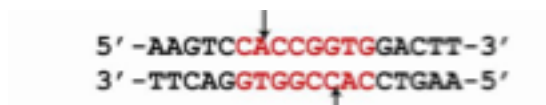
mutations are designed to destabilize the domain swapped tetrameric form of SgrAI, and if the hypothesis that the HMWS is composed of domain swapped tetramers is correct, then HMWS formation should be reduced in the case of the mutant enzymes relative to wild type. Further, if the hypothesis that the HMWS is the activated form of the enzyme is correct, and the mutations do in fact disrupt the HMWS, then the ability of the enzymes to be stimulated in DNA cleavage assays by the addition of PCP should be reduced with the mutant enzymes relative to wild type.

Materials and Methods

Protein and DNA Purification:

Purified P27W was provided by New England Bio Labs and Horton Lab. WtSgrAI and P27G were provided courtesy of Horton Lab. Primary site DNA (18-1) was purified by PAGE. PCP and 18s2, secondary site DNA, were purified via HPLC.

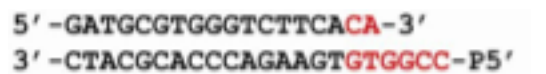
Sequence for 18-1:



Sequence for 18s2:



Sequence for PCP:



Recognition sites shown as red, cut sites denoted by arrows [from 12].

Radioactive Labelling of DNA:

1 pM of DNA is labeled through a reaction mixture consisting of [γ - 32 P]-ATP 10 uCi (Perkin-Elmer, Inc.), PNK T4 Kinase in PNK Buffer A (New England Biolabs). Excess ATP was removed with G-30 spin columns (Biorad Laboratories, Inc.)

Native gel electrophoresis:

Samples were prepared in binding buffer [20 mM Tris-OAc, 50 mM KOAc, 1 mM DTT, 10 mM Ca(OAc), 10% glycerol] with constant concentrations of SgrAI (1 μ M), 32 P labeled 18-1 or 18s2 (1 nM), PCP concentration ranged from 10, 30, 60, 100, 200, 300, 400, 500, 600, and 1000 nM at 4°C and incubated at 4°C for 30 minutes prior to electrophoresis. An 8% native gel was prepared

using (29:1) 6 mL of a 40% solution of acrylamide:bisacrylamide (29:1 ratio) in water, with 3 mL 10x TB (0.9 M Tris Base, 0.9 M boric acid), 0.3 mL of 1 M CaCl_2 , and 20.7 mL H_2O and polymerized with 0.25 mL 10% APS and 25 μL TEMED. Gels were prerun for one hour at 190V with recirculation of the running buffer (89 mM Tris Base, 89 mM boric acid, 10 mM $\text{Ca}(\text{OAc})_2$, pH 8.3). Samples were loaded onto the gel while undergoing electrophoresis at 300V, followed by electrophoresis of the gel for an additional 2 hours at 197V at 4°C. Native gels exposed were to GE Healthcare and Molecular Dynamics phosphor screens for 12-24 hours, and the screens scanned using Typhoon Phosphorimager (GE Healthcare) with 100 microns pixel size.

Single Turnover Assays:

All reactions were performed in SgrAI kinetic buffer [20 mM Tris-OAc pH 8.0, 50 mM KOAc, 10 mM $\text{Mg}(\text{OAc})_2$, 1 mM DTT]. DNA and PCP were combined in one test tube while enzyme was prepared in a separate test tube. The solutions were incubated at 37°C prior to mixing, and reactions were also performed at 37°C. The reaction was initiated by mixing enzyme the enzyme solution with the DNA (and PCP) solution. Endpoint samples were collected at various timepoints, t (seconds), and quenched in equal amounts of 8M Urea with 50 mM EDTA, 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue (dyes added for visualization during electrophoresis). Samples were electrophoresed using denaturing PAGE [14 g Urea, 7 mL of 10x TBE (0.9 M Tris Base, 0.9 M boric acid, 2 mM EDTA), 16 mL of 40% (19:1 bis:acrylamide), 7 mL H_2O , 250 μL APS, and 50 μL TEMED]. Denaturing gels were exposed to phosphor screens without drying (GE Healthcare and Molecular Dynamics). Phosphor screens were scanned with Typhoon Scanner scanning software on Typhoon Scanner with a pixel size of 100 microns. Densitometry of product and substrate bands were done with ImageQuant Software (Molecular Dynamics) (Figures 3). Product and substrate bands were added, and fraction of product cleaved

