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DNA Binding and Properties of Lac Repressor

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CHEM 154 Section 1C

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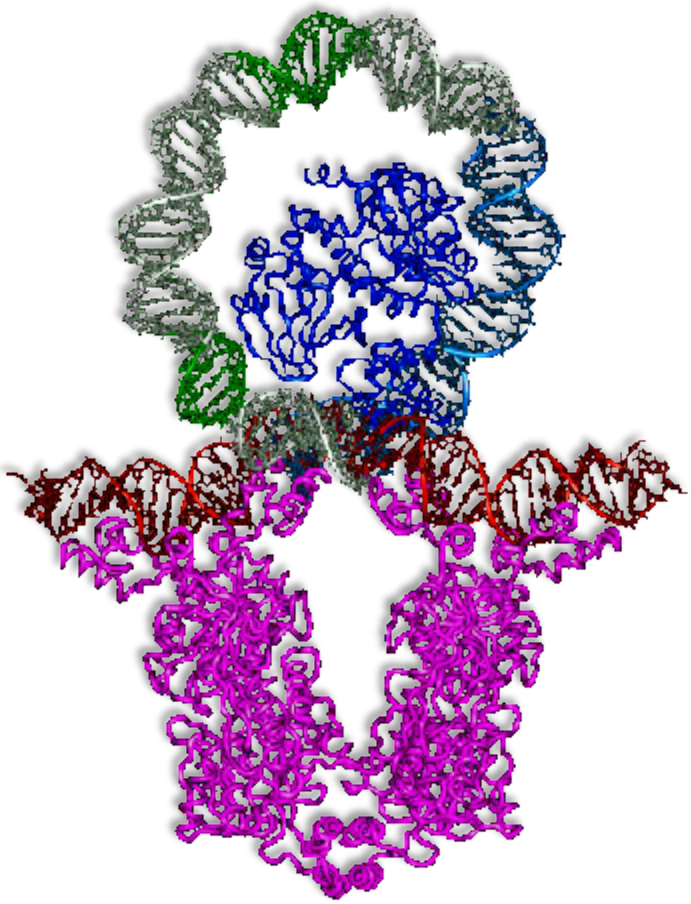

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Abstract

The binding of lacI to the lac operon is studied through in vivo and in vitro studies of variants of repressor and operator. Site directed mutagenesis on DNA coding for tyr-17 and arg-22 of lacI was cloned to pGEX-2T, over expressed, and the repressor protein was purified. Expression was performed with varying concentrations of IPTG. Gel shift assay with double mutant repressor showed affinity for pWB94464 operator sequence and no specificity for pWB910 operator when compared to nonsense sequence. β-galactosidase assays with different combinations of nucleotide changes in operator sequence and different residue mutant repressors demonstrate the importance of tyr-17 interaction with guanine-4 in DNA binding and thus repression. SDS-PAGE and HPLC determine subunit and native molecular weight of the repressor protein tetramer.

Introduction

The lac operon is a well studied example of prokaryotic gene regulation consisting of structural genes, promoter, terminator, regulator, and operator. The structural genes include β-galactosidase (Lac Z) which cleaves lactose to glucose and glactose, β-glactosidase permease (Lac Y) which is a lactose transport protein, and β-galactosidase transacetylase (Lac A) which transfers acetyl group.[2]

In the presence of glucose, the lac repressor protein (Lac I) is bound to the operator preventing binding of RNA polymerase and thus preventing transcription of structural genes including β-galactosidase. As glucose is depleted and lactose is an available nutrient source, allolactose will bind to the repressor protein causing a conformational change and allow binding of RNA polymerase. Allolactose's β1-6 bond can be cleaved by β-galactosidase, so as transcription increases for β-galactosidase it cleaves allolactose from binding up repressor. This free repressor is now able to bind to the operator again and stop transcription, providing a mechanism for transcriptional regulation.[2,3]

Specific residues of the operator and regulator have been shown to have an important role in protein-DNA interaction.[4] The lac repressor protein is a homotetramer with a core region that binds

allolactose, a DNA-binding region, and a tetramerization region which binds the subunits together.[5] The lac repressor is often referred to as a dimer of dimmers since two of the four subunits binds to the major groove of an operator so that each repressor can bind two operators forming a DNA repression loop.[4,5] The binding of the lac repressor to operator DNA follows a helix-turn-helix motif.[5]

Materials and Methods

The protocol was followed as described in the course reader[1] with slight modification. See figure 1 for a procedural flowchart. While transforming the E. coli with recombinant plasmid, growth at 37°C was allowed for 40 minutes rather than the full hour. The master mix prepared for day four screening was created at larger scale for 5 reactions as to not run out of master mix for the 4 reactions. For induction with IPTG, the 48 hour reactions were not prepared or measured. Preparation of the SDS-PAGE resolving and stacking gel used 150µL instead of 75 µL of ammonium persulfate solution. The IPTG induction was performed with 10mM IPTG. Individual task responsibility is outlined in tables below.

