CHAPTER IV

IN VITRO & IN VIVO
CHARACTERIZATION OF
lac REPRESSOR MUTANTS
IV.A.INTRODUCTION

lac repressor is an allosteric protein capable of binding two ligands: a DNA molecule called OPERATOR and a sugar molecule called INDUCER. The repressor operator interaction is a stronger interaction with an equilibrium binding constant of $2 \times 10^{12} \text{M}$, which is decreased 1000 fold upon binding the inducer. Since, the binding of inducer decreases the affinity of repressor towards another ligand: the operator, and the message of inducer binding is communicated from the inducer binding site to the operator binding site 45 Å away, this is termed as an allosteric interaction. However, the pathways or residues through which the signal travels have not been worked out well. Detailed studies on the interaction of lac repressor with the operator, inducer and factors affecting the conformational equilibrium in the protein are available (Chakerian et al. 1985),(Muller-Hartmann and Muller-Hill 1996), (Daly and Matthews 1986a) but the molecular intricacies governing the allosteric response still remain elusive. Still, some investigations as detailed below have provided critical insights on the allosteric equilibrium of lac repressor.

Hartmann et al (Muller-Hartmann and Muller-Hill 1996) demonstrated that substitution at just a single residue Alanine 110 could alter the allosteric equilibrium to either HIGH operator, LOW inducer affinity state in case of A110K mutation or LOW operator, HIGH inducer affinity state with A110T mutation. Evidence in the form of molecular modeling was provided suggesting a stabilized operator bound conformation in case of A110K mutant and a stabilized inducer bound conformation in case of A110T mutant. Therefore, A110 residue might be playing a crucial role in the allosteric equilibrium of lac repressor.

Daly et al (Daly et al. 1986) investigated the alteration of allosteric equilibrium by modification of one of the cysteine residues by Methyl Methane
Thio Sulfonate (MMTS) and concluded that the equilibrium between T and R states of the repressor depends upon whether cys-281 is capable of protonation and deprotonation. The same group performed an *in silico* analysis using Targeted Molecular Dynamics (TMD) simulation (Flynn et al. 2003) using the PDB structures of lac repressor bound to the operator and inducer respectively. Their study led to the inference of an asymmetry between the two monomers with respect to inducer binding and existence of at least three pathways working within the protein during the whole allosteric transition from the DNA bound state to the Inducer bound state.

The above reports are merely, initial steps in the construction of a detailed allosteric pathway of lac repressor. This paucity of mechanistic details on the allosteric response and general principles of protein-protein interactions hampered the exploration of the enormous potential of these molecular switches. Their ability to couple input signals such as effector binding, temperature, etc to modulation of output functions such as binding affinity, catalytic activity etc. can be translated into creation of excellent novel molecular switches.

Engineering allosteric proteins has been attempted by traditional methodologies like rational designing, discovery of new allosteric sites by high throughput screening followed by X ray crystallography (Hardy and Wells 2004) or creation of new allosteric sites by domain insertion (Guntas and Ostermeier 2004). Despite the success achieved by the conventional methods of engineering, there are serious limitations like the narrow diversity created, nonexistence of inventive strategies towards development of novel response regulators, inability to direct the expected outcome and requirement of additional parameters for optimizing the existing switches. In such cases, blind evolutionary approaches such as directed evolution of allosteric enzymes and regulatory proteins can lead to the discovery of molecular switches with novel properties. Our efforts in this direction have already been mentioned in the previous chapters and this chapter outlines the *in vivo* and *in vitro*
characterization of the lac repressor mutants showing altered allosteric response obtained by directed evolution.
CHAPTER 4: Characterization of lac I mutants

IV.B. MATERIALS AND METHODS

IV.B.1. Expression and purification of lac repressor

IV.B.1.a. Cloning the lac repressor gene under T7 promoter

The lac repressor gene was amplified from the vector pET21-D using the primers OEF (5'-ATTACGGGTGGCATATGGTGAAACCAGTAAC-3') and OER (5'-GAGATCCCGGTGCCTAGGGAGTGAGCTAAC-3') having NdeI and BamHI sites respectively. The vector pET3a (Novagen) was also digested with NdeI and BamHI enzymes. The above vector and insert were ligated using T4 DNA ligase overnight at 16°C. Subsequently, the ligation product was transformed into DH5α competent cells and plated on LB ampicillin (100μg/ml) plates. The plasmid was isolated from the clones to check for the presence of insert. Positive clones showing the presence of insert were sequenced using primer combinations as shown in figure 3.1.