was determined by dividing product by total (product+substrate), fraction product = product / total. Data was fit to first-order kinetics in Kaleidagraph:

First-order reaction (unimolecular reaction) rate law:

$$d[\text{product}]/dt = k[\text{product}]$$

Integrated rate law:

$$\ln [\text{product}] = kt + \ln [\text{product}]_0$$

$$[\text{product}] = [\text{product}]_0 e^{kt}$$

KaleidaGraph Equation for Integrated Rate Law:

Product = $m_2 * (1 - \exp(-m_1 * m_0)) + m_3$; where m_0 is time (seconds), m_1 is k , m_2 is a constant for the maximum value of the product band intensity, m_3 is a constant describing the baseline product band intensity

Results

Native Gel Electrophoresis

Ca^{2+} ions allow for high affinity binding of labeled primary and secondary sequences. Through native gel electrophoresis we see a HMWS that barely enters the gel (Figure 4 & 5). Bands directly below HMWS bands are dimers bound to a single DNA molecule. Disperse bands at the very bottom of the gel correspond to dsDNA and ssDNA. All bands correspond to locations of labeled DNA, either 18-1 or 18s2. PCP was not labeled and therefore is not visible.

Native gel electrophoresis shows that wtSgrAI forms a high molecular weight species (HMWS) as PCP concentrations are increased (Figure 4). PCP concentration increases from left to right for the first 10 lanes, and contains radioactively labeled 18-1, primary site. Likewise, the second 10 lanes contain radioactively labeled 18s2, secondary site. Native gel electrophoresis shows that P27W does not respond to PCP in the same fashion as wtSgrAI (Figure 5). PCP concentration increases from left to right for the first 10 lanes and second 10 lanes, the first 10 lanes contain radioactively labeled 18-1, primary site, the second 10 lanes contain radioactively labeled 18s2, secondary site. HMWS formation for WtSgrAI seems to be dependent upon PCP concentration, where PCP concentrations range from 10, 30, 60, 100, 200, 300, 400, 500, 600, and 1000 nM. However, HMWS formation for P27W does not seem to be dependent upon concentration of PCP.

Interestingly, P27W dimers are able to bind 18-1 as evidenced by the single band directly below the HMWS. However, in our native gel electrophoresis assays, no band corresponding to the dimeric form of P27W bound to labeled DNA is evident, indicating either no binding, or a complex that does not withstand the electrophoresis (Figure 5). P27W SgrAI affinity for 18s2

might be low enough for dissociation to take place before samples enter their respective lanes in the gel. Hence, dissociation of P27W SgrAI from labeled 18s2 results in multiple bands.

Random formation of HMWS could possibly be due to precipitation of protein. The HMWS is reduced with increasing PCP in the case of P27W SgrAI, thereby following an opposite pattern relative to the wild type enzyme, and may be due to the repulsion of aggregating enzymes upon binding to the added PCP. These results cannot be explained by any reduction in PCP binding by P27W SgrAI, since binding affinity measurements have determined that P27W SgrAI binds to PCP with the same affinity as does the wild type enzyme [11].