Day 1

Name	Solution Preparation	Plasmid Preparation	DNA Amplification by PCR	Restriction Digest of pGEX-2T Plasmid
Le Aye	x			
Heather		x	X	x
Ming-Hsiang	x			

Day 2

Name	CIP-treat Digested pGEX-2T	Analysis of PCR product	Digest Amplified DNA	Column Purify CIP-treated pGEX-2T	Ligation Reactions
Le Aye	x		X	x	x
Heather		x			x
Ming-Hsian	x		X	x	x

Day 3

Name	Transforming E.*coli* with Recombinant Plasmid
Le Aye	X
Heather	X
Ming-Hsiang	X

Day 4

Name	Screen Transformed E. coli	Preparation for Exponential Growth of Transformed E. *coli* Mutants
Heather	x	X
Ming-Hsiang	x	X

Day 5

Name	Measure Absorbance @600 nm	Assembly and Test of the β-Galactosidase Assay	Analyze PCR from Day 4
Le Aye	x	X	
Heather	x	X	x
Ming-Hsiang	x	X	

Day Before and 2 hours before Day Six

Name	Inoculate Colonies for Protein Expression	Incubate an overnight culture @ 37°C in incubator
Tim and Toby	X	x

Day 6

Name	Centrifuge and measure absorbance @600 for the cell culture for time 0, time 1.5 hours	Centrifuge and measure absorbance @600 for the cell culture for time 3 hrs and 48 hrs
Le Aye	X	
Heather	X	
Ming-Hsiang	X	
Tim & Toby		x

Day 7

Name	Sonicate Samples	Purification of GST fusion proteins
Le Aye		X
Heather	x	X
Ming-Hsiang		X

Day 8

Name	Prepare SDS-polyacrylamide gel	Analyze Fusion Protein Expression Preparing Samples	Running EMSA Gel
Le Aye	x	X	
Heather			x
Ming-Hsiang	x	X	

Results

The pGEX-2T expression vector (figure 2A) was digested and CIP treated with BamH1 and EcoR1. PCR products of wildtype amplification and double mutants (primers figure 2B) were also treated with BamH1 and EcoR1. These were ligated and transformed by heat shock to E. coli. Two colonies were selected and successful transformation was checked by PCR (figure 2C). Colonies that confirmed successful transformation were grown in LB and used for β-galactosidase assays and protein expression. Since the selected colonies did not show 1.1kb band on PCR (figure 2C), a successfully transformed recombinant plasmid was supplied for subsequent steps.

The β-galactosidase assays observed a visible yellow color in tubes 1, 6, 9, 11, 16, 17, 18, and 19 with ten minute incubation (table I, II, III). Protein expression was done with varying IPTG amounts with group assignment to a particular concentration of IPTG. The protein expression was performed with 10mM IPTG and A600 was taken at varying times form induction. t=0 (A600=1.040), t=1.5 (A600=1.423), and t=3(A600=2.139). After sonication and protein purification of t=0, t=1.5, and t=3 samples, the A420 of the combined samples was measured (A420=0). Since this low IPTG concentration resulted in A420 of zero and thus little to no expressed protein, a 20µL aliquot from group 3's higher concentration IPTG induction was used for protein purification.

SDS-PAGE of each t=0 induction, t=1.5 induction, t=3 induction, protein purification steps, and purified protein was run (figure 3A). The t=0, t=1.5, and t=3 samples were from 10mM IPTG induction. A graph of logMW of standards versus distance traveled (Figure 3B) allows interpolation of sample bands. A very faint band in lane 6 (supernatant) and lane 7 (wash 1) with 1.0cm distance traveled interpolates to a molecular weight of 58.2kD. The only band in lane 10 with 4.0cm distance traveled interpolates to 42.7kDa.

HPLC (figure 4) used pharmacia superdex 200HR 10/30 column with 10mm diameter, 30cm bead height, and bed volume of 24mL, flow rate 0.5mL/min, injection amount 100µL, and exclusion limit of $1.6x10^6$. Standards were dissolved into the column buffer and done in duplicate. Native molecular

weight of 128kD was obtained by interpolation of elution time of 23.127 minutes to standard curve. When neglecting higher molecular weight blue dextra and thyroglobulin from the standard curve, the native molecular weight is interpolated to be 129kD. EMSA with radioactive P^{33} was run with varying concentrations of purified double mutant repressor along with ideal, double mutant, and nonsense operators (figure 5).

