UNIVERSITY OF ILLINOIS

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Thomas P. Primiano

ENTITLED: The Fluorescence Quenching of the Lactose Operon Repressor

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Chemistry

Professor Gregorio Weber
Instructor in Charge

APPROVED:

HEAD OF DEPARTMENT OF
Professor Larry Faulkner
THE FLUORESCENCE QUENCHING OF THE LACTOSE OPERON
REPRESSOR PROTEIN BY POTASSIUM IODIDE

BY

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THESIS
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DEDICATED
TO MY MOTHER
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I. INTRODUCTION

The genes that control the metabolism of lactose or other β-galactosides are constructed to function at highly variable rates in that their mRNA molecules are made only when certain signals present themselves to specific nucleotide sequences. The compounds that transmit these signals are called inducers. Certain proteins are responsible for controlling the transcription of mRNA and are termed repressors (Jacob and Monod, 1961). The most extensively studied of these repressor systems in vivo and in vitro is the lac repressor system of Escherichia coli (Beckwith and zipser, 1970).

According to the model, the lac repressor protein, a 154,000 D tetramer consisting of identical subunits (Muller-Hill, 1971), binds specifically to a 21 base pair sequence termed the operator. Binding of the repressor to this DNA region inhibits synthesis of the mRNA of the three inducible enzymes responsible for lactose metabolism: namely, β-galactosidase, galactoside permease and galactoside transacetylase. Lactose is structurally altered to form 1,6-allolactose (Jobe and Bourgeois, 1972), an inducer, which binds to the repressor subsequently changing the quaternary structure and reducing its affinity for the operator. Transcription then proceeds, producing the three metabolizing proteins. This form of genetic regulation is termed negative control.

Steitz et. al. (1974) through powder X-ray diffraction and electron microscopy outlined a 222 symmetry and a plane rectangular structure of dihedral subunit arrangement for the repressor tetramer. Each subunit is composed of a core protein and an N-terminal domain directed from the center of symmetry (Figure 1). The core protein has the ability to bind inducer and operator DNA. The amino terminal region has been implicated in binding
to the operator DNA (Adler et al., 1972). However, the N-terminal region is responsible for nonspecific binding of repressor to the operator, while the core protein is responsible for specific binding to the operator (O'Gorman et al., 1980a; Matthews, 1979; Dunaway and Matthews, 1980).
FIGURE 1: Lactose repressor protein bound to operator DNA. The DNA sequence shown is the operator DNA sequence protected from DNase digestion. Diagonal lines indicate N-terminal regions. Vertical lines depict inducer binding sites. Checkered area portrays core protein contacting the operator sequence. (Reproduced from Dunaway et al., 1980).
The lac repressor is released from the operator sequence by specific inducers such as isopropyl-\(-\text{D}-\text{thiogalactoside (IPTG)}\) (Muller-Hill, 1971). O'Gorman et. al. (1980b) found that IPTG bound noncooperatively to repressor in the absence of operator DNA, and conversely, bound cooperatively in the presence of operator. Repressor binds two molecules of DNA and the affinity of the complexed repressor is altered through the binding of only two inducer molecules in their respective binding sites (Dunaway et. al., 1980).

The amino acid sequence of the repressor protein is known (Beyreuther et. al., 1973). Each repressor monomer contains 8 tyrosyl and 2 tryptophanyl residues. The fluorescence of the tyrosyl side chains is quenched with respect to the tryptophanyl groups, as is the case with most proteins (Teale, 1960; Weber and Rosenheck, 1964; Cowgill, 1976).

It is known that tryptophyls exposed to solvent or located in the binding sites are preferentially quenched by iodide anion \((I^-)\) (Lehrer, 1967). Quenching of tryptophyl model compounds by iodide (Lehrer, 1971; Burstein, 1968; Teale and Badley, 1970) were found to follow the classical Stern-Volmer law (1919), predominantly involving collisional quenching.