Single Turnover Cleavage Assays

Single turnover cleavage assays were performed at 37°C with Mg^{2+} present as a cofactor for the enzyme. Mg^{2+} was sufficient for cleavage of DNA as indicated by two separate bands that are seen on a denaturing gel (Figure 6). Densitometry of bands was done with via software. Product and substrate bands were added together for a total, fraction cleaved was calculated by dividing product by total. The fraction cleaved was plotted against time in seconds, the independent variable. Fits of a single exponential equation to our kinetic data showed correlation coefficients of 0.99 or above. Because SgrAI possesses a nanomolar affinity for the DNA used in the kinetic assays, and the SgrAI concentration is 1 μ M in the assays, SgrAI is expected to completely saturate the DNA. In addition, the rate of DNA binding is expected to be very fast (< 1 msec). Thus, binding of substrate is relatively fast compared to the catalysis of substrate. Furthermore, due to quenching of the reaction by urea, which induces unfolding, we assume that rate of the release of products is also not measured by our assay.

Kinetic activity assays reveal fairly similar reaction rate constants for wtSgrAI, P27W, and P27G under non-stimulating conditions, i.e. 0 nM PCP (Figure 8). Any difference in the

reaction rate constants for the different enzymes is less than the error associated with the measurement of the rate constants, and therefore insignificant. Figure 5 shows the reaction rate constants of wtSgrAI, P27W, and P27G under stimulating conditions, i.e. in 1 μM PCP. The reaction rate constants for wtSgrAI in 1 μM PCP requires rapid quench techniques [12]. When assayed without this equipment, results revealed a reaction rate constants higher than 20 min^{-1} (Figure 9), a stimulation of more than 200 fold. In contrast, the reaction rate constants of DNA cleavage by P27W and P27G SgrAI enzymes was not stimulated significantly, rather remained similar to the unstimulated rate constants (Figure 9).

Discussion

Wild type SgrAI responds to a change in PCP concentration in two ways: first, higher PCP concentrations increase the proportion of HMWS as seen through native gel electrophoresis; second, PCP concentration also increases DNA cleavage activity of SgrAI. Given this correlation, it has been proposed that the high activity form of the enzyme resides in the HMWS [12].

We tested the effects of two mutations, P27W and P27G, on the ability of SgrAI to both form HMWS and to be stimulated by PCP in DNA cleavage assays. The mutations were designed based on the crystal structure of two DNA bound SgrAI dimers interacting via domain swapping [11]. We specifically wanted to investigate the potential role of this observed interaction in the formation of the HMWS. In addition, we wanted to further investigate the relationship by HMWS formation and the activation of DNA cleavage activity by SgrAI. Pro 27 resides in a segment of the SgrAI enzyme that is the only portion of the enzyme that changes conformation between the swapped and unswapped forms. By mutating Pro 27 to Trp or to Gly we hoped to interfere with the swapped conformation and thereby destabilize the domain swapped configuration. If domain swapping is important to HMWS formation, we predicted that the mutant enzymes should show reduced HMWS formation. Indeed, measurement of HMWS formation by P27W SgrAI showed an altered pattern as a function of added PCP than that formed by wtSgrAI (Fig. 4-5). The HMWS formed by P27W SgrAI was anomalous and unreproducible as subsequent measurements showed formation only at very low PCP, and added PCP above 10 nM resulted in no HMWS formation [11]. The loss of HMWS formation with

increasing concentration of PCP could be disruption of nonspecific aggregation of enzymes bound to enzyme/DNA complexes as a greater proportion of the enzyme becomes bound to PCP.

We also predicted that if the HMWS is the activated species of SgrAI, and that the mutations P27W and P27G disrupted HMWS formation, then the mutations should also show a reduced capacity to become stimulated in DNA cleavage activity in the presence of 1 μ M PCP. Comparing Figures 8 and 9 it is clear that PCP concentration accelerates the reaction rate constants for wtSgrAI but does not seem to affect P27W/G SgrAI to the same extent.

Together, the results with P27W and P27G SgrAI enzymes suggest that Pro 27 is important for HMWS formation, presumably via domain swapping in SgrAI, and that HMWS is responsible for the accelerated cleavage of DNA by SgrAI.

Future directions of this project include the investigation of the origin of the loss of affinity of P27W and P27G for secondary site DNA. In addition, characterization of the detailed structure of the HMWS, and the activated conformation of the enzyme will also require further studies.

