RICE UNIVERSITY

MODIFICATION OF LACTOSE REPRESSOR PROTEIN WITH
2-CHLOROMERCURY-4-NITROPHENOL

by

Diana Sou Tung Yang

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Thesis Director's signature:

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The reaction of the lactose repressor protein of
Escherichia coli with 2-chloromercuri-4-nitrophenol (MNP)
has been investigated. MNP reacts specifically with thiol
groups in proteins. When a thiol reacts with a mercuri-
nitrophenol, the ionization state of nitrophenol may be
altered due to environmental changes, and this in turn
affects its spectral characteristics. It is possible from
the spectral changes observed to detect altered ionization
and/or absorption characteristics of the chromophore and
thereby to detect conformational changes in the protein.

Titrations of lactose repressor with MNP were carried
out under a variety of conditions. Similar results were
obtained in the presence of inducer, anti-inducer, or
neutral ligand. The equivalence point of the titration curve
corresponded to 2 moles MNP/mole repressor monomer. This
implies that there are 2 sulfhydryl groups which react with
MNP under all conditions. The third sulfhydryl group in
native repressor monomer did not react and is apparently
completely buried under all conditions. A slight difference
of environment between the two sulfhydryl groups which bind to MNP has been observed. The binding activities of the modified protein were measured under various conditions to determine any effects of modification. No significant loss of IPTG binding activity or operator binding activity was observed. Therefore, the two cysteine residues which MNP can modify do not interfere with either the inducer binding site or the operator binding site.

Difference spectra of MNP-repressor in the presence of inducer have been measured. The spectral changes apparently result from a conformation change which alters the environment of nitrophenol group. However, when anti-inducer is bound to the repressor no spectral changes are observed. Since inducers and anti-inducers have same binding site, this implies inducer binding and anti-inducer binding have different effects on the protein structure. The pK of free MNP was determined to be 6.75. The combined pK of the two mercurinitrophenol groups bound to cysteine residues was found to be ~8.0; and the combined pK of MNP-repressor in the presence of IPTG was found to be ~7.9. Reaction rates of MNP with repressor were measured. No significant differences were observed in the reaction rates in the presence of inducer or anti-inducer. When the concentration of MNP was increased, the reaction also increased. It can be determined from these measurements that MNP reacts with the 2 sulfhydryl groups with different rate constants.
ACKNOWLEDGMENT

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TABLE OF CONTENTS

I. Introduction 1

II. Materials and Methods 11

III. Results
   1. MNP Titration of Repressor 27
   2. Specificity of the Reaction 34
   3. Differentiation of the Cysteines 39
   4. Effects of Modification on Activity 44
   5. Difference spectra of MNP-repressor 51
   6. Determination of the pK of MNP groups 69
   7. Kinetics of the Reaction of MNP
      with Repressor 83

IV. Discussion / Conclusion 90

V. References 95
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Genetic regulation of Lactose Repressor</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Isolation of Lactose Repressor</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Amino Acid sequence of Lactose Repressor</td>
<td>7</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Titration of repressor with MNP</td>
<td>28</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Titration of repressor with MNP in the presence of inducer</td>
<td>30</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Titration of repressor with MNP in the presence of anti-inducer</td>
<td>32</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Titration of repressor with MNP at pH 9.0</td>
<td>35</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Titration of repressor with MNP in 8M guanidine solution</td>
<td>37</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Titration of DTNB reacted repressor with MNP</td>
<td>40</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Difference spectrum of titration of repressor with MNP at pH 9.0</td>
<td>42</td>
</tr>
<tr>
<td>Figure 11</td>
<td>a. IPTG binding activity of MNP-repressor at increasing concentrations of MNP at pH 7.5</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>b. IPTG binding activity of MNP-repressor at increasing concentrations of MNP at pH 9.0</td>
<td>45</td>
</tr>
<tr>
<td>Figure 12</td>
<td>a. IPTG binding activity of MNP-repressor at different times at pH 7.5</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>b. Repressor activity at pH 9.0</td>
<td>47</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Operator binding activity curves of repressor and MNP-repressor</td>
<td>49</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Difference spectrum of MNP-repressor in the presence of galactose</td>
<td>52</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Difference spectrum of MNP-repressor in the presence of glucose</td>
<td>55</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>Difference spectrum of MNP-repressor in the presence of IPTG</td>
<td>57</td>
</tr>
<tr>
<td>Figure 17.</td>
<td>Difference spectrum of MNP-repressor in the presence of ONPF</td>
<td>59</td>
</tr>
<tr>
<td>Figure 18.</td>
<td>Difference spectrum of MNP-repressor in the presence of APTG</td>
<td>61</td>
</tr>
<tr>
<td>Figure 19.</td>
<td>Difference spectrum of MNP-repressor in the presence of PTG</td>
<td>63</td>
</tr>
<tr>
<td>Figure 20.</td>
<td>Difference spectrum of MNP-repressor in the presence of mannose</td>
<td>65</td>
</tr>
<tr>
<td>Figure 21.</td>
<td>Difference spectrum of MNP-repressor in response to IPTG with increasing amount of MNP</td>
<td>67</td>
</tr>
<tr>
<td>Figure 22.</td>
<td>The absolute spectrum of MNP at various pH values</td>
<td>70</td>
</tr>
<tr>
<td>Figure 23.</td>
<td>The absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer with various pH values at wavelength 396nm</td>
<td>72</td>
</tr>
</tbody>
</table>
Figure 24. The absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer with various pH values at wavelength 410nm

Figure 25. The absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer with various pH values at wavelength 440nm

Figure 26. The wavelength of maximum absorption of absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer at various pH values

Figure 27. The difference spectrum of MNP-repressor with inducer versus MNP-repressor at various pH values

Figure 28. Reaction rates of MNP and repressor with repressor alone or in the presence of inducers or anti-inducers

Figure 29. Reaction rates of MNP and repressor with two different concentrations of MNP

Figure 30. A plot of log absorbance versus time of the reaction rate
LIST OF ABBREVIATIONS

MNP  2-Chloromercuri-4-nitrophenol
IPTG  Isopropyl-$\beta$-D-thiogalactoside
ONPF  $\alpha$-Nitrophenyl-$\beta$-D-fucoside
APTG  $\beta$-Aminophenyl-$\beta$-D-thiogalactoside
PTG  Phenyl-$\beta$-D-thiogalactoside
I. INTRODUCTION

The lactose repressor protein obtained from *Escherichia coli* is one of the few genetic control proteins which has been isolated. In 1961, Jacob and Monod(1) proposed a system of genetic regulation (Figure 1) based on data they had obtained during studies on sugar metabolism in *E. coli*. According to this model, a soluble protein called the repressor binds to DNA at a region called the operator to prevent transcription of RNA (and subsequent protein synthesis) corresponding to the structural genes for the operon.

The lactose operon contains three sites z, y, and a which code for the production of the enzymes β-galactosidase, lactose permease, and thiogalactoside-transacetylase respectively. In the presence of lactose, a metabolite of lactose called an inducer interacts with the lactose repressor, probably causing a conformation change, to allow release of the DNA. Transcription of RNA may then occur, followed by enzyme synthesis. The total process is called induction. There are also other sugars which competitively inhibit the repressor release from DNA, preventing transcription; these sugars are called anti-inducers. (Table 1).