Discussion/Conclusions

Neither of the selected colonies on figure 2 showed successful transformation due to an absence of a 1.1kb fragment aligned with the positive control. This was not due to experimental error since other groups picked different colonies from the same plate containing the transformed plasmid. Unfortunately, there is always a chance of picking a colony that did not successfully transform, though the experiment could be modified to transform by a more effective technique such as electroporation or chemical transformation rather than heat shock.

A confirmed transformed recombinant plasmid was used from another group's colony picking to perform β-galactosidase assays and induction. The assays take advantage of β-galactosidase's ability to cleave clear ONPG to yellow colored ONP. The A600 measured yellow due to ONP and adjustments were made to account for turbidity (1.75xA550) and cell density (A=420; see Table 1). The enzyme activity, total protein, and specific activity were calculated (table II). The control was a plasmid with 140bp N-terminal deletion that cannot function as a repressor, so 100% expression is expected. Operator 944 with mutant repressor N6000 showed expression of 132% and operator 964 with H1000 showed expression of 36.7% as compared to control. Though these values would be ideal close to 100%, they are definitely not the repressor pair that represses β-galactosidase activity. Some of the absorbencies are not within the 0.1-1.0 which degrades the reliability of the absorbance readings. It would also be advantageous to do these in duplicate, especially the controls, to establish a more reliable 100% expression specific activity to compare to.

Operator 944 with mutant repressor H1000 and operator 964 with repressor N6000 are the repressor pairs with relative activities to their controls of 31.6% and 10.4% (table III). The 94464 has no repression when paired with wildtype, H1000, and N6000 repressor due to the fact that both the 4 and 6 position are mutated which does not allow to bind, thus the high specific activity for β-galactosidase activity. The double mutant repressor; however, does bind and β-galactosidase is repressed. The ideal operator 910 has repression for wildtype, H1000, N6000, and H1N6000; however, the single mutants are far more successful in repression than the double mutant as expected. The 944 operator is moderately repressed with wildtype at 50.4% β-galactosidase expression and has better repression with H1000 mutant with 31.6% β-galactosidase expression. The 964 operator is moderately repressed by wildtype with 42.4% β-galactosidase expression. The H1000 does a slightly better job repressing at 36.7% activity, though this difference is negligible. The N6000 and H1N6000 represses significantly better than wildtype with 10.4% and 6.1% expression respectively signifying strong binding with 964 operator and N6000 or H1N6000 mutants (table III).

It can be proposed that for strong repression to occur, tyr-18 must be able to have an interaction with guanine-4 in the operator (figure 6). When operators without guanine-4 (944 and 94464) were paired with repressor with tyr-18 (wildtype and N6000), there is no ability for the guanine-4 and tyr-18 interaction to occur. This results in no protein-DNA binding, no repression, and 132% and 105% β-galactosidase activity for N6000 with 944 and 94464 respectively. When operators containing guanine-4 (910 and 964) are paired with repressor without tyr-18 (N1000 and H1N6000), repression still occurs but is poor when compared to wildtype and controls.

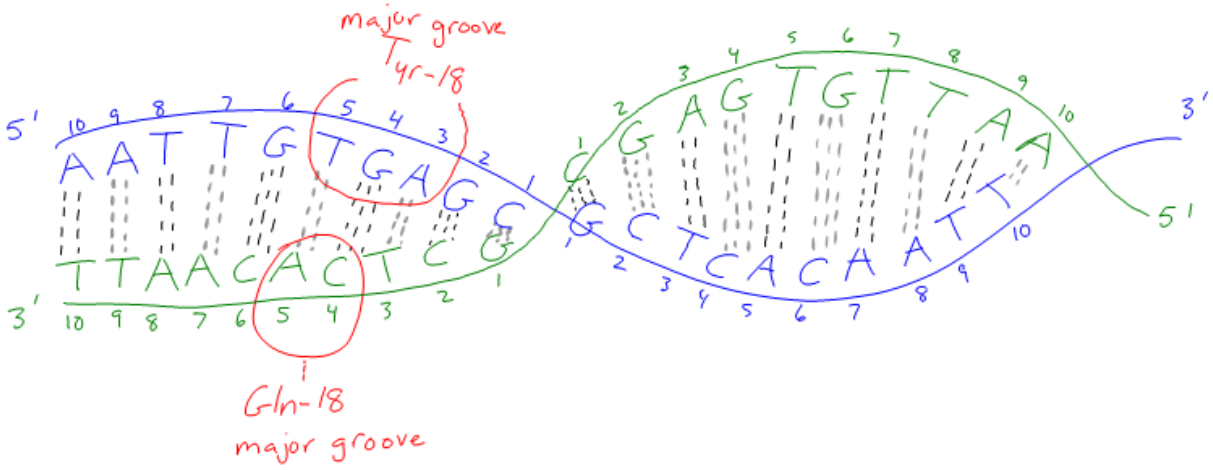

Figure 6: Proposed interaction of wildtype repressor to ideal operator.