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Figure Legends

1. Figure 1. SgrAI tetrameric structure comparison. Domain swapped regions shown as spacefill and duplex DNA shown as black helix taken from Park, *et al* [11]. (A) Dimeric crystal structure for SgrAI [7]. (B) Tetrameric model of SgrAI with domain swapped regions interacting between SgrAI dimers. NgoMIV like interactions notably absent. (C) NgoMIV tetramer with duplex DNA shown in black.
2. Figure 2. SgrAI model of self-modulation. Upon binding of DNA the SgrAI dimer can oligomerize with another dimer forming a tetramer. Dimers and tetramers can interact with each other leading to a shift in conformation with higher activity, denoted by grey monomers. Domain swapping occurs between one subunit and a subunit on an opposing dimer. Product of cleavage has sticky ends.
3. Figure 3. Single Turnover Assay. Enzyme and DNA in separate aliquots are incubated at 37°C. Samples are taken before and after mixing at various time points followed by subsequent quantization via denaturing gel electrophoresis and autoradiography.
4. Figure 4. wtSgrAI Native Gels. Native gel electrophoresis of wtSgrAI with increasing concentrations of PCP, radioactively labelled 18-1* and 18s2* concentrations remain constant. HMWS bands denoted by red arrow. PCP concentrations range from 10, 30, 60, 100, 200, 300, 400, 500, 600, and 1000 nM. Unbound dsDNA and ssDNA indicated by arrows.
5. Figure 5. P27W Native Gels. Native gel electrophoresis of P27W SgrAI with increasing concentrations of PCP, radioactively labeled 18-1* and 18s2* concentrations remain constant. HMWS bands denoted by red arrow. PCP concentrations range from PCP, 10,

30, 60, 100, 200, 300, 400, 500, 600, 1000 nM. Unbound dsDNA and ssDNA indicated by arrows.

6. Figure 6. Denaturing gel separation of product from substrate. Endpoint samples were run over a denaturing gel separating labeled product from substrate as indicated by arrows. Densitometry done via software, ImageQuant (Molecular Dynamics).
7. Figure 7. Single Turnover Assay data fitting. Fraction cleaved was plotted against time and fit to a single exponent for first-order kinetics.
8. Figure 8. Rate constants for 0 nM PCP. Rate constants (min^{-1}) for WtSgrAI, P27W SgrAI, and P27G SgrAI when mixed with 1 nM 18-1, 0 nM PCP, and 1000 nM of the respective enzyme. Standard deviation shown as error bars.
9. Figure 9. Rate constants for 1000 nM PCP. Rate constants (min^{-1}) for WtSgrAI, P27W SgrAI, and P27G SgrAI when mixed with 1 nM 18-1, 1000 nM PCP, and 1000 nM of the respective enzyme. Standard deviation shown as error bars. *Exact rate measurement requires rapid quench method [12].

Figures

Figure 1:

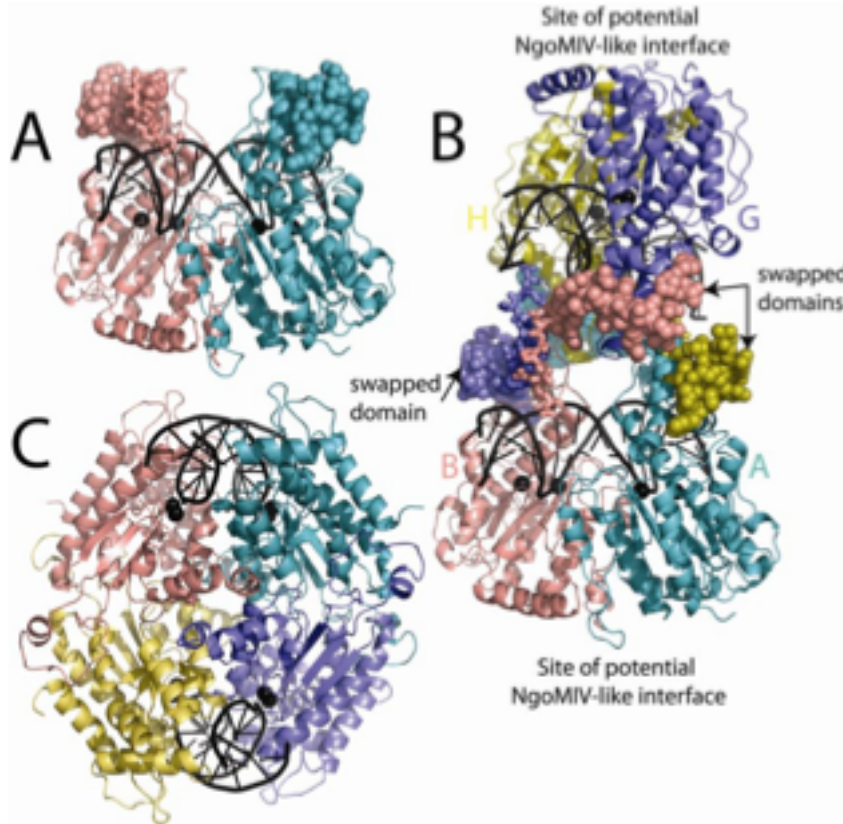


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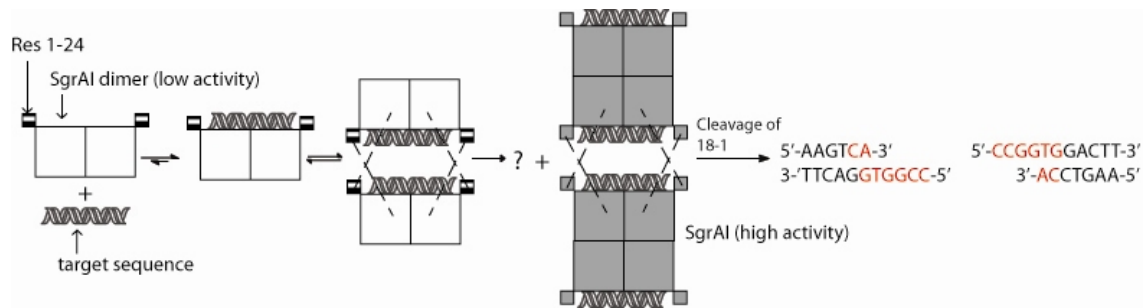


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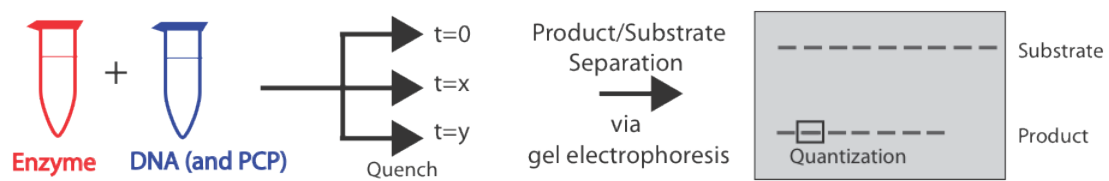