The isolation of lactose repressor was first carried out by Gilbert and Müller-Hill in 1966(2). They showed that the protein they had isolated would bind to isopropyl-β-D-thiogalactoside (IPTG) (3), an *in vivo* inducer, and would
The lactose operon is transcribed from DNA to mRNA. The repressor binds to the operator (o) region, preventing translation of the messenger RNA (mRNA). With lactose (I) as an inducer, the repressor binds to lactose enzymes, which then bind to the operator (o), allowing translation of the mRNA. The enzymes are synthesized, and the repressor is released. Without lactose, the repressor remains bound to the operator, preventing translation.
<table>
<thead>
<tr>
<th>INDUCER</th>
<th>ANTI-INDUCER</th>
</tr>
</thead>
<tbody>
<tr>
<td>GALACTOSE</td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>IPTG</td>
<td>ONPF</td>
</tr>
<tr>
<td>APTG</td>
<td>PTG</td>
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</tbody>
</table>
also bind to DNA containing lactose operator. This binding to operator DNA was destroyed by the addition of IPTG. The initial problem of the small amount of repressor which is usually found in bacterial cells has been overcome by the development of strains which increase the level by 2000 fold\(^4\). This overproduction is achieved by a mutation in the promoter region for the repressor, which increases the frequency of binding of RNA polymerase, thereby increasing transcription of the repressor gene. Introduction into the genome of the \textit{E. coli} of a transducing phage which carries the lactose operon also increases production; the phage begin multiplication upon short heat treatment at 44°C, but are lysis inhibited. An isolation procedure has been worked out by Gilbert, Beyreuther and Müller-Hill \(^5\) to obtain large amounts of repressor very easily. (Figure 2).

The repressor is a tetrameric protein of molecular weight 150,000 with an isoelectric point of pH 5.6\(^7\). There are four inducer/anti-inducer binding sites per tetramer, and a single DNA binding site \(^7\). The amino acid sequence has been determined \(^8\). (Figure 3).

Studies have been done on the interaction of repressor and operator \(^9 - 13\). The half-life of dissociation for repressor and operator in the absence of inducers is about 20 minutes\(^10\), while in the presence of anti-inducers the half-life increases to 80-160 minutes. In the presence of inducer, the half-life decreases to less than 4 minutes.
ISOLATION OF LACTOSE REPRESSOR

E. coli cells

+ 2 x wt. of BB buffer
+ 2 mg of DNAse

Stir well

+ 1 x wt. of BB buffer

Stir to homogenize

Spin at 10,000 rpm for 50'

ppt

Supernatant

+ 23 g/100 ml (NH₄)₂SO₄

Sit in ice for 1 hour

Spin at 10,000 rpm for 40'

supernatant

ppt

Suspend in 0.1M tris-Cl, 0.2M KCl, 10⁻⁴M DTT

+ eq. volume of water to solution

Dialyze against 0.12M KP overnight

Put onto phosphocellulose column

Elute with 0.12M KP, check OD₂₈₀, majority of protein elutes first

Start gradient (0.12M KP and 0.24M KP)

Do IPTG assays on fractions

Pool the IPTG-active peak

+ 23 g/100 ml (NH₄)₂SO₄

Figure 2
Sit in ice for 1 hour
Spin at 10,000 rpm for 40'
Suspend in 1M tris-Cl, 10^{-4}M DTT
Dialyze against same buffer overnight.
Met-Lys-Pro-Val-Thr-Leu-Tyr-Asp-Val-Ala-Glu-Tyr-Ala-Gly-Val-
Ser-Tyr-Gln-Thr-Val-Ser-Arg-Val-Val-Asn-Gln-Ala-Ser-His-Val-
Ser-Ala-Lys-Thr-Arg-Glu-Lys-Val-Glu-Ala-Ala-Met-Ala-Glu-Leu-
Asn-Tyr-Ile-Pro-Asn-Arg-Val-Ala-Gln-Gln-Leu-Ala-Gly-Lys-Gln-
Ser-Leu-Leu-Ile-Gly-Val-Ala-Thr-Ser-Ser-Leu-Ala-Leu-His-Ala-
Pro-Ser-Gln-Ile-Val-Ala-Ala-Ile-Lys-Ser-Arg-Ala-Asp-Gln-Leu-
Gly-Ala-Ser-Val-Val-Ser-Met-Val-Glu-Arg-Ser-Gly-Val-Glu-
Ala-Cys-Lys-Ala-Ala-Val-His-Asn-Leu-Leu-Ala-Gln-Arg-Val-Ser-
Gly-Leu-Ile-Ile-Asn-Tyr-Pro-Leu-Asp-Asp-Gln-Asp-Ala-Ile-Ala-
Val-Glu-Ala-Ala-Cys-Thr-Asn-Val-Pro-Ala-Leu-Phe-Ile-Ile-Phe-
Ser-His-Gln-Asp-Gly-Thr-Arg-Leu-Gly-Val-Glu-His-Leu-Val-Ala-
Leu-Gly-His-Gln-Gln-Ile-Ala-Leu-Leu-Ala-Gly-Pro-Leu-Ser-Ser-
Val-Ser-Ala-Arg-Leu-Arg-Leu-Ala-Gly-Trp-His-Lys-Arg-Leu-Arg-
Arg-Asn-Gln-Ile-Gln-Pro-Ile-Ala-Gln-Arg-Glu-Gly-Asp-Trp-Ser-
Als-Met-Ser-Gly-Phe-Gln-Gln-Thr-Met-Leu-Asn-Glu-Gly-Ile-Val-
Pro-Thr-Ala-Met-Leu-Val-Ala-Asn-Asp-Gln-Met-Ala-Leu-Gly-Ala-
Met-Arg-Ala-Ile-Thr-Glu-Ser-Gly-Leu-Arg_Val-Gly-Ala-Asp-Ile-
Ser-Val-Val-Gly-Tyr-Asp-Asp-Thr-Glu-Asp-Ser-Ser-Ser-Cys-Tyr-Ile-
Pro-Pro-Leu-Thr-Thr-Ile-Lys-Gln-Asp-Phe-Arg-Leu-Leu-Gly-Gln-
Thr-Ser-Val-Asp-Arg-Leu-Leu-Gln-Leu-Ser-Gln-Gly-Gln-Ala-Val-
Lys-Gly-Asn-Gln-Leu-Leu-Pro-Val-Ser-Leu-Val-Lys-Arg-Lys-Thr-
Thr-Leu-Ala-Pro-Asn-Thr-Gln-Thr-Thr-Pro-Arg-Ala-Leu-Ala-
Asp-Ser-Leu-Met-Gln-Leu-Ala-Arg-Gln-Val-Ser-Arg-Leu-Glu-Ser-
Gly-Gln

Figure 3
Platt, Files and Weber (14) found that lactose repressor digested with trypsin under native conditions yields a trypsin-resistant core molecule. This core has lost its amino terminal ~60 amino acid residues but it is still a tetramer. It exhibits full inducer binding activity, but has lost operator binding activity.

Using ultraviolet spectral techniques, Matthews et al., (15), (16) and Ohshima et al. (17) observed a change in the UV spectrum on addition of inducer, while no change occurred on addition of anti-inducer (15). Similar results were obtained with glycerol perturbation spectra (16). UV difference spectra of the core protein derived from trypsin treatment of the lactose repressor exhibits alterations in the aromatic residues upon addition of inducers. These changes are identical with those of whole repressor protein, indicating that the conformation change which occurs upon binding of inducers involves segments of the protein which are in the core protein and not in the C- and N-terminal regions which are removed by trypsin treatment.

Chemical modification has been used on proteins to determine the differences in exposed residues and changes in introduced chromophores due to conformation changes in the proteins. One chemical reagent used in these experiments is the organomercurial, 2-chloromercuri-4-nitrophenol (CMN). In 1969, McMurray and Trentham (19) found CMN to be useful as a chemical reagent that will react specifically with thiol
groups in protein. The magnitude of the spectral change observed when a thiol reacts with a mercurinitrophenol is a function of the local environment. The ionization state of the nitrophenol can be changed due to environmental changes, and this in turn affects its spectral characteristics. The ionized and unionized states exhibit different spectra and the degree of ionization can thus be determined spectrally.