SDS-PAGE is a denaturing gel used to measure subunit molecular weight. Due to varying charges on different proteins, negatively charged SDS binds to all proteins negating any charge difference to make them uniformly negatively charged. This allows protein samples to be separated on an acrylamide gel based on each proteins respective size. When comparing distance traveled on the gel to known molecular weight standards, the approximate molecular weight of samples can be determined.

The SDS-PAGE was run with 10mM IPTG at t=0, t=1.5 hours, and t=3 hours as well as protein purification steps (figure 3A). Induction did not occur due to absorbance showing no protein in combined t=0, t=1.5 and t=3 samples. The SDS-PAGE further supports induction did not occur with lanes 3, 4, and 5 lacking any bands with molecular weights that would correspond to a fusion protein weight of 67.6kDa. Due to low IPTG concentration not inducing production of fusion protein, an aliquot was used from another group for protein purification. Lane 6 (supernatant) and lane 7 (wash 1) showed a faint band that interpolates to 58.2kDa (figure 3B). This is likely the fusion protein of theoretical weight of 67.6kDa. Additional lower molecular weight bands show in lanes 6 and 7 which fade through subsequent washes in lanes 8 and 9, which is expected since the purification removes these impurities (figure 3A). Lane 10 contains the purified protein that has GST cleaved so theoretical weight of 38.6kDa compares to the interpolated value of 42.7kDa (figure3B). The experimentally obtained subunit

repressor molecular weight does compare to theoretical weight but with high error. One reason for error is that the image shows scrunching of the leftmost lanes, which effected measurement of distance traveled.

HPLC determines native molecular weight of the repressor by size exclusion. Large globular proteins exit the column at a faster rate since they spend less time in inclusion volume. The smaller globular proteins can enter and exit small porous beads, so they exit the column at a slower rate. The purified protein was run with known molecular weight standards and interpolated to a logMW vs. time graph (figure 4). The native molecular weight was interpolated to 123kDa with inclusion of all standards in graph. Since the theoretical native molecular weight is 160kDa[5], the largest standards were neglected since they may fall outside linear range to give new interpolation of 129kDa. It is possible that instead of four subunits aggregating upon synthesis only three folded together although the stability and occurrence of this is not reported elsewhere. More likely error is due to the fact that HPLC operates under the assumption that the protein being studied is perfectly globular, and any variations in size will effect elution time. If the protein was not globular and had irregular folding, this could cause a discrepancy. Different standards could be used that are closer to 160kDa to have a linear range that is more reliable, though the non globular folding would still occur. Alternative ways to measure native molecular weight could be investigated such as techniques that rely on protein density. Both SDS-PAGE and HPLC appeared to have a moderate amount of error, though it seems reasonable that a tetramer of 129kDa with four subunits of 42kDa if the native molecular weight is under reported and subunit weight is over reported.

EMSA measured repressor binding to radio labeled phosphate in operator DNA. Any lane with hot operator showed a radioactive mark at the bottom of the gel shift assay (Figure 5). Lane 2-4 contained only radio labeled operator, and as expected had bands only at the bottom of the gel. Lane 5 with double mutant and 0.1µg repressor showed no band because repressor concentration was too low

to visualize. Lane 6 had double mutant with 1.0µg repressor which showed DNA binding and of a DNA-protein complex midway through the gel. Lane 7 contained double mutant with 10µg repressor and a smear of DNA-protein due to the especially high concentration of repressor. Lane 8 (910 operator) and 10 (nonsense operator) did not show binding of with 1.0µg of repressor. Lane 9 (910 operator) and lane 11 (nonsense operator) showed the same slight binding to 10µg of repressor, so the double mutant repressor binds just as poorly to 910 as it does a nonsense sequence. Lane 12, 13, and 14 are mixed with cold operators that compete for binding with repressor. Binding to cold operator effectively reduces available repressor for binding to radio active operator that will show on assay. Lane 13 shows a darker band than 14 because 14 had cold 910 which did not bind well to the repressor. Lane 15 and 16 were combined with inducer IPTG which theoretically binds to the repressor and causes its release from DNA binding; however, the intensity of the bands did not decrease. It is possible a higher concentration of IPTG was required to obtain a different result.

In conclusion, the binding of tyr-17 of the repressor to guanine-4 of the operator sequence is crucial to the repression of lac structural genes due to the results observed from β-galactosidase assays. EMSA data showed that the double mutant repressor binds strongly to pWB94464 operator sequence and binds just as poor to pWB910 operator as nonsense DNA. Further investigation for the method of binding of repressor to operator DNA could be investigated with NMR studies and X-ray crystallography, though not reasonable for the scope of this laboratory class. Different mutants could be investigated since other repressor residues have been shown key to successful DNA binding. [5]

References

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Appendix