Quenching of a heterogenous distribution of fluorophores in a protein shows modified Stern-Volmer characteristics leading to effective quenching constants and bimolecular rate constants (Lehrer, 1971). Similar studies were performed on the lac repressor protein to indicate quencher accessibilities to tryptophan side chains. By monitoring the spectral shifts and fluorescence intensities, in the presence and absence of IPTG, as a function of increasing quencher concentration, information regarding immediate fluorophore environments may be obtained (Laiken et. al., 1972).

Collisional and static quenching processes are resolved by measuring corresponding phase and modulation lifetimes with increasing concentration.
of quencher. Adherence to the relation—

$$\frac{F_0}{F} = \frac{T_0}{\tau}$$

—would indicate an exclusively collisional process (Perrin, 1929).

Deviations from the above relation would implicate formation of some fluorescent complex predating excitation (Baughn and Weber, 1970).

Quenching curves using three dilutions of repressor with equal concentrations of iodide were recorded to detect dissociation of the tetramer in more dilute solutions. This is addressed by noting an increase in the Stern Volmer slopes; which is equated with iodide becoming more accessible to buried tryptophans exposed upon dissociation.

Several other DNA binding proteins retain similar structures as the *lac* repressor. A study such as this could be applicable for multimers containing buried tryptophyls detectable by fluorescence methods. This will be discussed in greater detail later.
II. MATERIALS AND METHODS

A. Chemicals

KI, and KCl were from Mallinckrodt, Inc. NaCl was purchased from Baker Chemical Company. Dithioerythritol (DTE) was obtained from Sigma Chemical Co. Triu Ultrlot from Calbiochem-Behring was used as buffer.

The lac repressor tetramer was a generous gift from Dr. Kathleen Matthews of Rice University, Houston, Texas. The protein was isolated and purified from E. coli according to the methods of Rosenberg et. al. (1977) with modifications described by O'Gorman et. al. (1980b). Protein was stored in aliquots of 200µl at -70°C.

B. Instrumentation

Fluorescence emission spectra of the lac repressor protein were measured using a microprocessor controlled photon counting scanning fluorimeter (Royer, 1985). The fluorimeter had the distinct advantage of subtracting away background noise, solvent scattering and Raman peaks which was essential to monitoring fluorescence of dilute solutions.

Ultraviolet measurements were made using a Beckman Acta MVI Spectrophotometer using quartz cuvettes.

Lifetime measurements were performed using an SLM Instruments device with specifications according to Spencer and Weber (1969). Data was digitized and computed using an accommodating Hewlett-Packard calculator.

pH was measured using a Metrohm/Brinkman pH-103 apparatus with a hydrogen ion selective glass electrode.

C. Methods

Repressor was thawed on ice, diluted into 0.1M Tris-Cl, pH 7.5, 0.1M DTE and 0.1M KCl and dialysed against the same buffer overnight at 4°C.
Concentrations were determined using ultraviolet absorption and calculated assuming a molar extinction coefficient of $2.25 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$. All glassware was washed with nitric acid before use and millipore filtered water (18 MΩ) was boiled before making buffers.

Quenching measurements were made on 1.0, 0.5 and 0.1 μM dilutions of lac repressor containing increasing amounts of KI (0-0.2M). Corresponding additions of NaCl were used to maintain an ionic strength of 0.2M. Stock solutions of KI, NaCl and buffer were prepared by diluting into volumetric flasks. A small amount of $S_2O_3^{2-}$ (1mM) was added to iodide stock solutions to prevent $I_3^-$ formation. KI was also stored in a dark flask to prevent photochemical formation of $I_3^-$. This ion absorbs in the wavelength region of tryptophanyl fluorescence (filter effects). Solutions were equilibrated at 4°C, using a thermal jacketed cuvette holder, to prevent thermal decomposition and/or dissociation.

Excitation wavelength was 295nm and emission scanned from 300-500 nm. Lifetime measurements employed a 295 nm excitation wavelength modulated at 30MHzs and passed through a Corning 754 blue filter. A bandpass emission Schott WG320 filter eliminated scattering from the sample. A neutral density filter was used to align the scattered light from the glycogen (in H2O) scattering solution onto the detector and to select an optimal intensity of the scattered light. Lifetimes were recorded five times for each sample and the results and standard deviations averaged.