Since both the degree of ionization and the absorption characteristics of the chromophoric group are dependent on the environment, any changes in the protein structure in the region surrounding the chromophore may be detected if they affect the ionization or the energy levels of the spectral transitions. By this method, it is possible from the spectral changes to detect the ionization characteristics of the chromophore and any conformational change in the protein in response to various stimuli.

In these studies, the lactose repressor protein has been reacted with MNP. The label is specific for cysteine residues, with no apparent side reactions. There are 3 cysteines per monomer in the repressor protein, and all three are in the core region. The extent of reaction with repressor under a variety of conditions was determined. Changes in spectral characteristics of MNP-repressor in response to ligands were detected, as well as the effects of reaction on the inducer and operator binding activities of the protein. The pK's of the MNP chromophore attached to the repressor
were determined under a variety of conditions. The rate of reaction of the repressor with MNP was measured under a variety of conditions.
II. MATERIALS AND METHODS

1. Growth of bacteria and plating methods

The solutions and media used were:

**Lactose-Tetrazolium Red plates (Lac-Tet plates)**

Per liter:

- 25.5 g antibiotic medium 1 (Difco)
- 0.05 g 2,3,5-triphenyl-tetrazolium chloride
- 950 ml H$_2$O

The medium is heated to dissolve it before autoclaving. The medium is then autoclaved for 22 minutes. Then 50 ml of 20% d-lactose (Sigma L-3625) are added. The solution is mixed and poured into plastic sterile petri plates (15 ml each).

The plates are allowed to cool and harden and are then inverted and stored in cold room.

**Trytone (T-broth) for growing strains**

- 8 g tryptone
- 5 g NaCl per liter H$_2$O

The solution is autoclaved for 17 minutes, then poured into sterile disposable tubes.

**Tryptone Yeast Extract (TYE) medium**

Per liter:

- 16 g tryptone
- 10 g yeast extract
- 5 g NaCl
The medium is diluted to volume with H₂O and adjusted to pH 7.0. The medium is autoclaved for 17 minutes. No more than 1/3 volume of the flask should be used.

a. Bacteria Strain

The strain of *E. coli* was M96, B⁻Δ(lac pro)
Sm^SΦ80AC^I857 h80t 68dlaci^Sq^-z^- This strain carries both i-gene promoter mutation, i^Sq_, and λ80 phage and, therefore, produces high amounts of lac repressor. Phage are induced by heating to 45°C for 17 minutes. Phage DNA is transduced into *E. coli* DNA near the lac operon; when temperature is increased to 45°C, phage λDNA is excised out of *E. coli* DNA, is reproduced including the *E. coli* DNA fragment containing the lac operon. When the temperature is decreased to 32°C, the fragment of DNA corresponding to lac repressor is transcribed giving large amount of mRNA of lac repressor, which is then translated into lac repressor protein. (20).

b. Selecting Culture

Before beginning growth of bacteria, a colony of M96 is selected. The appropriate colonies appear red with a white halo on lac-tet plates. In order to minimize the number of revertants when using any of the above strains, colonies should be used to grow innocula in tryptone broth, which are then replated on lac-tet plates; this process is repeated several times until a satisfactory
c. Plating Method

An innoculating needle is used to streak plates: the loop is flamed to sterilize it, then it is put into tryptone broth. The loop is then moved back and forth across half of the surface of plate. The loop is sterilized, then plunged into agar to cool; plate is rotated 90°, and the loop streaked through culture and over an additional quarter of plate. Loop is flamed again, cooled, and streaked through second quarter of the plate to cover the remaining quarter of plate. Plate is covered, inverted and incubated overnight.

Culture solutions are prepared from streaked plates by sterilizing the loop in the flame, cooling, and picking a single colony off plate then suspending it in the medium. The culture is placed in an incubator overnight.

d. Growth Protocols for M96

A colony of M96 is selected and put into 50ml culture, grown overnight at 32°C. Growth cultures are put into a shaking water bath at 32°C and OD_{590} is read until OD_{590} = 1.0. The temperature is increased as rapidly as possible to 45°C for 20 minutes to induce phage, then returned to 32°C and growth is continued for 3 hours. The culture is divided into two tubes (A, B), and the cultures spun down at 7,000 rpm for 15 minutes. (Pellet is frozen overnight or frozen/
thawed several times). Pellet is suspended in 0.2 ml TMS and a drop of DNAse solution (1 mg/ml) is added. 0.2 ml of A solution is added for IPTG assay to A tube, 0.2 ml B solution to B tube. Assays are run to determine the culture with greatest activity. The M96 inoculum chosen (in 50ml), is added to several (~3) 2 liter batches of TYE broth in 6 liter flasks. OD\textsubscript{590} is monitored continuously. 100 liters of medium is inoculated with 5 liters of culture. The medium is stirred and aerated vigorously at 32°C in fermentation machine, grown to OD\textsubscript{590} = 5. It is then induced at 45°C cooled to 32°C and growth continued for 3 hours. The cells are collected and frozen.

2. Isolation of Lac Repressor

Solutions for Lac Repressor purification:

a. BB buffer: 0.2M Tris-Cl, pH 7.6
   
   0.2M KCl
   0.01M Mg acetate
   3 x 10\textsuperscript{-4}M DTT
   5\% glycerol

b. 0.1M tris-Cl, pH 7.6:
   
   0.2M KCl
   2 x 10\textsuperscript{-4}M DTT (need 30 ml)

c. 0.12M KP (per liter):
   
   100 ml 1M K\textsubscript{2}HPO\textsubscript{4}
   20 ml 1M KH\textsubscript{2}PO\textsubscript{4}
3 ml 0.1M DTT
50 ml glycerol
pH 7.4, 7.6, \( \eta = 1.3425 \)

d. 0.24M KP (per liter):
200 ml 1M \( K_2HPO_4 \)
40 ml 1M \( KH_2PO_4 \)
3 ml 0.1M DTT
50 ml glycerol
pH = 7.4, \( \eta = 1.3446 \)

e. 2M KCl
f. \( 10^{-4} \) M DTT

Frozen cells are broken into pieces (cells must be broken up with hammer into manageable pieces) and thawed in twice their weight of BB buffer; the suspension is stirred with a spatula, 1ml DNAse solution (2 mg/ml) is added (or same amount of solid DNAse), the solution is stirred well, breaking up all clumps. BB buffer is added up to 3 x weight of cells and the solution is stirred gently until it is relatively homogenous. (This takes 1-2 hours; if necessary additional DNAse may be added). Cell debris is removed by centrifugation. The supernatent is precipitated with 23 g/100 ml ammonium sulfate. The precipitated protein is removed by centrifugation and precipitate suspended in 0.1M tris-Cl, 0.2M KCl, \( 10^{-4} \)M DTT (as small volume as possible). Repressor solution is dialyzed against 0.12M KP overnight with at least three changes of buffer.
The solution after centrifugation is layered onto phosphocellulose column and washed with 0.12M KP. Fractions are collected (250 drops/tube). The OD at 280nm of the fractions is measured after all the "yellow" color has eluted. When the OD is less than 0.2, the gradient is started (0.12M to 0.24M KP). For large preparations 250 - 500 ml each of 0.12M KP and 0.24M KP should be used. After the gradient, 0.24M KP is used to elute the repressor. The IPTG active peak is pooled and precipitated with ammonium sulfate (33% saturation). After centrifugation, the precipitate is resuspended in 1M tris-Cl, 10^{-4}M DTT, pH 7.6, and then dialyzed against the same buffer before storage.