Monitoring protein over-expression on SDS-PAGE

Since, most of the E.coli based over-expression systems are based on the lac repressor- IPTG regulation; we could not utilize this system for overexpressing lac repressor. So, we chose to over-expresss the lac repressor using salt (NaCl) inducible over-expression system and the over-expression protocol was followed exactly as specified by Bhandari and Gowrishankar (Bhandari and Gowrishankar 1997). The lac repressor gene on pET-3a after confirmation by sequencing was transformed into the host GJ 1158 and grown on LBON medium (LB medium without NaCl) plates containing 100μg/ml. A single colony was inoculated in 3 ml LBON and grown overnight at 37°C, 250 rpm. A secondary culture was inoculated using 1% inoculum from the above culture
and grown in LBON at 37°C, 250 rpm till the OD_{600} reaches 0.4. At this stage, the culture was induced using 0.3% final concentration of NaCl. After induction, the culture was grown for 2-3 hrs more before harvesting. At the end of 2-3 hrs, 1ml of the culture was taken in a fresh tube and centrifuged briefly at 10,000 rpm for 10 mins. This pellet of cells was resuspended in 100μl of autoclaved MilliQ water and vortexed thoroughly. To this, 4X gel loading dye was added and mixed thoroughly. The sample was boiled for 5-10 mins. and then loaded on 12% SDS-PAGE. The gel was run at 30mA constant current and when the dye front reached the bottom of the gel, the supply of current was stopped. The gel was subsequently stained using Comassie stain and destained using destainer (45% MQ + 45% Methanol + 10% Glacial Acetic Acid).

**IV.B.1.b. Partitioning of lac repressor in soluble and insoluble phases**

The lac repressor expressed as described above was harvested by centrifuging and resuspended in cell lysis buffer. Cell lysis was tried by using various agents like lysozyme, sonication and freeze-thaw cycles. The cell lysate was then centrifuged at 14,000 rpm for 20 mins. The supernatant containing the soluble fraction was taken in a fresh tube and separated from the pellet containing the insoluble fraction. The volume of supernatant was measured and the pellet resuspended in same volume of MQ. Equal volumes of the soluble and insoluble phases were loaded on a 12% SDS-PAGE and coomassie staining done.
CHAPTER 4: Characterization of lac I mutants

**IV.B.1.c. lac repressor protein purification**

*lac* repressor gene was cloned under the lacI^q^ promoter on pBSSK vector as described in chapter 3. This plasmid was transformed into the host SL1 and used for purifying the protein. A single SL1 transformant colony was used to inoculate a primary culture in 2XYT medium. This primary culture was grown at 37°C, 250 rpm overnight. 1% of primary inoculum was used to inoculate a secondary culture in 2X YT medium and grown at 37°C, 250 rpm for 12-16 hrs. Subsequently, the culture was harvested by centrifugation and the weight of cell pellet noted down. This cell pellet was resuspended in 3X volume of cell lysis buffer. Lysozyme and sodium deoxycholate were added to a final concentration of 2mg/ml and 2mg/g cell pellet respectively. Following cell lysis, sonication was done to shear the released genomic DNA. The sonicated sample was spun at 14,000 rpm for 30 minutes to separate the insoluble from the soluble portion. Ammonium sulphate to a final concentration of 23% (w/v) was added to the soluble portion and precipitated at 4°C for 1 hour. Then, the precipitate was centrifuged at 14,000 rpm for 30 mins. and the pellet taken for dialysis. The pellet was resuspended in dialysis buffer and dialysed against the same to remove ammonium sulphate. The dialysate was spun at 10,000 rpm for 10 mins. to remove any particulate matter before loading onto the column. Meanwhile, the phospocellulose column was packed and equilibrated with equilibration buffer. The supernatant of dialysate was loaded onto the column and washed with equilibration buffer till the A_{280} reaches 0.01. At this stage, elution was started using a gradient of 0.12M -0.3M potassium phosphate buffer, pH7.4. The eluted fractions were concentrated and protein concentration estimated using absorbance at A_{280} and BCA protein estimation kit (PIERCE co.).
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**IV.B.2. Intrinsic tryptophan fluorescence studies**