D. Calculations

Treatment of quenching data followed the methods of Lehrer (1971) and will briefly be recapitulated here.

The quantum yield was calculated by comparing the integrated fluorescence intensity of the lac repressor tetramer with that of L-Trp in H2O.
corrected to the same absorption at the exciting wavelength and using a value of 0.13 as the quantum yield for L-Trp (Chen, 1967).

For a heterogeneous distribution of fluorophores, adherence to the Stern-Volmer law for each fluorophore is assumed. The protein fluorescence quantum yields in the absence and presence of quencher, \( F_0 \) and \( F \), respectively, and then given by—

\[
F_0 = \frac{\Sigma F_{o1}}{n}
\]

\[
F = \frac{\Sigma F_{11}}{n} = \frac{1}{n} \Sigma \frac{F_{o1}}{1+K_{q1}(X)}
\]

where the sums are taken over the \( n \) fluorophores of the molecule. \( K_{q1} \) is the effective quenching constant of each fluorophore and \( (X) \) the concentration of quencher. The difference is—

\[
\Delta F = F_0 - F = \frac{1}{n} \Sigma \frac{F_{o1} K_{q1}(X)}{1+K_{q1}(X)}
\]

and—

\[
\frac{F_0}{\Delta F} = \left[ \frac{\Sigma f_1 K_{q1}(X)}{1+K_{q1}(X)} \right]^{-1}
\]

where \( f_1 = F_{o1}/F_0 \). Equation 4 represents the effect of collisional quenching on the protein fluorescence quantum yield in terms of the fluorescence and quenching constants associated with the \( n \) fluorescent side chains. For \( m \) accessible fluorophores—

\[
\frac{F_0}{\Delta F} = \frac{1}{(X) f_a K_q} + \frac{1}{f_a}
\]

where \( f_a = \Sigma f_1 \) summed over \( m \) is the fractional maximum accessible protein fluorescence. A certain fraction of the protein fluorescence, \( f_b \), is associated with buried side chains, and therefore \( f_a + f_b = 1 \). A plot of \( \frac{F_0}{\Delta F} \) vs. \( 1/(X) \) will yield a straight line of slope \( (f_a K_q)^{-1} \) and
and intercept $1/f_a$, with $K_q = \text{intercept/slope}$. This is considered the effective quenching constant. From this and knowledge of the fluorescence lifetime in the absence of quencher, a biomolecular rate constant can be calculated by the relation——

$$K_q = k \tau_0$$

which is a good approximation when only collisional quenching is involved.

Stern-Volmer plots and their modified forms were associated with theoretical Stern-Volmer plots for various mixed systems by the method of Badley (1975). Particularly, the total fluorescence emitted by a protein molecule was presumed to contain two heterogeneous components, each contributing some fraction $\alpha$. The total fluorescence would then be

$$F = F_1\alpha + F_2(1-\alpha)$$

$F_1$ and $F_2$ are the calculated fluorescence intensities of each heterogeneously emitting fluorophore. Such values may be obtained by assuming the initial total emission intensity to be unity, and by incorporating representative values for quenching constants $K_1$ and $K_2$ to the following expressions.

$$\frac{1}{F_1} = 1 + K_1[Q], \quad \frac{1}{F_2} = 1 + K_2[Q]$$

Likewise, theoretical values of were also selected and results plotted in Figure 18. Indexing multiple values shows that a large difference in k values is necessary for the Stern-Volmer plot to become evidently curved. Also, the points will converge asymptotically to a maximum value as one constant approaches zero. The case shown in Figure 18 is that of $K_1 = 8, K_2 = 1$ at the values of shown.
III. RESULTS

Fluorescence emission spectra and phase and modulation lifetime experiments were performed on three separate dilutions of lac repressor: 1.0x10^-6 M, 5.0x10^-7 M and 1.0x10^-7 M each with and without a 4000 fold excess concentration of IPTG. The results are presented in Tables I-VI.