3. IPTG Assay for Repressor

Solutions for IPTG assay are:

a. TMS:
   0.01M Tris-Cl, pH 7.4
   0.001M EDTA
   0.01M MgCl$_2$
   10^{-4}$M$ DTT
   0.2M KCl

b. 70% saturated ammonium sulfate

c. 10% TCA

d. Scintillation Fluid
   2,100 ml toluene
   1,230 ml EtoH (100%)
12 g PPO
0.3 g POPOP

100 ml TMS + 0.1 ml of $^{14}$C-IPTG (1 mM/1 ml)

50 ml

"A" + 11.9 mg IPTG "B"

A = TMS + $10^{-6}$M $^{14}$C-IPTG
B = TMS + $10^{-6}$M $^{14}$C-IPTG + $10^{-3}$M $^{12}$13C-IPTG

A series of duplicate tubes, one containing A, the other B, are used with 0.2 ml per tube. A sample volume totalling 0.2 ml is added to both A & B. These solutions are incubated at room temperature for 5 minutes. Then 3.0 ml 70% saturated ammonium sulfate is added. Carrier protein may be used if necessary. Centrifugation is used to remove the precipitate. Supernatant is removed by aspiration. The pellet is then dissolved in 0.5 ml TMS, and 0.5 ml 10% TCA is added. After centrifugation, 0.2 ml supernatant is placed in 15 ml scintillation fluid and counted. The amount of IPTG specifically bound can be determined from the difference between A and B, and this can be correlated with the amount of repressor present.

4. Lowry Assay

Solutions:
a. 60 g Na₂C₀₃, 8 g NaOH in 2 liters of water (H₂O)

b. 2% CuSO₄·5H₂O in H₂O

c. 4% Na tartrate in H₂O

d. Phenol reagent 1:1 in H₂O

1 ml (b) + 1 ml (c) dilute with (a) 100 ml = Solution Z

Protein sample (0.25 ml) is mixed with 2.5 ml solution Z, and the mixture is allowed to sit at room temperature for 10 minutes. Then, 0.25 ml of (d) are added. The solution is shaken immediately and vigorously. After ten minutes, the OD at 660nm is determined. A standard curve is determined using pure repressor.

5. Isolation of ³H-DNA

Bacteria strain:

MBC5: (lac-pro) Δ B₁-T⁻ nal A⁺(λCl₈₅⁷S₇plac₅⁻z⁺y⁻)

(Thymine-Reducing Mutant):

<table>
<thead>
<tr>
<th>Medium for Agar plates</th>
<th>Stock Solutions</th>
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<tbody>
<tr>
<td>0.7% K₂HPO₄</td>
<td>1.75 g / 235 ml</td>
</tr>
<tr>
<td>0.3% KH₂PO₃</td>
<td>0.75 &quot;</td>
</tr>
<tr>
<td>0.1% NH₄Cl</td>
<td>0.25 &quot;</td>
</tr>
<tr>
<td>0.08% Na₂SO₄</td>
<td>0.20 &quot;</td>
</tr>
<tr>
<td>0.25% MgSO₄·7H₂O</td>
<td>0.0625 &quot;</td>
</tr>
</tbody>
</table>
0.12% \( \text{Na}_3\text{Citrate} \cdot 2\text{H}_2\text{O} \) \( 0.3 \text{ g/235 ml} \) \\
1.5% Agar \( 3.75 \text{ "} \) \\
0.5% glucose \( 1.25 \text{ g/10 ml} \) \( \text{B} \) \\
40 \( \mu\text{g/ml} \) "X Gal" \( 10 \text{ mg/2.5 ml} \) \( \text{C} \) \\
0.005% Thiamine \( 125 \text{ mg/10 ml} \) \( \text{D} \) \\
5 \( \mu\text{g/ml} \) Thymidine \( 12.5 \text{ mg/10 ml} \) \( \text{E} \) \\
20 \( \mu\text{g/ml} \) proline \( 50 \text{ mg/10 ml} \) \( \text{F} \)

Colonies are blue on this medium.

Combine 235 ml A + 10 ml B + 1 ml C + 1 ml D + 1 ml E + 1 ml F for agar plate media.

Growth Medis:

\[ \begin{align*}
3\% \text{Casamino Acid} & \quad 0.75\text{g/25ml} \quad \text{G} \\
\text{Na}_2\text{HPO}_4 & \quad 1.4\text{g/94ml} \\
\text{KH}_2\text{PO}_4 & \quad 0.6 \quad " \\
\text{NH}_4\text{Cl} & \quad 0.2 \quad " \\
\text{NaCl} & \quad 0.1 \quad " \\
\text{MgSO}_4 & \quad 2.47\text{g/10ml} \quad \text{I}
\end{align*} \]

50 ml 3\% Casamino acids + 47 ml \( \text{NH}_4\text{Cl} \) + 0.5 ml \( \text{MgSO}_4 \) + 2 ml 5\% Glucose + 0.4 ml 0.005\% Thiamine + 0.4 ml 5\( \mu\text{g/ml} \) Thymidine.

A blue colony is added to 100 ml of media and grown at 32°C for 8 hours. \( 0 \text{D}_{590} \) is followed until it reaches 1.0. Induction at 45°C for 10-12 minutes is carried out and \( 3\text{H} \)
labeled thymidine (5μCi/ml) is added. Growth is continued for 3-4 hours at 32°C. The culture is left overnight allowing cells to lyse. Then 1 ml 1.0M MgSO₄ and 2 mg RNAse are added and allowed to incubate at 32°C for 15 minutes. After centrifugation, the supernatant is put in nitrocellulose tubes and centrifuged to remove phage. After suspension in 0.01M Tris (pH 7.4), and 0.01M MgSO₄, Cesium gradients are run.

The solutions are spun for approximately 40 hours. Using auto densi-flow fraction collector, the gradient is collected, and the radioactive peaks located. Active fractions are pooled and diluted with 0.01M Tris, 0.01M MgSO₄ before dialysis against the same buffer. Phenol saturated with 0.01M Tris, 0.01M EDTA, 0.3M NaCl and washed with "TE" buffer (0.01M Tris, pH 7.6, 0.001M EDTA) is used to extract the DNA from the phage. The final aqueous solution is dialyzed against "TE" buffer. DNA is characterized by measuring OD at 280nm, 260nm, and 230nm and determining radioactivity.

6. Operator Assay for Repressor

Solutions for operator assay:

a. BB' - 0.01M MgAc use 500 ml FB to make up BB'

0.01M KCl
10⁻⁴M EDTA
10⁻⁴M DTT
5% DMSO
0.01M Tris-Cl, pH 7.4

50 mg/ml BSA

b. FB = BB' minus BSA

c. $^3$H-DNA = 0.05 $\mu$g/ml

d. 1 mM MNP

e. 0.1M IPTG

$^3$H-DNA concentration in this experiment is 0.00025 $\mu$g/ml. Repressor is 0.00005 - 0.0001 $\mu$g/ml. MNP concentration is 1 mM and the volume ratio of MNP and repressor is 2:5.

Repressor (5 $\mu$l repressor + 2 $\mu$l MNP) is diluted into 5 ml BB' buffer, then diluted further using 20 $\mu$l of this solution in 1 ml BB' buffer.