WT lac repressor and mutants were taken at a final concentration of 165μg/ml, mixed with inducer binding buffer and appropriate concentration of IPTG. This mixture was left for equilibration overnight at 4°C on the rotor torque. Fluorescence measurements were done on HITACHI 4500 fluorimeter. The samples were excited at 295 nm and the emission spectra were collected from 310 - 400 nm. The excitation and emission slit widths were 5 nm. Following the collection of spectra, area under the curve was calculated for each spectrum between the wavelengths 315 -395 nm.

**IV.B.3. Binding isotherm calculations**

Change in area under the curve is represented here as change in fluorescence. Change in fluorescence is in turn used for calculating fractional saturation.

Fractional saturation \([R]\) was calculated for each concentration of IPTG as follows:

\[ R = \frac{\text{change in fluorescence at a given conc. of IPTG}}{\text{total change in fluorescence at saturating conc. of IPTG}} \]

The binding isotherms were thus, plotted using Fractional saturation \([R]\) on Y axis and concentration of IPTG on X axis.
CHAPTER 4: Characterization of lac I mutants

IV.B.4. Secondary structure analysis by Far-UV Circular Dichroism spectroscopy

The secondary structure analysis was performed on WT lacI and mutants 2-1A5, 2-2D10, 2-5A1 and 2-5D2 in the absence and presence of IPTG using far UV CD spectroscopy. The protein samples in the presence of IPTG were incubated with 2mM IPTG for 4-5 hrs at 4°C and then taken for measurement. The samples in the absence of IPTG were treated similarly and taken for measurements.

The proteins were used at a concentration of 165 μg/ml in the buffer containing 10 mM Tris Cl pH 7.4, 200mM KCl, 0.1mM DTT, 1mM EDTA, and 10mM MgCl₂. The measurements were carried out on JASCO J-715 spectropolarimeter. The path length used is 0.5cm and the data represents three accumulations for each sample. The data has been converted from ellipticity values into molar ellipticity values. Molar ellipticity [θ] has been calculated as:

\[ [\theta] \text{(deg.cm}^2\text{.dmol}^{-1}) = \left( \frac{\theta \times \text{MRW}}{10 \times \text{C} \times \text{L}} \right) \]

where,

\[ \theta = \text{ellipticity in mdeg} \]

\[ \text{MRW} = \text{Mean Residue Weight} \]

\[ \text{C} = \text{concentration in g/ml} \]

\[ \text{L} = \text{path length in cm} \]
IV.B.5. Electrophoretic mobility shift assay

The 40 bp operator was formed by annealing two oligos representing the two strands of the duplex whose sequence is as follows: 5’-TGTGGGACGGAATGGCTGAGCGGATAACAAATTTCACACAGGG-3’. This annealed oligo was labelled at the 5’ end with γ- P 32 ATP using polynucleotide kinase enzyme (NEB). The labeled operator was purified using Nucleotide purification kit from (QIAGEN). The purified operator was quantified using a DNA standard curve made by using DNA standards of known amount and quantified using SYBR Green I (MOLECULAR PROBES).

The repressor protein was used at a concentration of (1.5-2)*10^{-11} moles and labeled operator DNA at 1.3*10^{-15} moles. IPTG concentration was varied from 1*10^{-11} moles to 5*10^{-8} moles for WT repressor and for the mutants it was varied from 1*10^{-13} moles to 5*10^{-10} moles.

Tubes containing the operator DNA and protein mixture were incubated for a period of 1 hour at RT and then IPTG added at the required concentration and again incubated at RT for another 1 hour. Subsequently, gel loading dye containing trace amounts of Bromophenol blue and Xylene Cyanol was added and loaded on a native 8% gel polyacrylamide gel made in 1X TBE. The gel was run at 10mA in 1X TBE till the dye front reached the bottom of the gel. At the end of gel run, the gel was lifted onto a Whatman sheet of the same dimensions and exposed in a FUJI Phosphor Imager cassette. Phosphor imaging of the gel was done after an exposure of 2-3 hrs and the images collected for data analysis.
IV.B.6. IPTG concentration profile of β gal in mutants

WT lac I and mutants were transformed into SL1 host and grown in the presence of varying concentrations of IPTG. The β gal levels were then estimated for each sample using the protocol of Miller and as described before. The IPTG concentrations thus, checked include: 0, 10, 25, 50, 100, 150, 250, 350 and 500 μM.
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IV.C. RESULTS and DISCUSSION

IV.C.1. Expression and purification of lac repressor

The WT lac repressor protein was cloned under T7 promoter in pET 3a as described under materials and methods. Since the protein to be over-expressed is lac repressor, IPTG based induction format could not be undertaken. So, a salt inducible over-expression method as described in materials and methods was used. The results of over-expression are shown in the figure 4.1 where a clear over-expressed thick band of desired size is visible. Attempts to purify protein from such a cell lysate were not successful as the over-expressed protein partitioned into the insoluble fraction as shown in the figure 4.2. Repeated attempts to salvage the repressor from insoluble fraction by decreasing the temperature, changing the lysis conditions etc were not fruitful. Purification from inclusion bodies was not favoured as it involves unfolding and refolding the protein. This would impose a limitation while purifying the mutants due to the inability to validate complete and correct refolded state of the mutants.

Thus, other alternatives were explored and one of the options was to express the lac repressor constitutively under a high strength lacI promoter on a high copy number vector. Such a system was presumed to provide sufficient intracellular concentrations of the repressor to enable purification. The results as shown in the figure 4.3 proved our supposition and finally WT lac repressor as well as the four mutants 2-1A5, 2-2D10, 2-5A1 and 2-5D2 were purified using this scheme of over-expression and the protocol as described in materials and methods.
FIGURE 4.1. lac repressor overexpression in *E.coli* using salt inducible over-expression system

M: Low molecular weight marker
I: Induced cell lysate
UI: Uninduced cell lysate
FIGURE 4.2. Partitioning of lac repressor into the insoluble fraction

M: Low molecular weight marker
Sol: Soluble fraction
Isol: Insoluble fraction
FIGURE 4.3. Various fractions showing purified lac repressor eluted from phosphocellulose column

Lane 1: Showing overexpressed lac repressor in a total cell lysate

Lanes 2-10: Fractions showing purified lac repressor at the same position of overexpressed lac repressor
IV.C.2. Inducer binding properties of the WT lac I & mutants

The inducer binding property of lac repressor has been investigated using the following approaches:

1. Equilibrium dialysis using C-14 IPTG (Ohshima et al. 1974)
2. Filter binding assay using C-14 IPTG (Riggs and Bourgeois 1968) (Barry and Matthews 1997)
3. Blue shift in intrinsic tryptophan profile (Laiken et al. 1972)

Due to the unavailability of C-14 IPTG, we chose to investigate inducer binding properties of mutants using blue shift in intrinsic tryptophan fluorescence.

lac repressor protein has two tryptophan residues in each monomer at positions 201, 220 respectively (Farabaugh 1978). The conformational change observed in the protein during the allosteric transition from the DNA bound state to the inducer bound state is reflected in the intrinsic tryptophan profile as a blue shift (Sommer and Lu 1976). Laiken et al (Laiken et al. 1972) suggested that the fluorescence spectral shift is a consequence of an increase in the hydrophobicity of environment of the indole fluorophore upon inducer binding due to burial of the tryptophan residue. This burial in turn occurs due to the conformational transition and rotation of N sub-domains upon inducer binding. The location of W220 proximal to the inducer binding site shown by genetic and biochemical studies (Laiken et al. 1972), (Sommer and Lu 1976), (Gardner and Matthews 1990), (O'Gorman and Matthews 1977) has been confirmed by crystallographic structures also (Lewis et al. 1996). Substitutions at W220 abolish this fluorescence spectral shift while substitutions at W201 do not affect. This suggests that of the two tryptophan residues, W220 due to its proximity to the inducer binding site is the major residue responsible for the shift in fluorescence (Barry and Matthews 1997).
Intrinsic tryptophan profile was monitored with complexes of purified protein & IPTG and binding isotherms were drawn as described in Materials and Methods. As shown in the figure 4.4, the WT lacI binding constant of $K = 2 \times 10^6$ matched with the reported value (Laiken et al. 1972) (Falcon et al. 1997) and the binding constant for the mutant 2-5D2 calculated similarly with the binding isotherm as shown in figure 4.5 was $K = 9.5 \times 10^7$. The other three mutants 2-5A1, 2-1A5 and 2-2D10 showed no change in the tryptophan emission profile in the absence and presence of IPTG as shown in figure 4.6. Further, with these three mutants, the emission profile in the absence of IPTG was similar to the profile with saturating concentrations of IPTG characterized by an emission maximum at 330-332 nm. Unfortunately, the absence of a shift in the emission maximum after the addition of inducer in the mutants and also unavailability of radioactive C-14 IPTG precluded us from calculating the inducer binding constants for these mutants.

The absence of the shift could be due to the inability of mutant proteins to bind IPTG which can be ruled out by evidences from the far UV CD data (section IV.C.4) and $\beta$ gal assay with increasing concentration of IPTG (section IV.C.5). Both these results indicate that the mutant lac repressors do possess the ability to bind IPTG. So, another reason for the absence of shift could be due to an altered conformation in these mutants with respect to the WT. lac repressor has been postulated to have two alternate conformations: DNA bound state versus inducer bound state or T versus R state and MONOD-WYMAN-CHANGUEX model has been utilized to describe the allosteric behaviour of lac repressor protein (O'Gorman et al. 1980). There are many reports available in the literature suggesting alteration in the allosteric equilibrium of lac repressor such that one of the conformations is stabilized.

Daly and Matthews (Daly et al 1986) showed that by modifying the Cysteine residue at 281, this equilibrium could be altered towards the R state. Not only modifications but even high pH was shown to shift the conformational equilibrium towards the T state. They concluded that the
FIGURE 4.4. Inducer binding studies of WT lac I titrated with IPTG

A. Intrinsic tryptophan fluorescence profile

B. IPTG binding isotherm

Equilibrium Dissociation constant $K = 1.94 \times 10^{-6} \pm 9.36 \times 10^{-8}$
FIGURE 4.5. Inducer binding studies of mutant 2-5D2

A. Intrinsic tryptophan profile

Panel A

- No IPTG
- 2 * 10^-6 M
- 2 * 10^-5 M
- 2 * 10^-4 M
- 2 * 10^-3 M

B. IPTG binding isotherm

Panel B

Equilibrium Dissociation constant \( K = 9.45 \times 10^{-7} \pm 3.66 \times 10^{-8} \)
FIGURE 4.6 Intrinsic tryptophan profile of mutants 2-1A5 & 2-2D10

**2-1A5**

![Graph showing fluorescence intensity vs. wavelength for mutant 2-1A5 with different concentrations and conditions.]

**2-2D10**

![Graph showing fluorescence intensity vs. wavelength for mutant 2-2D10 with different concentrations and conditions.]

Contd..
FIGURE 4.6 Intrinsic tryptophan profile of mutant 2-5A1
protonation/deprotonation state of cys-281 can decide the quarternary conformational status of the lac repressor.

Hartmann et al (Muller-Hartmann and Muller-Hill 1996) showed in an excellent example that the substitutions at a single residue Alanine 110 could shift the lac repressor into one of the conformations. They substituted A110 with lysine (K) and threonine (T) and calculated the \textit{in vivo} as well as \textit{in vitro} binding constants of repressor-operator and repressor-IPTG interactions. These measurements proved that A110K could be stabilized in an operator bound conformation while A110T could be stabilized in an inducer bound conformation. Similarly, Chakerian et al showed that modification of Alanine at position 81 to Valine is capable of locking the protein in the induced or sugar bound conformation. (Chakerian et al.1985).

These examples suggest that in the case of these mutants also, the conformational equilibrium could have shifted towards the inducer bound conformation or R state. Since, the the T state is characterized by high operator/low inducer affinity and R state is characterised by high inducer/low operator affinity, these mutants should exhibit reduced ability to bind operator as compared to the WT. To investigate the DNA binding ability of these mutants as well as WT lacI, electrophoretic mobility shift assay (EMSA) was done whose results have been discussed below.

\textbf{IV.C.3. Operator DNA binding properties WT lacI and mutants}

The second generation clones obtained by shuffling the mutations generated by error prone PCR showed a phenotype of leaky expression in the mutants while retaining the ability to respond to the inducer. This behavior is suggestive of reduced repression \textit{in vivo} due to the inefficient complex formation between operator DNA and the mutant repressor molecules. As suggested above, it is possible that the mutant repressors have compromised DNA binding ability. If the above supposition holds true and the leaky background expression is indeed due to the reduced DNA binding ability of
mutants, then the mutant proteins should demonstrate compromised DNA binding \textit{in vitro} also.

To investigate this, \textit{in vitro} DNA binding assay was attempted with radiolabelled operator fragment and purified repressor protein as described in materials and methods. The results as shown in the figure 4.7 clearly demonstrate that wild type binds to the operator efficiently and there is no free DNA left upon complex formation. In the case of mutants, although some fraction of operator DNA goes into the bound form, a major fraction is present as free DNA even when the protein concentration is in molar excess. Upon addition of saturating concentrations of IPTG however, WT as well as mutant lac repressors are observed to be dissociated from DNA characterized by a large amount of free DNA.

To check the response of wild type and mutants towards IPTG while bound to the operator, increasing concentration of IPTG was added to these complexes expecting release of operator DNA in an IPTG concentration dependent manner. As the concentration of IPTG is increased, we expected to observe a proportionate increase in the free DNA released. The results as shown in figure 4.8 show that in the case of WT, the free DNA released continues to increase with increase in concentration of IPTG till at saturating concentrations of IPTG almost all the bound DNA is released into the free form of DNA. Addition of increasing concentrations of IPTG in mutants did not release the bound DNA in a graded fashion but instead releases the bound DNA abruptly into the free form at a particular concentration of IPTG in case of mutants.

To verify, whether the purified mutant lac repressor proteins possess any structural abnormalities due to purification or other reasons, leading to reduced DNA binding ability and inability to show a shift upon binding IPTG, secondary structure analysis was performed on the mutant proteins as well as WT lac I.
FIGURE 4.7. EMSA gel showing DNA association and dissociation by WT lac repressor and mutants

Lane 1: Only DNA
Lane 2: WT (DNA + Protein)
Lane 3: WT (DNA + Protein + IPTG)
Lane 4: 2-1A5 (DNA + Protein)
Lane 5: 2-1A5 (DNA + Protein + IPTG)
Lane 6: 2-5A1 (DNA + Protein)
Lane 7: 2-5A1 (DNA + Protein + IPTG)
Lane 8: 2-2D10 (DNA + Protein)
Lane 9: 2-2D10 (DNA + Protein + IPTG)
Lane 10: 2-5D2 (DNA + Protein)
Lane 11: 2-5D2 (DNA + Protein + IPTG)
FIGURE 4.8. Effect of IPTG on DNA dissociation by WT lac repressor and mutants

EMSA gel showing unbound DNA fraction

Lane 1: Only DNA
Lane 2: DNA + Protein
Lane 3-10: DNA + Protein + IPTG
DNA conc.: $1.3 \times 10^{-15}$ moles
Protein conc.: $(1.5-2) \times 10^{-11}$ moles
IPTG conc.: $1 \times 10^{-11}$ moles to $5 \times 10^{-8}$ moles for WT and $1 \times 10^{-13}$ moles to $5 \times 10^{-10}$ moles for mutants
IV.C.4. Secondary structure analysis of WT lacI and mutants

The secondary structure of lac repressor has been determined by far UV CD spectroscopy as shown by Matsura et al (Matsuura et al. 1972; Oshima et al. 1972) in the absence as well as presence of IPTG. They estimated by Optical Rotatory Dispersion (ORD) and Circular Dichroism (CD) that lac repressor contains 33-38% of α helix and 18-27% of β sheet structure. Also, upon binding IPTG, there is a reduction in negative ellipticity. This decrease has been explained due to the conformational changes like rearrangement of sub-units and not entirely due to the loss of structure. Similar results were obtained by us in the secondary structure analyses of WT and mutant lac repressors in the absence and presence of IPTG by far UV CD. The results as shown in figure 4.9 clearly indicate the reduction in molar ellipticity upon IPTG binding for WT and mutants. This also implies that the mutant lac repressor proteins are capable of binding to the inducer IPTG and that this property has not been lost as might be suggested by intrinsic tryptophan fluorescence results and operator binding results.

IV.C.5. Mutant lac repressors as good molecular switches

The in vitro binding studies of WT lacI and mutant repressors with inducer-IPTG and operator show that the behaviour of mutants is drastically different from that of WT lacI. The WT lacI shows good operator and inducer binding as shown in the literature, while the mutants did not conform to WT standards of inducer or operator binding. These mutants were checked for induction at only a particular IPTG concentration during screening and therefore, we wanted to profile these clones with respect to increasing concentration of IPTG. This led us to the β gal assay of WT lac I and mutant repressors with respect to increasing concentration of IPTG whose results have been discussed below.
FIGURE 4.9. Far UV Circular dichroism spectra of WT lacI and mutants in the absence and presence of IPTG.
CHAPTER 4: Characterization of lac I mutants

The WT lacI as well as mutants were expressed on high copy number vector, pBSSK to reduce the leaky background expression from these clones as described in chapter III. These clones were then transformed into the host SL1 and β gal levels assayed in the presence of various concentrations of IPTG. The results as shown in figure 4.10 indicate that the response of WT lacI and mutants towards increasing concentration of IPTG is similar to the response shown during in vitro operator binding assays.

β gal gene expression is switched OFF in the absence of IPTG both for WT and mutants. In the presence of IPTG however, WT shows a gradual increase in expression of β gal which saturates above 250 μM IPTG. In the case of mutants, expression starts at 10 μM IPTG and saturates at 50 μM IPTG itself and addition of further IPTG does not increase gene expression levels. Thus, as compared to WT which shows an IPTG concentration dependent increase in expression of β gal, the mutants show three distinct steps of gene expression:

1) At 0 μM IPTG gene expression is switched OFF,

2) At 10 μM IPTG gene expression is switched ON,

3) At 50 μM IPTG gene expression reaches saturating levels.

Most of the E.coli based over-expression systems (Dubendorff and Studier 1991; Studier and Moffatt 1986) are based on lac repressor-IPTG based regulatory systems. For inducing protein expression by this system, the IPTG concentration used is generally in the range of 500μM - 1mM. The IPTG concentration at which the above mutant lac repressors can induce gene expression is 10μM which is 100 times lower than the usual concentration. Apart from inducing at low concentration, these mutant repressors also confer higher levels of gene expression as compared to the WT. WT lac I at saturating concentrations of IPTG, shows highest induced levels of β gal ~ 250 Miller Units while all of the mutants show higher levels than WT. Mutant 2-5D2 shows only 2 times higher expression while the mutant 2-1A5 shows the
FIGURE 4.10. Effect of IPTG concentration on β-gal level of WT lacI and clones 2-1A5, 2-2D10, 2-5A1 and 2-5D2.
highest induced β gal levels of > 2500 which is 10 times higher levels of expression than WT. Also, the constitutive β gal levels of the host strain SL1 profiled at similar IPTG concentrations has been shown which indicates that all the mutant proteins possess the feature of inducibility and are not constitutive for β gal expression.

It could be argued that WT confers better control than mutants over the levels of expression due to its graded response towards IPTG which is a desirable feature when expressing toxic proteins from *E.coli*. The window for controlling gene expression with WT lac I ranges from 50 μM - 500 μM while for the mutants it is much reduced and ranges from 5 μM- 50 μM IPTG. So, the mutants possess all the desirable features of a perfectly inducible gene expression system as compared to the existing system with WT lac I.

So we can conclude by saying that, the mutant lac repressors behave as perfect molecular switches for controlling gene expression, induce gene expression with 100 times lower concentration of IPTG and confer 2-10 times higher level of gene expression as compared to the WT.