Figures 2 and 3 display the effects of increasing iodide concentration on 1.0x10^-6 M repressor tetramer with and without saturating levels of IPTG, respectively. The fluorescence intensity decreases as quencher concentration is increased. Fluorescence intensity was assumed proportional to the number of photons counted. The center of mass was taken as the statistical average of the sum of the fluorescence intensities. Figure 2 also shows a relative blue shift in the center of mass (column 3, Table I) at higher concentrations of quencher. Such a shift suggests preferential quenching of exposed tryptophans (Lehrer, 1967, 1971; Elkan, 1968) implying that both of the subunit tryptophans lie in nonequivalent environments or one tryptophan contributes overwhelmingly to the observed emission (Laiken, 1971). Figure 3 does not portray a pronounced blue shift in the center of mass dictating an alternative mode of quenching.

Figure 4 depicts the corresponding Stern-Volmer plot for the two samples mentioned. A greater slope pertains to a more accessible fluorophore and therefore a larger extent of quenching. Figures 5 and 6 show a decrease in fluorescence yield upon dilution of the tetramer for bound and unbound inducer, respectively. Accompanying decrease in quantum yield was a slight red shift in the spectral center of mass for the samples not containing inducer. This phenomenon is expected for tryptophyls that are exposed to a more polar environment and hence more accessible to quenching (Brand and Wittlich, 1967; Anderson, Brunori and Weber, 1970). Tryptophyl
### TABLE I

**1.0 x 10^{-6} M Lac Repressor**

<table>
<thead>
<tr>
<th>(M)[KI]</th>
<th>Counts</th>
<th>(v_g) (KK)</th>
<th>(\lambda) (nm)</th>
<th>(\tau_p) (ns)</th>
<th>(\tau_o/\tau_p)</th>
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### TABLE II

**1.0 x 10^{-6} M Lac Repressor, 4 mM IPTG**

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<th>(M)[KI]</th>
<th>Counts</th>
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**KEY**

- a - ± 50 counts
- b - ± 1 x 10^{-4} KK
- c - ± 0.1 nm
- d,f - ± 0.01-0.04 ns
- e,g - ±0.002-0.01
### Table III

**5.0x10⁻⁷M Lac Repressor**

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<th>(M)[KI]</th>
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<th>τ₀/τₚ</th>
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### Table IV

**5.0x10⁻⁷M 2mM IPTG**

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### Table V

1.0x10⁻⁷ M Lac Repressor

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### Table VI

1.0x10⁻⁷ M Lac Repressor, 0.4 mM IPTG

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fluorescence does not shift to longer wavelengths with inducer bound to the protein leading one to believe that a different process is being observed. The blue shift of the inducer bound tetramer with respect to that in absence of IPTG is plotted in Figure 7.

Figures 8 and 9 establish reasonable evidence for interpreting the different fluorescence processes just mentioned. Figure 8 shows an increase in quenching upon dilution of the tetramer without inducer. The extrapolation of the $1.0 \times 10^{-6}$M concentrated sample suggests some electrostatic interaction between the negatively charged quencher and some acidic amino acid near the fluorophore in the inducer binding site. Comparison with Figure 9 (containing saturating levels of IPTG) shows that the two higher concentrations of tetramer are quenched by approximately an equal magnitude (see Table VII). The lowest concentration of tetramer shows greater quenching at low concentrations of iodide in absence of inducer as opposed to that with inducer present. Inducer facilitates dissociation of the tetramer or increase the ease of quenching by a change in the protein conformation.

The assumptions previously stated are true only in the case of collisonal quenching. Phase and modulation lifetime measurements akin to emission measurements insure that static quenching was not a factor in this determination. Figures 10 and 11 correspond with Figure 4 and show a direct proportionality between the dilutions for modulation and phase measurements respectively, indicating collisional quenching exclusively which is expected for the iodide ion. Figures 12 and 13 correspond with Figure 8; Figures 14 and 15 correspond with Figure 9.