Using series tubes, 2 $\mu$l, 5 $\mu$l, 10 $\mu$l, 20 $\mu$l, 50 $\mu$l, 100 $\mu$l, 200 $\mu$l of the final diluted repressor solution is mixed with 5 $\mu$l operator DNA solution in 0.5 ml BB' buffer. To another duplicate series of tubes, MNP-repressor is added instead of repressor. In addition, three tubes are made up with 10 $\mu$l, 100 $\mu$l, 200 $\mu$l of repressor solution in tubes with 0.1M IPTG and 5 $\mu$l operator DNA solution in 0.5 ml BB' buffer. These are incubated for 10 minutes at room temperature. Membrane filters (Millipore HAWP 45M) are saturated with FB buffer, before use, and the filter rate is adjusted to 0.5 ml/10 sec. The filters are placed onto a Buchner funnel, washed 2 times with 0.5 ml portions of FB buffer. Sample is
then placed on the filter and allowed to pass through; then
the filters are each washed with 0.25 - 0.30 ml FB buffer.
The filters are dried, and the amount of radioactivity de-
termined.

The operator activity is the difference between solutions
with IPTG and without IPTG. A saturation curve is deter-
mined.

7. SDS Polyacrylamide Electrophoresis

Solutions used:

a. Acrylamide solution
   22.2 Gm acrylamide
   0.6 Gm methylene bisacrylamide
   water to 100 ml

b. Gel buffer 0.2M, pH 7.2
   7.8 Gm NaH$_2$PO$_4$$\cdot$H$_2$O
   38.6 Gm Na$_2$HPO$_4$$\cdot$H$_2$O
   2.0 Gm SDS
   water to 1000 ml

c. Ammonium persulfate
   7.5 mg/ml in water should be made just prior
to use.

d. TEMED

e. Reservoir buffer
   1:1 gel buffer and water mixture
f. Sample buffer
   0.01M sodium phosphate, pH 7.2
   0.1% SDS
   0.1% 2-mercaptoethanol

g. 0.05% bromophenol blue in 0.01M phosphate buffer, pH 7.2

i. 10% TCA

j. mercaptoethanol

k. 0.05% Coomassie brilliant blue in 10% TCA

Gels preparation:

Well cleaned glass tubes are marked to indicate the required gel length (7 cm). One end of tubes is sealed with parafilm and placed in a vertical position on a rack. The gel solution is mixed according to the following order:
10.1 ml acrylamide solution, 3.4 ml water, 15.0 ml gel buffer, 1.5 ml ammonium persulfate, 0.045 ml TEMED.

After mixing gel solution, each tube is filled up to the mark. A small volume of water is layered on the top of the gel solution for polymerization.

The amount of repressor used should be 2 μg to 100 μg. Duplicates of each concentration of repressor are run. The sample is applied on the top of the gel and reservoir buffer is carefully layered over each sample with a disposable pipette. Four mAMP current is used until the samples have
entered the gels, then the current is increased to 8 mamp. The electrophoresis is stopped when the bromphenol blue tracking dye approaches the bottom of the gel. The gels are removed and the length of the gel and distance the tracking dye moved are measured.

Staining and Destaining:

The gels are fixed in 10% trichloroacetic acid and stained using Coomassie Brilliant Blue (in 10% trichloroacetic acid). The gels are placed in 7% acetic acid for storage and handling. The length of the gel and distance the protein moved are measured to calculate mobilities.

Calculation of Mobilities:

\[
\text{MOBILITY} = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by tracking dye}} \times \frac{\text{Length before staining}}{\text{Length after staining}}
\]

A plot of mobility versus logarithm of the molecular weight yields a standard plot/proteins of known molecular weight.

8. Amino Acid Analysis

a. Lactose repressor, 7 mg/ml in 1M tris-Cl, 0.1 mM DTT, pH 7.6.

b. 12N HCl

c. Sample buffer
Protein (20 µl) in a prepared hydrolysis tube is mixed with hydrochloric acid to 6 N. The evacuated, sealed tube is placed in an oven at 110°C for 20 hours. After evaporation of the 6NHCl, sample buffer is added for application to the amino acid analyzer (Beckman Model 120C).

9. Spectrophotometry

All absolute spectra and difference spectra were obtained using a Cary 118 Spectrophotometer.

a. Absolute spectrum

1 ml quartz cuvettes were used in obtaining absolute spectra, with path length of 1.0 cm. Repressor or repressor with ligand in 1M tris-Cl at pH 7.5 or pH 9.0 was put into the cuvette. Absorbance at 400nm was monitored each time after adding 2 µl increments of 1 mM MNP. For pH titrations of MNP-repressor, the same cuvette was used. Dilutions (1:20) of MNP-repressor or MNP-repressor-ligand into tris-Cl buffer of various pH's were used. The absorbance was scanned from 500nm to 350nm.

b. Difference spectrum

Using tandem cells of 0.876 cm pathlength (0.436 cm per compartment), the difference spectra were determined. The baseline was first scanned from 550nm to 350nm with MNP-repressor and ligand separated in both
cells. Buffer was 1M tris-Cl at pH 9.0; for pH
titrations, MNP-repressor and ligand were diluted
in same pH buffer. MNP-repressor and ligand were then
mixed in one cuvette; the other cuvette remained
unmixed as reference. The spectra were recorded
again from 550nm to 350nm to obtain difference
spectra.
III. RESULTS

1. MNP Titration of Repressor

The lactose repressor protein was reacted with MNP in the presence of various ligands. Figure 4 shows the titration of repressor with MNP at pH 7.5 in the presence of buffer alone. The equivalence point of the titration curve corresponds to 2 moles MNP/mole repressor. Figure 5 shows the titration of repressor in the presence of inducer (galactose). The equivalence point again is 2 moles MNP/mole repressor. Similar results (Figure 6) were obtained using an anti-inducer (glucose). A neutral sugar (mannose) which does not bind repressor gave identical results. This implies that there are 2 sulfhydryl groups which react with MNP under all conditions. The third sulfhydryl group in native repressor is not available for reaction. Additional inducers and anti-inducers which can bind to repressor were used for titration studies. The equivalence point of these titrations is once again 2 moles MNP/mole repressor. The third cysteine residue is apparently buried when these ligands are bound to native repressor.

Since the molar absorptivity at 400nm of the bound MNP moiety at pH 7 is less than that of MNP-C1, a change in the state of ionization must have occurred. There are two cysteines involved, both of which have altered ionization
Figure 4. Titration of repressor protein with MNP. Repressor 0.3 mg/ml, 1.0M tris-C1 buffer, pH 7.5, 1 mM MNP added in 2 µl increments. Absolute absorbance was measured at 400 nm. Reference solution was water. The same titration curves were observed in the presence of mannose.
Figure 5. Titration of repressor with MNP in the presence of inducer. Repressor 0.3 mg/ml, $10^{-3}$M IPTG, 1.0M tris-Cl buffer, pH 7.5, 1 mM MNP added in 2 μl increments. Absolute absorbance was measured at 400nm. Reference solution was water. The same titration curves were observed with galactose and APTG.
Figure 6. Titration of repressor with MNP in the presence of anti-inducer. Repressor 0.3 mg/ml, $10^{-3}$ M ONPF, 1.0 M tris-Cl buffer, pH 7.5, 1 mM MNP added in 2 μl increments. Absolute absorbance was measured at 400nm. Reference solution was water. The same titration curves were observed with glucose and PTG.
states. By increasing the pH, it might be possible to distinguish the cysteines, if their pK's are different. Therefore, titrations were carried out at pH 9.0. From Figure 7 it is apparent that a single break in the curve at 2 moles MNP/mole repressor was found. We cannot see any difference in these two sulfhydryl groups from this titration method. The pKs of those groups are apparently too close to distinguish at these pH's.

The titration curves at pH 9.0 of other inducers and anti-inducers which bind to repressor are similar to that shown in Figure 7. The result is identical with the finding at pH 7.0: no differences were observed. The equivalence point is 2 moles MNP/mole repressor monomer.