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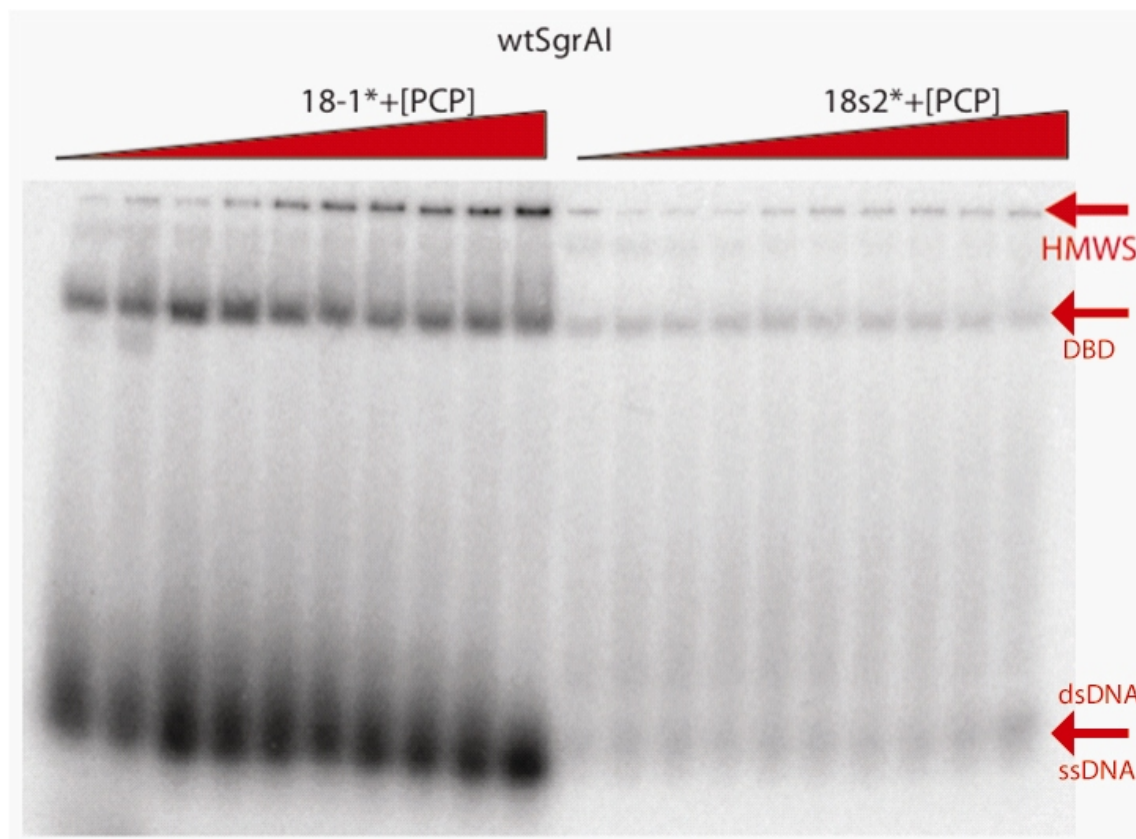


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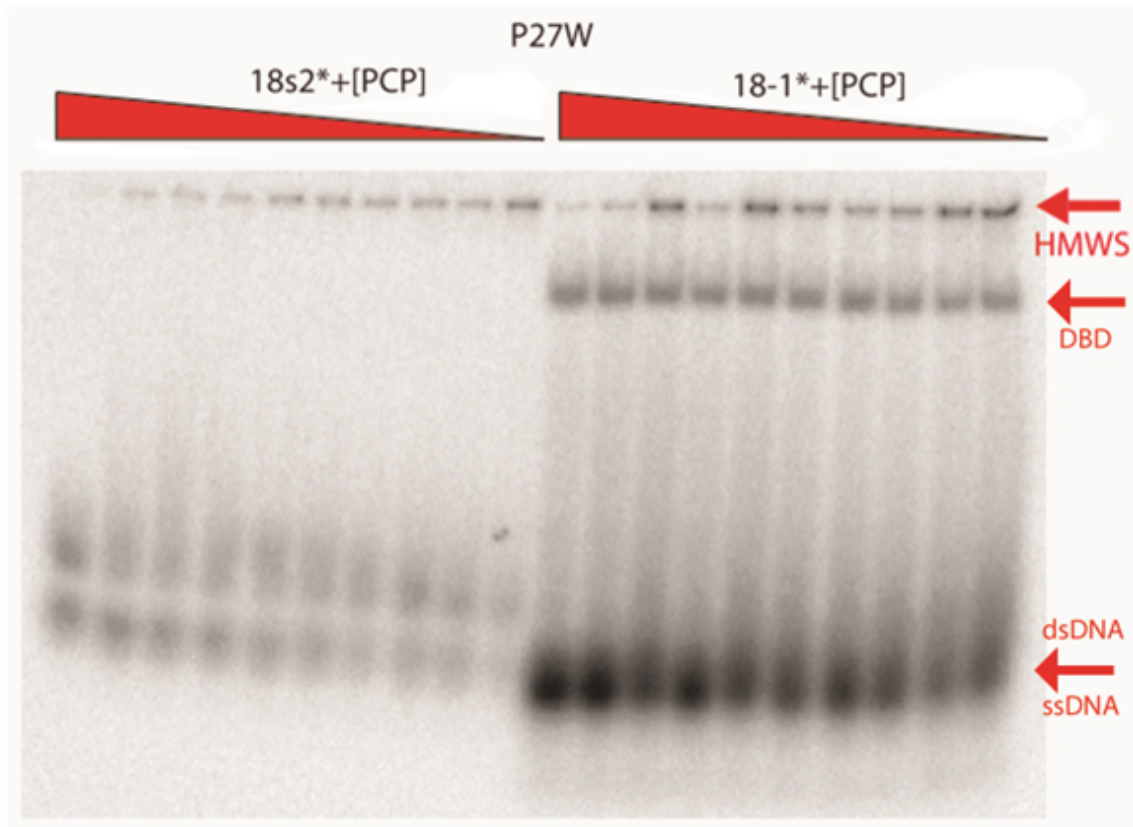