It has already been proposed that lac repressor subunits emit fluorescence from two heterogenous tryptophans. Weber (1981) described a method of resolution using lifetimes which ascribes a single exponential decay for each
FIGURE 2: Fluorescence emission spectra of 1.0 × 10^{-6} M lac repressor, 4 mM IPTG with increasing levels of iodide quencher. All measurements performed in 0.1 M Tris-HCl, 0.1 M KCl, 10^{-4}M DTE at pH 7.5 and 4°C with λ excitation=295nm unless specifically stated otherwise.
FIGURE 3: Fluorescence emission spectra of $1.0 \times 10^{-6}$ M lac repressor with increasing amounts of iodide quencher.
FIGURE 4: Ratio of fluorescence yield in the presence and absence of inducer with increasing levels of iodide quencher. (○-○) 1x10^-6M lac repressor. (●-●) 1x10^-6M lac repressor. 4mM IPTG.
RATIO: FLUORESCENCE YIELD vs. QUENCHER CONCENTRATION

$\frac{F_0}{F}$ vs. $[KI] (m)$

1.0 $\mu$M Lac

1.0 $\mu$M Lac + IPTG
FIGURE 5: Fluorescence emission spectrum of various concentrations of repressor tetramer with saturating levels of inducer and increasing levels of iodide quencher.
FIGURE 6: Fluorescence emission spectrum of various concentrations of repressor tetramer with increasing levels of iodide quencher.
FIGURE 6

Intensity

Wavelength

1.0 μM Lac

0.5 μM Lac

0.1 μM Lac

Intensity

380

400

420

460
FIGURE 7: Fluorescence emission spectra of 1.0x10⁻⁶ M lac repressor in presence and absence of 4.0mM IPTG.
FIGURE 8: Ratio of Fluorescence yield of various concentrations of protein with increasing levels of iodide quencher. (△-△) $1.0 \times 10^{-6}$ M lac repressor, (●-●) $5.0 \times 10^{-7}$ M lac repressor, (■-■) $1.0 \times 10^{-7}$ M lac repressor.
RATIO: FLUORESCENCE YIELD vs.
QUENCHER CONCENTRATION

\[ \frac{F_0}{F} \]

- 0.1 \( \mu \)M Lac
- 0.5 \( \mu \)M Lac
- 1.0 \( \mu \)M Lac

\([KI] (M)\)
FIGURE 9: Ratio of fluorescence yield of various concentrations of protein in presence of inducer with increasing levels of iodide quencher. (△-△) 1.0x10^-6M lac repressor, 4mM IPTG (○-○) 5.0x10^-7M lac repressor, 2mM IPTG. (●-●) 1.0x10^-7M lac repressor, 0.4mM IPTG.
FIGURE 9
RATIO: FLUORESCENCE YIELD vs.
QUENCHER CONCENTRATION
LAC + IPTG

0.1 μM Lac
0.5 μM Lac
1.0 μM Lac

\[ \frac{F_0}{F} \]

[KI] (M)
# TABLE VII

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## KEY

- A: ±0.04
- B: ±0.04
- C: ±0.05
separate class of fluorophores in a system. This method is very complex and simpler graphical methods were used by producing a modified form of the classical Stern-Volmer relation. From the reciprocal of the ordinate intercept, the fraction of accessible tryptophans may be calculated and an effective quenching constant formulated. Figures 16 and 17 display this determination for the three different dilutions with and without saturating amounts of inducer, respectively. Results of the calculations are presented in Table VIII. Biomolecular collisional constants calculated from the effective quenching constants were formulated using the average phase and modulation lifetimes specific for each concentration of protein.

Theoretical Stern-Volmer plots display manipulable quenching constant effects interrelating inaccessibility of one fluorophore (or group of fluorophores) with respect to another. There is agreement between modified plots, theoretical and, within experimental error, standard plots. Elucidation of the results suggests that quenching in the presence of inducer is due exclusively to one group of tryptophyls. Quenching in the absence of inducer produces curves indicating a slight heterogeneity of the emission, but once again due maximally to a single group.
FIGURE 10: Ratio modulation lifetime at increasing levels of thiodide
quantum, (p-o) 1.0x10^-8 M thiodide, (e-o) 1.0x10^-8 M 1-ac
expression and 10 mM IPTG.
RATIO: MODULATION LIFETIME vs. QUENCHER CONCENTRATION

\[ \frac{\tau_0}{\tau} \]

[\text{KI}] (M)

1.0 \mu M Lac

1.0 \mu M Lac + IPTG
FIGURE 11: Ratio of phase lifetimes of lac repressor with and without inducer with increasing levels of iodide quencher. (○-○) \(1.0 \times 10^{-6}\)M lac repressor, (▲-▲) \(1.0 \times 10^{-6}\)M lac repressor, 4mM IPTG.
RATIO: PHASE LIFETIMES vs.
QUENCHER CONCENTRATION

1 μM Lac

1 μM Lac + ITPG

[KI] (M)

0

0.1

0.2
FIGURE 12: Ratio of modulation lifetimes of various concentrations of protein with increasing levels of iodide quencher. (△-△) 1.0x10^-5M lac repressor. ( ■-□ ) 5.0x10^-7M lac repressor, (○-○) 1.0x10^-7M lac repressor.
FIGURE 12

RATIO: MODULATION LIFETIMES vs. QUENCHER CONCENTRATION

\[ \frac{\tau_0}{\tau} \]

0.1 \( \mu \)M Lac

0.5 \( \mu \)M Lac

1.0 \( \mu \)M Lac

[KI] (M)
FIGURE 13: Ratio of phase lifetimes of various concentrations of protein with increasing levels of iodide quencher. (■•) 1.0x10^{-8} M lac repressor. (○•) 5.0x10^{-7} M lac repressor (●●) 1.0x10^{-7} M lac repressor.
FIGURE 13

RATIO: PHASE LIFETIMES vs. QUENCHER CONCENTRATION

$\frac{\tau_0}{\tau}$

$0.1 \mu M$ Lac
$0.5 \mu M$ Lac
$1.0 \mu M$ Lac

$[KI]$ (M)
FIGURE 14: Ratio of phase lifetimes at various concentrations of protein with increasing levels of iodide quencher. (○-○) 1.0x10^{-6}M lac repressor, 4mM IPTG. (△-△) 5.0x10^{-7}M lac repressor, 2mM IPTG, (□-□) 1.0x10^{-7}M lac repressor, 0.4mM IPTG.
RATIO: PHASE LIFETIMES vs.
QUEENCHER CONCENTRATION
LAC + IPTG

\[ \frac{\tau_0}{\tau} \]

0.1 \( \mu \text{M} \) Lac

0.5 \( \mu \text{M} \) Lac

1.0 \( \mu \text{M} \) Lac

[KI] (M)
FIGURE 15: Ratio of modulation lifetimes at various concentrations of protein with increasing levels of iodide quencher. (△-△) 1.0x10^{-6} M lac repressor, 4 mM IPTG; (■-■) 5.0x10^{-7} M lac repressor, 2 mM IPTG; (●-●) 1.0x10^{-7} M lac repressor, 0.4 mM IPTG.
RATIO: MODULATION LIFETIMES vs.
QUENCHER CONCENTRATION
LAC + IPTG

\[ \frac{\tau_0}{\tau} \]

\[ [KI] \text{ (M)} \]

0.1 \mu M Lac

0.5 \mu M Lac

1.0 \mu M Lac
FIGURE 16: Modified Stern-Volmer plot $F_o/\Delta vsw. 1/[I^-]$ for samples containing saturating amounts of IPTG. (■-■) $1.0 \times 10^{-8}$ M lac repressor, 4 mM IPTG; (Δ-Δ) $5.0 \times 10^{-7}$ M lac repressor, 2 mM IPTG; (○-○) $1.0 \times 10^{-7}$ M lac repressor, 0.4 mM IPTG.
FIGURE 17: Modified Stern-Volmer plot ($F/F_0$ vs $1/[	ext{I}^-]$) for three separate dilutions of lac repressor. (•-•) $1.0 \times 10^{-6}$ M lac repressor; (△-△) $5.0 \times 10^{-8}$ M lac repressor; (△-△) $1.0 \times 10^{-7}$ M lac repressor.
FIGURE 17

MODIFIED STERN-VOLMER PLOT LAC

\[ \frac{F_0}{\Delta F} \]

against

\[ \frac{1}{[I^-]} \text{ M}^{-1} \]

for different concentrations of \( I^- \):

- 1.0 \( \mu \text{M} \)
- 0.5 \( \mu \text{M} \)
- 0.1 \( \mu \text{M} \)
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**KEY**

Q - ±0.08

5 - ±0.05 x 10$^9$
FIGURE 18: Theoretical Stern-Volmer plots using $K_1 = 8$ and $K_2 = 1$ for fractions of $F_1$ shown.
IV. DISCUSSION

The fraction of accessible tryptophan residues did not change when IPTG saturated its binding site. The simplest explanation proposes quenching of 50% of the tryptophans due to relatively exposed to solvent in their native conformation. The other 50% lie in or near the inducer binding site so that iodide cannot diffuse toward and contact these tryptophans. The increase in biomolecular rate constant from 1.3 to $1.6 \times 10^9 M^{-1}\text{sec}^{-1}$ could then be indicative of dissociation of the tetramer into dimers or some conformational change allowing collision with quenchers more rapidly. These numbers have an order of magnitude like that of a diffusion controlled process, and the biomolecular rate constant is proportional to the increase in fluorescence lifetime; since most iodide molecules would be able to diffuse toward a fluorophore with a longer lifetime. The fact that in the absence of inducer the fraction of accessible tryptophans increases upon dilution and that in the presence of inducer the constant decreases, suggests a leaking of iodide into the inducer binding site which becomes more pronounced by dissociation of the subunits and only electrostatic interactions imposed by local charges producing specific quenching kinetics.

In the presence of inducer, intrinsic fluorescence emission spectrum shifts blue, and narrows (See Figure 7) due to the protection of trp 220 which lies closer near the IPTG binding site (O'Gorman et. al., 1977; Sommer et. al., 1970, Chakerian et. al., 1985). The increase in quenching which is portrayed in the Stern-Volmer plots in the presence and absence of inducer is due predominantly to the exposure of trp 201 to the polar solvent cage and the vast amounts of quencher present. Since quenchers such as $I^-$ generally are able to quench the more exposed red
tryptophanyl residues, there will be a tendency for the fluorescence to be blue shifted on the addition of quencher.

Figure 19 (C. Royer, 1985) shows a red shift in the center of mass upon dilution down to 7.5x10^{-8} M lac repressor tetramer in the presence of inducer. This red shift could be explained by the increase in fluorescence lifetime and subsequent increase in collisional encounters that accompanies dissociation of the subunits. Figure 20 (Royer et al.) further implicates dissociation as the observed process. Figure 20A depicts a red shift incurred by pressure increase that is dependent on concentration of protein. For both concentrations, the average energy of emission shifts towards lower energies by approximately 500 cm^{-1} at increased pressures. At lower pressures, there is a definite dependence on protein concentration with some apparent dissociation at atmospheric pressure upon dilution. If IPTG dissociation was the only transition, then the profiles would be superimposed, because the only difference between each curve is a 10-fold decrease in tetramer concentration. Therefore below 1.5kbar, the major transition followed is dissociation of the tetramer, while above that pressure IPTG dissociation dominates.

Figure 20B outlines the same equilibria except the dissociation of IPTG from the binding site is removed to lower pressures due to its lower concentration.

The same group proposed a dissociation constant for tetramer to be 2.0x10^{-23} for 5.0x10^{-7} M tetramer and 0.5mM IPTG. Repressor binds 4 moles inducer/mole tetramer non-cooperatively in absence of operator DNA with a K_d of 4x10^{-6} (O'Gorman et al., 1980b) while the K_d for IPTG binding to native monomer has not been measured.

Polarization studies by Royer et al. (1985) determined that lac repressor tetramer dissociates under pressure from tetramer to monomer.
However, due to pressure dependence of polarization upon the dissociation of IPTG, part of the calculated volume change could be attributed to it.

It is evident that these studies define a microenvironment for the red tryptophan as being polar and accessible to solvent and that of the other tryptophan to be apolar and buried. Treatment using a modified Stern-Volmer law is possible only in such cases where two heterogenous fluorophores contribute to the intrinsic fluorescence.

Stern-Volmer plots show that an increase in slope with tetramer dilution indicates an increase in quenching with concentration of I⁻. Moreover, the linearity of lifetime plots in the presence of IPTG opposed to the non linearity of these results in the absence of quencher infers that complexity of the quenching is due to trp 220 which lies buried in the inducer binding site.
FIGURE 19. Shift in the center of mass of fluorescence emission upon dilution. 1.0x10^{-6}M lac repressor, 4mM IPTG. (Royer, 1985)
FIGURE 20: Effect of pressure upon the center of mass of fluorescence emission of lac repressor. Center of spectral mass ($\bar{\gamma}$) was measured for repressor complexed with ligands in 0.1M Tris-HCl, 0.1M KCl, 10^{-4} M DTT, pH 7.5 (except as indicated). Excitation wavelength was 280nm.

A, (••) 1.0x10^{-6}M lac repressor, 4 mM IPTG, (••) 1.0x10^{-7}M lac repressor, 4mM IPTG. (▲▲) 1.0x10^{-6}M lac repressor, 4mM IPTG, pH 9.0.

B, (●●) 1.0x10^{-6}M lac repressor, 1mM IPTG, (●●) 1.0x10^{-7}M lac repressor, 1.0mM IPTG. (Royer et. al., 1985)
V. CONCLUSION

Modified Stern-Volmer calculations have a limited application in conformational study. Only in the case in which a protein contains one fluorophore accessible to quencher and one totally inaccessible, conditions assured by Lehrer (1971) in his original derivation, can such plots be interpreted in a straightforward manner. Only a few proteins, such as liver alcohol dehydrogenase, or lac repressor protein, may approach this condition. In general, proteins will contain more than two fluorophores, and totally inaccessible tryptophanyl residues may be rare (this of course will depend on the quencher used). Also, problems such as tyrosinate emission, nonexponentiality of the decay of individual tryptophanyl residues, inefficiency of the quencher, and interaction between the quencher and the protein may further complicate the quenching process (Kftink and Ghiron, 1980).

If the emission of systems is heterogenous due to the existence of different conformations of the fluorophore, or due to relaxation processes occurring during the lifetime of the excited state, the quenching curves will be more complicated than predicted by equation 5.

To relate these quenching studies with some functional conformation of the repressor in terms of DNA binding or for any structurally effective protein, more specific tests such as isotope exchange (Woodward, Ellis and Rosenberg, 1975; Englander, 1975; Woodward and Hilton, 1979), magnetic resonance relaxation (Sander et al., 1975; Visscher and Gurd, 1975; Campbell et al., 1975), and Raman spectroscopy (Brown et al., 19720, studies of the temperature factors in protein crystals (Frauenfelder, Petsko and Teernogloc, 1978; Sternberg, Grace and Phillips, 1979) and analysis of empirical energy functions for proteins (McCoyron, Wolynes and Karplus, 1977). Such
Experiments remain to be accomplished concerning the lac repressor and are beyond the scope of this presentation.

Proteins such as the cro repressor, repressor and catabolite activator protein have similar structures to the lac tetramer and are proposed to bind to operator or promoter DNA in the same manner as lac repressor (Pabo, ct. al., 1982; Steitz et. al., 1982; Anderson et. al. 1981).

Fluorescence quenching studies would be difficult to accomplish if any one of the above stipulations is not tractable. One could, however, combine certain residues with a probe such as DANSYL or ANS which could offer efficient polarization, differential phase lifetime, high pressure or atmospheric quenching studies to yield some correspondence between structure and function.
REFERENCES


Royer, C. A., Daly, T. J. and Matthews, K. S. (in preparation).