Since the third cysteine did not react under native conditions, titration under denaturing conditions was carried out. Guanidine hydrochloride (8M) denatures the protein and unfolds the structure. If all 3 sulfhydryl groups react with MNP, the break of the titration curve should be increased to 3 moles MNP/mole repressor monomer. From Figure 8, it can be seen that the equivalence point is increased to 2.8 moles MNP/repressor monomer. This confirms that the unreactive cysteine is buried in the native conformation, but it can be exposed by denaturing conditions.

2. Specificity of the Reaction

The specificity of the reaction of MNP with cysteines
Figure 7. Titration of repressor with MNP at pH 9.0. Repressor 0.3 mg/ml, 1.0M tris-Cl buffer, pH 9.0, 1mM MNP added in 2 μl increments. Absolute absorbance was measured at 400nm. Reference solution was water. The same titration curves were observed in the presence of inducers and anti-inducers.
Figure 8. Titration of repressor with MNP in 8M guanidine solution. Repressor 0.3 mg/ml, 1.0M tris-Cl buffer, pH 7.5, 8.0M guanidine solution. 1 mM MNP added in 2 μl increments. Absolute absorbance was measured at 400 nm. Reference solution was water.
in repressor was confirmed by two methods. First, repressor was reacted with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), which reacts specifically with sulfhydryl groups. This DNTB-modified repressor was titrated with MNP to determine if reaction occurred. Figure 9 shows the titration curve; it coincides with the blank curve. Since DTNB has bound to available sulfhydryl groups and MNP reaction is prevented, MNP must therefore react with thiol groups.

The second confirmation of specificity is the ability of reagents containing free sulfhydryl groups (e.g. mercaptoethanol, dithiothreitol) to prevent reaction. Curves similar to those in Figure 6 are observed if mercaptoethanol or dithiothreitol is included in the reaction buffer. Furthermore, addition of either of these mercaptans to MNP-repressor results in spectral changes corresponding to release of MNP from repressor protein.

3. Differentiation of the Cysteines

The absolute spectra of the titration at pH 7.5 and pH 9.0 did not indicate any differences in the sulfhydryl groups on the protein. Difference spectra of MNP-C1 in buffer and MNP-repressor were carried out at pH 9.0 to explore this further. The difference spectrum of MNP titration carried out at pH 9.0 (Figure 10) shows that there is a slight difference of environment between these two sulfhydryl groups which bind to MNP. There are two breaks on the curve. Appar-
Figure 9. Titration of DTNB reacted repressor with MNP.

100 µl repressor (1.5 mg/ml) was reacted with 50 µl DTNB solution (4 mg/ml) in 1.0M tris-Cl buffer, pH 7.5, dialyzed against the Tris buffer overnight. 1 mM MNP was added in 2 µl increments. Absolute absorbance was measured at 400nm. Reference solution was water.
Figure 10. Difference spectrum of titration of repressor with MNP at pH 9.0. Repressor (0.3 mg/ml), 1.0M tris-Cl buffer, pH 9.0, 1 mM MNP added in 2 μl increments. Difference absorbance was measured at 400nm. Reference solutions were unmixed repressor and MNP.
ently the first sulfhydryl group reacting with MNP results in a lower pK value for the phenol, so that more intensive absorption at pH 9.0 occurs. However, the second group shifts the MNP pK slightly higher so that the absorption is slightly decreased compared to the first one. The two groups must therefore have somewhat different environments.

4. Effects of Modification on Activity

The activity of the modified protein was measured under various conditions to determine any effects of modification on the function of the protein. IPTG binding activity of repressor at increasing concentrations of MNP at both pH 7 and pH 9 is shown on Figure 11. No significant loss of binding activity is observed at any excess of MNP. However, a time course was carried out to determine if any long-term (compared to reaction time) effects could be found. This is shown in Figure 12. Again, no loss in IPTG binding activity of MNP-repressor occurs within 30 minutes. Apparently MNP binding to the two cysteine residues does not affect the inducer binding site. This implies that these residues are not an essential part of the inducer binding site.

Operator binding curves for repressor and MNP-repressor are shown in Figure 13. Repressor and MNP-repressor have essentially the same operator binding activity; therefore, the two cysteine residues which MNP can modify are either not a part of the operator binding site or do not interfere
Figure 11a. IPTG binding activity of MNP-repressor at increasing concentrations of MNP at pH 7.5. Repressor 0.0025 mg for each assay. 1 mM MNP, 1.0M tris-Cl buffer, pH 7.5, IPTG binding assay (in Materials and Methods).

11b. IPTG binding activity of MNP-repressor at increasing concentrations of MNP at pH 9.0. Repressor 0.01 mg for each assay. 1 mM MNP, 1.0M tris-Cl buffer, pH 9.0. IPTG binding assay (in Materials and Methods).
Figure 12a. IPTG binding activity of MNP-repressor at different times at pH 7.5. Repressor 0.01 mg for each assay, 1 mM MNP, 1.0M tris-Cl buffer, pH 7.5. IPTG binding assay (in Materials and Method).

12b. Repressor 0.005 mg for each assay. 1 mM MNP, 1.0M tris-Cl buffer, pH 9.0, IPTG binding assay (in Materials and Methods).
Figure 13. Operator binding activity curves of repressor and MNP-repressor. Repressor ($1.4 \times 10^{-4}$ mg/ml), MNP ($2 \times 10^{-8}$ M), $1M$ tris-Cl buffer, operator binding assay (in Materials and Methods).
OPERATOR BINDING $\Delta$CPM

([REPO] OR [MNP-REPO] (UL))
with operator binding when reacted.

5. Difference spectra of MNP-repressor

Repressor has been shown by a variety of methods to undergo a conformational change on binding to inducers but not anti-inducers. Introduction of MNP into the repressor molecule provides an opportunity to observe any changes in the environment of these reporter groups which may result from inducer binding. Alterations in the surrounding environment can cause changes in either the pK or the absorption properties of the MNP which can be observed spectrally.

Figure 14 shows MNP-repressor in the presence of inducer at pH 9.0. Apparently when inducer binds to repressor, the conformation change alters the environment of the nitrophenol groups bound to the cysteine residues. This change appears to be toward slightly more polar conditions since the shift is toward shorter wavelength (21). The pK value of nitrophenol group may decrease slightly so that nitrophenol group is more ionized and gives a small increase in absorption as well as the shift. This would account for the larger magnitude of the 396nm peak as opposed to the 440nm trough. Theoretically a simple shift in wavelength of maximum absorption with no change in pK would give a trough and peak of equal magnitude. This difference spectrum was determined to be maximum near pH 9.0 (Figure 24). Since the MNP bound to repressor is close
Figure 14. Difference spectrum of MNP-repressor in the presence of galactose. After mixing, repressor \(4.4 \times 10^{-6}\) M, MNP \(10^{-5}\) M, galactose .1M, 1.0M tris-Cl buffer, pH 9.0. Difference spectrum measured from 550nm to 350nm. Reference solutions were unmixed MNP-repressor and galactose.
to its pK, small shifts in ionization can be observed at pH 9.0.

On the other hand, Figure 15 shows that anti-inducer bound to the repressor causes no spectral changes. MNP-repressor still binds anti-inducers, since competition of these molecules with IPTG is still observed. Since genetic evidence indicates inducers and anti-inducers bind at the same site, this means inducer binding and anti-inducer binding have different effects on the protein structure. Figures 16-19 show the difference spectra for other inducers and anti-inducers. Figure 20 shows that no spectral change occurs in the presence of mannose which is neither an inducer nor anti-inducer. Therefore, only inducers cause a change in the protein structure of sufficient magnitude to be reflected in the spectral characteristics of bound MNP.

Difference spectra of MNP-repressor in response to IPTG with increasing amounts of MNP (pH 9.0) are shown in Figure 21. In this case, 30 μl 1 mM MNP (2 equivalents per repressor monomer) saturates the sulfhydryl groups. As Figure 21 shows, the maximum absorption corresponds to this point. Addition of more MNP does not increase the absorption further. This is the additional evidence that MNP only reacts with 2 sulfhydryl groups per repressor monomer. Excess MNP does not alter the spectral changes in response to inducer.
Figure 15. Difference spectrum of MNP-repressor in the presence of glucose. After mixing repressor $4.4 \times 10^{-6}\text{M}$, MNP $10^{-5}\text{M}$, glucose 0.1M, 1.0M tris-Cl buffer, pH 9.0. Difference spectrum measured from 550nm to 350nm. Reference solutions were unmixed MNP-repressor and glucose.
Figure 16. Difference spectrum of MNP-repressor in the presence of IPTG. After mixing, repressor $4.4 \times 10^{-6}$ M, MNP $10^{-5}$ M, IPTG $10^{-3}$ M, 1.0 M tris-Ch buffer, pH 9.0. Difference spectrum was measured from 550 nm to 350 nm. Reference solutions were unmixed MNP-repressor and IPTG.
Figure 17. Difference spectrum of MNP-repressor in the presence of ONPF. After mixing, repressor $4.4 \times 10^{-6}\text{M}$, MNP $10^{-5}\text{M}$, ONPF $10^{-3}\text{M}$, 1.0M tris-Cl buffer, pH 9.0. Difference spectrum was measured from 550nm to 350nm. Reference solutions were unmixed MNP-repressor and ONPF.
Figure 18. Difference spectrum of MNP-repressor in the presence of APTG. After mixing, repressor $4.4 \times 10^{-6} \text{M}$, MNP $10^{-5} \text{M}$, APTG $10^{-2} \text{M}$, 1.0M tris-Cl buffer, pH 9.0. Difference spectrum was measured from 550nm to 350nm. Reference solutions were unmixed MNP-repressor and APTG.
Figure 19. Difference spectrum of MNP-repressor in the presence of PTG. After mixing, repressor $4.4 \times 10^{-6}$ M, MNP $10^{-5}$ M, PTG $10^{-2}$ M, 1.0 M tris-Cl buffer, pH 9.0. Difference spectrum was measured from 550 nm to 350 nm. Reference solutions were unmixed MNP-repressor and PTG.
Figure 20. Difference spectrum of MNP-repressor in the presence of mannose. After mixing, repressor $4.4 \times 10^{-6}$M, MNP $10^{-5}$M, mannose 0.1M, 1.0M tris-Cl buffer, pH 9.0. Difference spectrum was measured from 550nm to 350nm. Reference solutions were unmixed MNP-repressor and mannose.
Figure 21. Difference spectra of MNP-repressor in response to IPTG with increasing amount of MNP. After mixing, repressor $7.5 \times 10^{-6}$ M, IPTG $10^{-3}$ M, 1.0 M tris-Cl buffer, pH 9.0, 1.0 M MNP was added. Difference spectrum was measured from 550 nm to 350 nm. Reference solutions were unmixed MNP-repressor and IPTG.
6. Determination of the pK of MNP groups

The absolute spectrum of MNP at different pH values was determined in order to obtain a pK value for free MNP. The pK of the free mercurinitrophenol was determined from this pH titration (Figure 22) to be 6.75. This value is close to the value of 6.5 determined by McMurray and Trentham(19).

The absolute spectra of MNP-repressor and MNP-repressor in the presence of inducer were also determined at different pH values. Plots of absorbance versus pH for several wavelengths are shown in Figures 23, 24, and 25. Three different wavelengths, 396nm, 410nm, 440nm were chosen. The difference spectrum of MNP-repressor in response to inducer has a maximum at 396nm and a minimum at 440nm. The maximum absorption of absolute spectra of MNP-repressor and MNP-repressor with inducer were at 410nm. From Figure 24, the combined pK of the two mercurinitrophenol groups bound to cysteine residues is found to be ~8.0; however, the pK of MNP-repressor in the presence of IPTG was found to be ~7.9. In the presence of inducer, the pK may decrease slightly, giving more absorption at pH's below the pK compared to MNP-repressor alone. This phenomenon can also be seen in the difference spectra of MNP-repressor (Figures 14-19). The shift of the pK of MNP from 6.75 to 8.0 when bound to repressor indicates that the cysteines are either in a hydrophobic environment, or some specific interaction (for both cysteines) stabilizes the unionized form of the phenol.
Figure 22. The absolute spectrum of MNP at various pH values. 0.005 mM MNP, 1.0M tris-Cl buffer of different pH values. Absolute absorbance was measured at 410nm. Reference solution was water.
ABSORBANCE 410NM

pH

0.02 0.04 0.06 0.08

3.0 4.0 5.0 6.0 7.0 8.0 9.0
Figure 23. The absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer with various pH values at wavelength 396nm. Repressor 0.22 mg/ml, IPTG $10^{-3}$M, MNP 0.009 mM, 1.0M tris-C1 buffer of different pH values. Absolute spectrum was measured at 396nm. Reference solution was water.
Figure 24. The absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer with various pH values at wavelength 410nm. Repressor 0.22 mg/ml, IPTG $10^{-3}$M, MNP 0.009 mM, 1.0M tris-Cl buffer of different pH values. Absolute spectrum was measured at 410nm. Reference solution was water.
Figure 25. The absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer with various pH values at wavelength 440nm. Repressor 0.22 mg/ml, IPTG $10^{-3}$M, MNP 0.009 mM, 1.0M tris-Cl buffer of different pH values. Absolute spectrum was measured at 440nm. Reference solution was water.
The former explanation would appear most likely, particularly in view of the kinetic data (see below).

A comparison of the wavelength of maximum absorption versus pH values for the spectra of MNP-repressor and MNP-repressor with IPTG is shown in Figure 26. The maximum absorption wavelength shifts from 394 nm to 414 nm as the pH increases. This is likely due to ionization of side chains on the protein which are sufficiently near the cysteine residues to interact with the MNP (18). Both curves indicate such a group with a pK of 7.0. The pK of another group on the protein appears to be 9.25 in the absence of inducer. In the presence of inducer, the pK appears to be 9.0. (An alteration of this type could be accounted for by the conformation change which occurs when repressor binds to inducer). A shift to longer wavelength of maximum absorption generally involves decreasing polarity of the environment (21), implying that a shift from ionized to unionized has occurred for the side chain on the protein. The pK~7 could be a histidine residue, and the ε-amino group of lysine would appear to be the most likely candidate for the pK near 9.

Difference spectra of MNP-repressor with inducer versus MNP-repressor at various pH values from 7.5 to 9.5 were determined. Figure 27 shows the maximum of the difference spectrum is at pH 9.06. It is not possible to determine a pK from this curve. The problems of this method
Figure 26. The wavelength of maximum absorption of absolute spectrum of MNP-repressor and MNP-repressor in the presence of inducer at various pH values. Repressor 0.22 mg/ml, IPTG $10^{-3}$M, MNP 0.009 mM, 1.0 tris-Cl buffer of different pH values. Absolute spectrum was measured from 550nm to 350nm. Reference solution was water.
Figure 27. The difference spectrum of MNP-repressor with inducer versus MNP-repressor at various pH values. Repressor 0.4 mg/ml, IPTG $10^{-3}$M, MNP 0.015mM, 1.0M tris-Cl buffer. Difference spectrum was measured from 550nm to 350nm. Reference solutions were unmixed MNP-repressor and inducer solutions.
are that the absorbance change of the difference spectrum is quite small (less than 0.01) and the tris-Cl does not buffer if pH is above 9.5 or below pH 6.5. Therefore, the pH changes dramatically after mixing the solutions in the tandem cuvettes, resulting in a pH of the solution in a sample cuvette which is different from that in reference cuvette. For this reason absolute spectra were used for pK determinations, (Figures 22-26), instead of difference spectra.

7. Kinetics of the reaction of MNP with repressor

Some kinetic studies have been done. Reaction rates of MNP and repressor were determined under a variety of conditions. In Figure 28, no significant difference in reaction rate can be seen between repressor alone or in the presence of inducers or anti-inducers. When the concentration of MNP is increased, however, the reaction rate is increased, as shown in Figure 29. From a plot of log absorbance versus time (Figure 30), it can be determined that there are 2 phases, implying that MNP reacts with the 2 sulfhydryl groups with two different rate constants. The data are not sufficient to determine rate constants in these cases, and further investigation is required.
Figure 28. Reaction rates of MNP and repressor with repressor alone or in the presence of inducers or anti-inducers.
Repressor 0.28 mg/ml, $10^{-3}$M IPTG (or ONPF), 0.015 mM MNP, 1.0M tris-Cl buffer, pH 9.0. Reaction rates were measured by a Durrum-Gibson stopped-flow spectrophotometer at 400nm.
Figure 29. Reaction rates of MNP and repressor with two different concentrations of MNP. Repressor 0.28 mg/ml, $10^{-3}$M IPTG (or ONPF), 0.015 mM MNP, 1.0M tris-Cl buffer, pH 9.0. Reaction rates were measured by a Durrum-Gibson stopped-flow spectrophotometer at 400nm.
Figure 30. A plot of log absorbance versus time of the reaction rate. Repressor 0.28 mg/ml, 0.015 mM MNP, 1.0M tris-Cl buffer, pH 9.0. Reaction rate was measured by a Durrum-Gibson stopped-flow spectrophotometer at 400nm. Log absorbance versus time of the reaction rate is plotted.
IV. Discussion / Conclusion

The spectral data show that MNP provides an excellent probe for thiol groups in repressor protein. The change of the pK of the MNP residues on binding to repressor appears to be the result of either introduction of anionic species into the immediate vicinity of the phenolic group (19) or increased hydrophobicity of the surrounding environment (19). Both of these factors may exhibit changes if the protein structure is altered; therefore, chromophoric changes resulting from conformational changes may be monitored in the spectrum.

Titration studies carried out at pH 7.5 and pH 9.0 indicate that only 2 moles MNP reacted per mole repressor monomer in the presence of inducer or anti-inducer. This implies that there are 2 sulfhydryl groups which react with MNP under all conditions. The third cysteine in native repressor does not react; this cysteine residue is apparently buried in native repressor whether free or bound to ligands. When the titration was carried out under denaturing conditions (guanidine hydrochloride (8M) denatures the protein), the equivalence point is increased to 2.8 moles MNP/mole repressor monomer, which is very close to the 3 moles of cysteine found in the protein.

DTNB which reacts specifically with sulfhydryl groups was used to check the specificity of the reaction of MNP with
cysteines in repressor. DTNB-modified repressor was titrated with MNP. The protein curve coincides with the blank curve. Since DTNB has bound to available sulfhydryl groups and MNP reaction is prevented, it is concluded that MNP must also react with thiol groups.

The absorbance spectra of MNP titration did not show any differences in the sulfhydryl groups on the protein. Difference spectra of MNP titration of MNP-C1 and MNP-repressor were carried out at pH 9.0. The titration curve has two equivalence points. Apparently, the first sulfhydryl group reacting with MNP results in a lower pK value for the phenol, so that it exhibits more intensive absorption at pH 9.0. The reaction of the second sulfhydryl results in a slightly higher pK for MNP and therefore lower absorbance compared to the first one. We can conclude that these two thiol groups have somewhat different environments. The activity of the modified protein was measured, and no significant loss of either IPTG binding activity or operator binding activity was observed. Apparently MNP binding to the two cysteine residues does not affect the inducer/anti-inducer or operator binding sites. This implies that these residues are not an essential part of those binding sites. Difference spectra of MNP-repressor in response to ligands were measured to discover any absorption changes due to conformational changes of repressor on binding to ligands. When inducer binds to repressor, a conformation change occurs
which alters the environment surrounding the nitrophenol group bound to cysteine residues. The environment appears to change to more polar conditions, since the shift is toward shorter wavelength. The pK value of nitrophenol group seems to decrease slightly so that nitrophenol group is more ionized, and a small increase in absorbance is observed. On the other hand, anti-inducer bound to the repressor causes no spectral changes. Mannose which is neither an inducer nor anti-inducer gives no spectral alterations. This phenomenon is likely due to the different effects on protein conformation which inducer and anti-inducer binding produce.

The pK of free MNP was determined from pH titration studies to be 6.75. The pK values of MNP-repressor and MNP-repressor in the presence of inducer were also determined. These values are 8.0 and 7.9, respectively. The pK of MNP-repressor decreases slightly in the presence of inducer, giving more absorption at pH's below the pK compared to MNP-repressor alone. Again, it was shown that pK of MNP-repressor bound to inducer is slightly lower than that of MNP-repressor. The same conclusion was reached using data on difference spectra of MNP-repressor.

From the plot of the wavelength of maximum absorption versus pH values for the spectra of MNP-repressor and MNP-repressor with IPTG, it can be seen that the maximum absorption wavelength shifts from 394nm to 414nm as the pH increases. The environment appears to decrease in polarity,
since the shift is toward longer wavelength. This might be due to changes in the ionization state of side chains on the protein which are sufficiently near the cysteine residues to interact with the MNP. Apparently the side chain shifts are from ionized to unionized form. The pK of one side-chain group appears to be 9.25 in the presence of inducer; in the absence of inducer, the pK appears to be 9.0. This shift in pK for one group from 9.25 to 9.0 could be accounted for by the conformational change which occurs when repressor binds to inducer. The residue involved is likely to be lysine. A similar shift is seen at pH 7.0. The group involved in this case may be histidine.

Kinetic studies have shown no significant difference in reaction rates of MNP and repressor between repressor alone or in the presence of inducer or anti-inducer. However, when the concentration of MNP is increased, the reaction rate is increased. The rates are several orders of magnitude slower than rates observed for exposed sulfhydryls (22). A plot of log absorbance versus time demonstrates that the reaction is biphasic. Further investigation is necessary to determine rate constants.

In summary, two cysteines of the repressor protein monomer react with MNP under all conditions studied. The alterations of the pK of the MNP group on binding to protein and on protein binding to inducer have been investigated. Difference spectra of the MNP moiety in the presence of
inducer have been determined. The cysteine residues are apparently in a hydrophobic region of the molecule, since the pK shifts to significantly higher pH's on reaction of MNP with protein and since the rate of reaction of repressor with MNP is several orders of magnitude slower than rates observed for free thiols (22). The cysteine residues are also perturbed by the conformation change which occurs in repressor on addition of inducer, and this results in a shift to slightly more polar environment for at least one of the cysteines. Current studies using peptide mapping to locate the site of reaction should shed further light on the charged residues (lysine, histidine) which apparently interact with the MNP and result in shifts in the wavelength of maximum absorption.
V. REFERENCES

6. Mary Clark, personal communication.
20. Jeffrey, H. Miller, "Experiments in Molecular Genetics".