Figure 6:

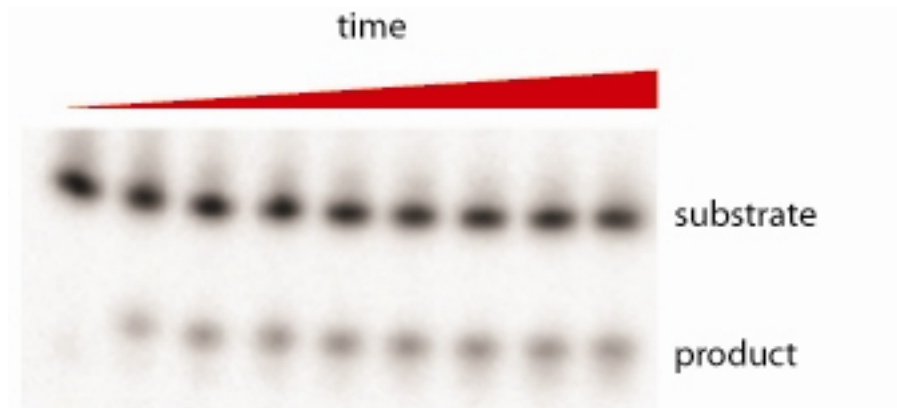


Figure 7:

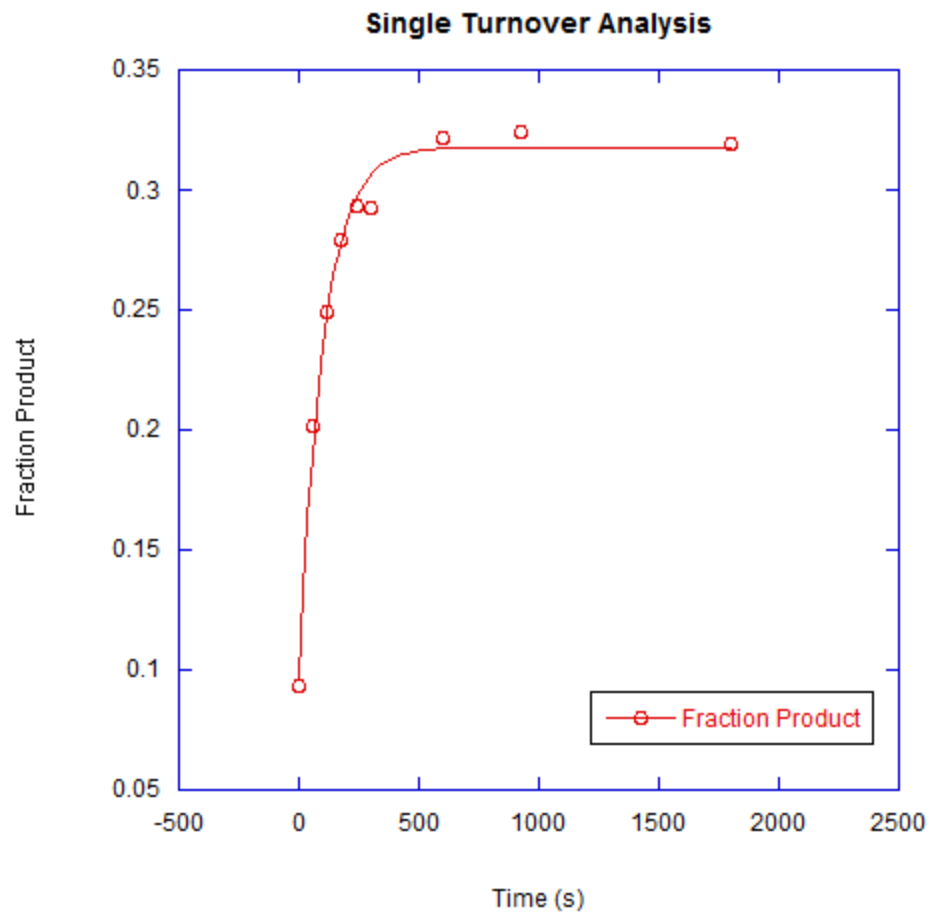


Figure 8:

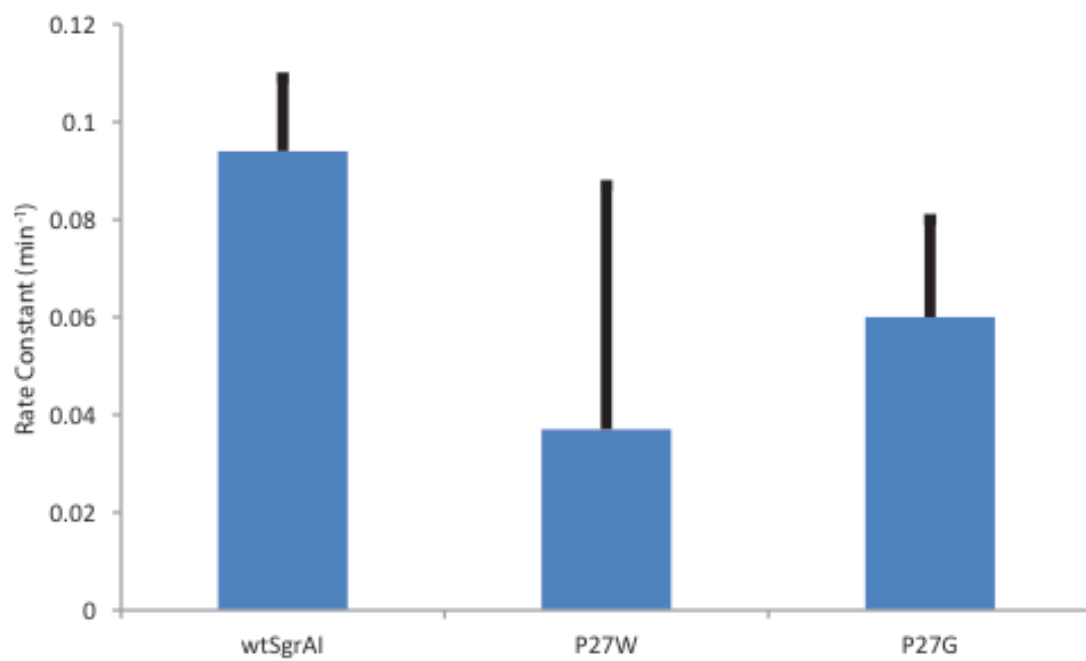


Figure 9:

