Role of the C-Terminal Tail Region in the Self-Assembly of λ-Repressor†

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ABSTRACT: Acrylamide quenching of the tryptophan fluorescence of the λ-repressor at different protein concentrations indicates that one of the three tryptophan residues, W129, W142, and W230, undergoes a change in environment upon self-assembly, from dimer to associated species. Quenching data suggest that this tryptophan residue is inaccessible to low concentrations of acrylamide and is blue-shifted in the associated form. In the dimer, this tryptophan residue is highly accessible to acrylamide and is red-shifted. NBS oxidation, at protein concentrations which favor the associated form, showed that this tryptophan is also significantly protected from NBS oxidation. HPLC peptide mapping of NBS-oxidized λ-repressor, amino acid analysis, and sequencing indicate that the protected, blue-shifted tryptophan is tryptophan 230. A mutant repressor (F235C) was specifically labeled at Cys 235 with an environment-sensitive probe, acrylodan. The acrylodan fluorescence of the labeled F235C λ-repressor undergoes a significant blue-shift, accompanied by fluorescence enhancement, upon protein association. Along with other genetic evidence, these results suggest involvement of the C-terminal tail region in the self-assembly of the λ-repressor.

Regulation of gene expression not only involves correct recognition of DNA sequences by regulatory proteins but also involves homologous and heterologous protein−protein interaction (Guerente *et al.*, 1982; Kuldell & Hochschild, 1994; Adhya, 1989). It is becoming increasingly clear that protein−protein interactions among various regulatory proteins are crucial in the regulation of gene expression (Brenowitz *et al.*, 1991; Ishihama, 1992, 1993; Li *et al.*, 1994). The regulatory system of bacteriophage λ is a model system for the study of the roles of such protein−nucleic acid and protein−protein interactions in the regulation of gene expression.

Bacteriophage λ uses a genetic switch to choose between the lytic and lysogenic modes of development (Ptashne, 1986). λ-Repressor and three contiguous operator sites (O_R^1, O_R^2, and O_R^3) constituting O_R are important components of this genetic switch (Ptashne, 1986). λ-Repressor binds to these operator sites with alternate pairwise cooperativity (Johnson *et al.*, 1979; Senear *et al.*, 1986). Contacts between operator site bound protein dimers are responsible for binding cooperativity (Hochschild & Ptashne, 1986). Thus, an understanding of the structural nature and energetics of the protein−protein interaction is important for elucidating the nature of cooperativity and functioning of the genetic switch.

Because of the obvious importance of protein association in the regulation of gene expression, there is a resurgence of interest in the self-assembly of λ-repressor. Previous ultracentrifugation and electron microscopic studies (Brack & Pirotta, 1975; Chadwick *et al.*, 1970) have been supplemented by more detailed ultracentrifugation and fluorescence anisotropy studies (Laue *et al.*, 1993; Banik *et al.*, 1993). Studies of noncooperative mutants have also appeared (Beckett *et al.*, 1993), but for now they lack detailed structural characterization. Despite significant progress, we still do not have an overall idea about protein−protein contact sites in the λ-repressor.

Fluorescence quenching studies with collisional quenchers may provide information on the accessibilities of tryptophan residues in protein and under favorable circumstances on the change of accessibilities that may occur upon protein−protein and protein−nucleic acid association. λ-Repressor has three tryptophan residues that are located either in or close to the C-terminal domain (W129, W142, and W230) (Sauer & Anderegg, 1978). Since the C-terminal domain is primarily involved in all homologous protein−protein contacts in the λ-repressor (Pabo *et al.*, 1979), fluorescence quenching may be used to probe the change of tryptophan accessibilities that may occur upon protein association.

In the present paper, on the basis of fluorescence spectroscopic and quenching studies, we suggest that Trp 230 and the C-terminal tail region of the λ-repressor may be involved in the protein−protein contact that forms upon self-association.

EXPERIMENTAL PROCEDURES

Materials. Acrylamide, isopropyl β-D-thiogalactopyranoside (IPTG),[1] NBS, poly(ethylenimine), TPCK-trypsin, soybean trypsin inhibitor, PMSF, BSA, DTT, calf thymus DNA, DNase, and RNase were purchased from Sigma Chemical Co. (St. Louis, MO). Bacto-tryptone, Bacto-agar, and yeast

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; DTT, DL-dithiothreitol; NBS, *N*-bromosuccinimide; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate; NEM, *N*-ethylmaleimide; PM, pyrenylmaleimide; AC, acrylodan (6-acryloyl-2-dimethylaminonaphthalene); Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

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extract were purchased from Difco Laboratory (Detroit, MI). 6 N HCl for amino acid analysis was from Pierce Chemical Co. (Rockford, IL). β-Mercaptoethanol and glycerol were purchased from Aldrich Chemical Co. (Milwaukee, WI). All materials for amino acid analysis were from Waters Associates (Milford, MA). Pyrenylmaleimide and acrylodan were purchased from Molecular Probes Inc. (Eugene, OR). All other reagents were of analytical grade.

Repressor Isolation. λ-Repressor was isolated from a strain of *Escherichia coli* RR1 15 Δ *lac* Z carrying the plasmid pEA 305 which contains the wild-type cI gene under the control of the *tac* promoter. The details of the purification procedure were given in Saha *et al.* (1992). The native repressor concentration was determined using $E^{1\%}_{280} = 11.3$. The molar concentration of the repressor was always calculated in terms of the monomer subunit unless mentioned otherwise. For all studies, the repressor was dialyzed against 0.1 M phosphate buffer, pH 8.0, unless specifically mentioned otherwise.

Chemical Modification. For NBS oxidation, the λ-repressor was dialyzed overnight against 0.1 M phosphate buffer, pH 7.0, at 4 °C. NBS was used to modify the tryptophans of the λ-repressor. A freshly prepared high concentration of NBS in 0.1 M phosphate buffer, pH 7.0, was added to solutions of λ-repressor, and after 5 min, the extent of modification was monitored by quenching of the tryptophan fluorescence.

Acrylodan modification of F235C λ-repressor was done in 0.1 M phosphate buffer, pH 8.0; 1 μM λ-repressor, dialyzed against the same buffer, was incubated with 100 μM acrylodan (added as a DMF solution in such a way so that the final DMF concentration does not exceed 1%) for 30 min at 25 °C. The reaction was then quenched with 1 mM β-mercaptoethanol and dialyzed extensively against 0.1 M phosphate buffer, pH 8.0, to remove the excess reagent. The final incorporation was determined by using $\epsilon_{360} = 18\,000\ M^{-1}\ cm^{-1}$ (Haugland, 1992) and estimating the protein using the Bio-Rad protein assay (Bradford, 1976).

Fluorescence Methods. All fluorescence spectra were measured in a Hitachi F 3010 spectrofluorometer equipped with a computer for spectra addition and subtraction facility. The excitation and emission band-passes were 5 nm unless mentioned otherwise. Acrylamide quenching of tryptophan fluorescence was done by adding a freshly prepared high concentration of 3 times recrystallized acrylamide in 0.1 M potassium phosphate buffer, pH 8.0, to the protein solution. All fluorescence values were corrected for volume changes and the inner filter effect. Inner filter effect correction was made according to the equation:

$$F_{corr} = F_{obs}\ \text{antilog}[(A_{ex} + A_{em})/2]$$

where A_{ex} is the absorbance at the excitation wavelength, A_{em} is the absorbance at the emission wavelength, F_{corr} is the corrected fluorescence, and F_{obs} is the observed fluorescence. Absorbances at the excitation and emission wavelengths were measured before and after a titration, and intermediate values were obtained by interpolation. Appropriate blank spectra were always subtracted from the actual spectra. The protein concentration-dependent emission maximum shift experiments were conducted in 0.5 M acrylamide. The emission maximum of λ-repressor at various concentrations in 0.1 M phosphate buffer, pH 8.0, containing 0.5 M acrylamide was determined at 25, 30, and 37 °C. The scan speed was kept low at 15 nm/min, and the excitation and emission band-passes used were 5 and 1.5 nm, respectively.

Acrylodan-labeled F235C λ-repressor, at 0.5 μM, was titrated with increasing concentrations of wild-type λ-repressor in 0.1 M phosphate buffer, pH 8.0. The excitation wavelength was 390 nm, and the full emission spectrum at each repressor concentration was measured. The excitation and emission band-passes were 5 and 5 nm. Tempol quenching was also done in 0.1 M phosphate buffer, pH 8.0, at 0.5 μM AC-labeled F235C λ-repressor and at 0.5 μM AC-labeled F235C λ-repressor with 18 μM unlabeled wild-type λ-repressor. All the experiments with acrylodan-labeled λ-repressor were done at 25 °C.

Nonlinear Least-Squares Fit. Nonlinear least-squares fitting of the fluorescence data was done by a grid search procedure to the Stern−Volmer equation having three quenchable phases. Three K_{sv}s and two amplitudes were the independent parameters. They were varied systematically within a reasonable range of values. The parameters that gave the lowest χ^2 value were taken as the best-fit parameter.

Time-Resolved Emission. Fluorescence lifetimes of tryptophans of NBS-oxidized λ-repressor were determined in an Applied Photophysics, U.K., single photon counting apparatus using laser (Coherent Antares 76-YAG) as an excitation source. Excitation was done at 296 nm, and the emission was measured at 340 nm. The repetition rate was 0.95 MHz, and the response time was 60 ps. The data were fitted using an Applied Photophysics nonlinear least-squares fit program.

Peptide Mapping. A 30 μM sample of NBS-modified (quenched by addition of β-mercaptoethanol to a final concentration of 0.4 mM) and unmodified λ-repressor was dialyzed against 0.1 M phosphate buffer, pH 8.0, extensively. Solid guanidine hydrochloride was then added to a final concentration of 7 M. Repressor was then blocked at the sulfhydryl residues by addition of a 10-fold molar excess of *N*-ethylmaleimide. This was followed by dialysis against 0.2 M ammonium bicarbonate containing 2 M urea. TPCK-trypsin (in 1 mM HCl) was then added to a final ratio of 1:50 (w/w)(trypsin/repressor) and digested for 72 h. The reaction was quenched by the addition of a 1:1 molar ratio of trypsin to soybean trypsin inhibitor. The tryptic digest was analyzed on a μ-Bondapak C-18 reversed-phase column (Waters Associates) using a linear gradient of acetonitrile (0−60%) in 0.1% TFA for 60 min. The elution was monitored at either 295 or 220 nm. The fluorescence of the collected fractions was measured after dilution with 0.1 M potassium phosphate buffer, pH 8.0.

Amino Acid Analysis. All glasswares were washed with HCl followed by triple-distilled water and then were oven-dried. Gloves were always used during handling of the samples. The amino acid analysis was carried out by a precolumn derivative procedure using PITC as the derivatization reagent. The isolated peptide from the reverse-phase column was dried and subjected to hydrolysis at 110 °C using constant-boiling HCl (Pierce Chemical Co.), after the usual nitrogen flush. After hydrolysis, the samples were dried and derivatized with PITC in a 7:1:1:1 mixture of ethanol/triethylamine/water/PITC at 25 °C for 20 min. The samples were then dried thoroughly and analyzed on a Pico Tag column (Waters Associates) at 38 °C. The details of the

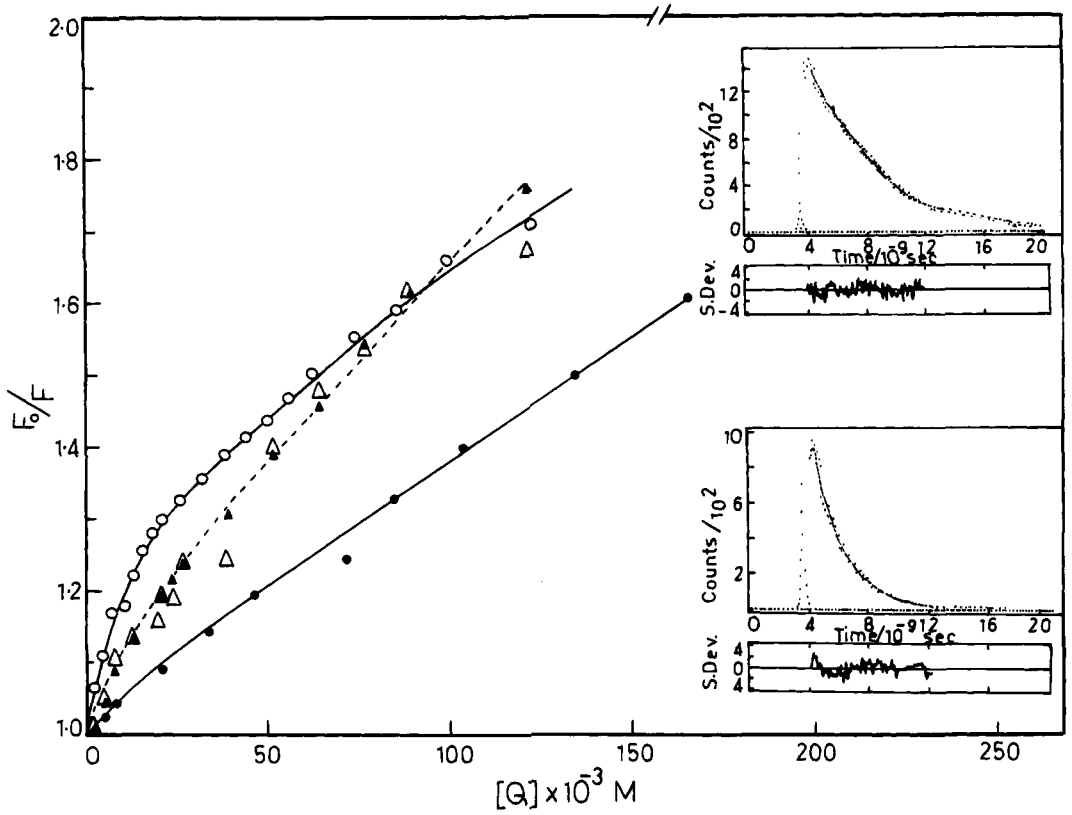

FIGURE 1: Stern–Volmer plot of acrylamide quenching of 10 μM (●) and 0.5 μM (○) λ-repressor and 15 μM (▲) and 0.5 μM (△) human serum albumin. The solution conditions are 0.1 M phosphate buffer, pH 8.0, 25 °C. The excitation wavelength was 295 nm, and the emission wavelength was 340 nm. The upper inset shows the time decay of tryptophan fluorescence of the λ-repressor. The lower inset shows the time decay of tryptophan fluorescence of the λ-repressor in the presence of 0.5 M acrylamide. The lines shown are the best-fit single-exponential curves. The excitation wavelength was 296 nm, and the emission wavelength was 340 nm. The solution conditions were same as above.

procedure are described in the Waters Associates amino acid analysis manual.

Peptide Sequencing. Sequencing was done on an Applied Biosystems 471A protein sequencer having an integrated isocratic HPLC system for PTH-AA detection. The peak collected from HPLC was directly loaded onto the Biobrene-treated membrane. The amino acids were identified from the elution position, and 19 PTH-AAs were used as standard. β-Lactoglobulin was sequenced for 20 cycles as a standard protein to establish the correctness of sequencing.

RESULTS

Acrylamide quenching of tryptophan fluorescence has been widely used to study tryptophan environments in proteins (Eftink & Ghiron, 1981). We have utilized acrylamide quenching to differentiate between emission spectra of different tryptophans at protein concentrations that favor the dimeric (0.5 μM) or the associated form (10 μM) (see Discussion for the nature of the associated species) (Banik *et al.*, 1993; Laue *et al.*, 1993). Figure 1 shows the Stern–Volmer plot of acrylamide quenching of the λ-repressor at 0.5 and 10 μM. The Stern–Volmer plots at two concentrations are different and show downward curvature, suggesting a protein concentration-dependent difference in the accessibilities of some tryptophans. As a control, we have done acrylamide quenching of human serum albumin at 15 and 0.5 μM protein concentrations. Human serum albumin is a known monomeric protein at both 15 and 0.5 μM. It is not expected to show a concentration-dependent Stern–Volmer plot. Figure 1 shows the Stern–Volmer plot of acrylamide quenching of human serum albumin at 15 and 0.5 μM under the same conditions as those of the λ-repressor. The two plots are almost identical within experimental error, suggesting that the observed difference in the Stern–Volmer plot of the λ-repressor is due to protein association. The insets show the time-resolved decay of fluorescence of λ-repressor at 10 μM concentration, in the absence and in the presence of 0.5 M acrylamide. Although the decay is probably multiexponential, the major part of the decay has been fitted to a single exponential to estimate the average lifetime. The average lifetime decreases dramatically from 6.5 ns in the absence of acrylamide to 2.1 ns in the presence of 0.5 M acrylamide. Such a large decrease in the lifetime in the presence of quencher and the lack of any upward curvature in the Stern–Volmer plot suggest dynamic quenching as the predominant mechanism (Lakowicz, 1986).

We have thus fitted the data to the Stern–Volmer equation with three different fluorescence components having three different Stern–Volmer constants. At 0.5 μM protein concentration, an attempt to fit the data to the Stern–Volmer equation with three quenchable phases resulted in convergence to two quenchable phases, with excellent χ^2 values. One of the phases has a high K_{sv} value of 81 ± 12 M^{-1}, and another has a low K_{sv} value of 3.2 ± 0.72 M^{-1}. The corresponding amplitudes were 0.31 ± 0.06 and 0.69 ± 0.06, respectively. The data at 10 μM, on the other hand, can be fitted to three quenchable phases with K_{sv} values of 50 ± 21, 3.12 ± 0.3, and 1.42 ± 0.17 M^{-1}, suggesting a change of accessibility for one tryptophan upon protein association.

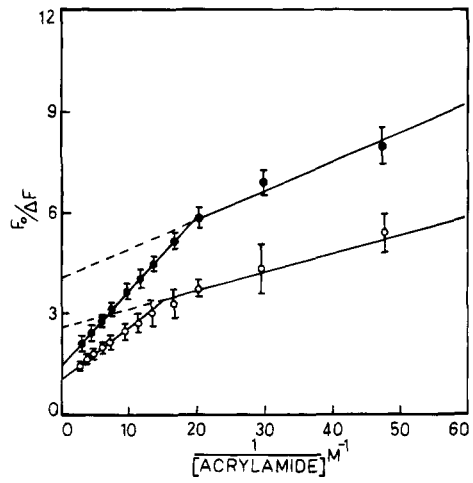

FIGURE 2: Lehrer plot of acrylamide quenching of 10 μM (●) and 0.5 μM (○) λ-repressor. The solution conditions were 0.1 M phosphate buffer, pH 8.0, 25 °C. The excitation and emission wavelengths were 295 and 340 nm, respectively. Each point is an average of four independent determinations.

The corresponding amplitudes are 0.11 ± 0.01, 0.63 ± 0.065, and 0.26 ± 0.07, respectively.

Plotting of these data according to Lehrer (1971) yields similar values. Figure 2 shows the Lehrer plot of the acrylamide quenching of the tryptophan fluorescence of λ-repressor at 0.5 and 10 μM. The initial part of the Lehrer plot when extrapolated cuts the y-axis at $F_o/\Delta F$ values of 2.5 and 4.0 for 0.5 and 10 μM, respectively. The corresponding K_{sv} values for the most quenchable phases were 45 and 38 M^{-1}, respectively. At higher acrylamide concentrations, a second linear phase of quenching cuts the y-axis at 1.4 on extrapolation at 10 μM repressor concentration. At 0.5 μM repressor concentration, the second linear phase cuts the y-axis at 1.1. Thus, approximately 40% of the fluorescence intensity at 0.5 μM is quenchable by low concentrations of acrylamide (K_{sv} = 45 M^{-1}), and the remaining 51% is quenchable with a much lower K_{sv} and 9% is nonquenchable. At 10 μM, approximately 25% of the fluorescence is quenchable at low acrylamide concentrations, and about 28% is not quenchable by low and moderate acrylamide concentrations. The rest (47%) is quenchable with K_{sv}s similar to the less quenchable phase at 0.5 μM. Since the aggregate fractional quantum yields at this wavelength are not protein concentration-dependent to a significant extent in this protein concentration range [i.e., the fluorescence value at 340 nm is approximately proportional to the protein concentration (data not shown)], the K_{sv} and intercept values suggest a highly quenchable tryptophan in the dimeric state becomes very inaccessible upon protein association (Table 1).

Figure 3 shows the shift of the emission maximum of the λ-repressor as a function of added acrylamide. At 10 μM protein concentration, i.e., a condition favoring the associated form, the wavelength maximum shifts from 340.0 to 335.8 nm at a final 0.5 M acrylamide concentration. At 0.5 μM protein concentration, i.e., which favors the dimeric form, the emission maximum shifts from 340.4 to 337.8 nm at a final 0.5 M acrylamide concentration. The difference spectra suggest that the highly quenchable part of the fluorescence originates from the emission of tryptophans having emission maxima around 345 nm (Banik *et al.*, 1992). At around 0.5 M acrylamide, the emission spectra would be dominated by spectra of the least quenchable tryptophan. This suggests that at 0.5 μM protein concentration the least quenchable tryptophan has an emission maximum around 338 nm, whereas at 10 μM protein concentration the emission maximum of the least quenchable tryptophan is around 335 nm. If one of the highly quenchable tryptophans at 0.5 μM shifts to a highly inaccessible environment at 10 μM, then the corresponding emission maximum shift is from around 345 to 335 nm.

If the self-assembly of λ-repressor leads to a blue-shift of the emission maximum of one tryptophan, then the shift of the emission maximum should occur at about the same range of protein concentrations as required for the self-assembly. Previously, it has been shown that self-assembly of the λ-repressor is temperature-dependent and higher temperature shifts the assembly profile to higher concentrations (Banik *et al.*, 1993). Thus, the emission maximum change profile should also shift to higher protein concentrations at higher temperatures. Figure 4 shows the shift of the emission maximum as a function of the λ-repressor concentration in 0.5 M acrylamide at three different temperatures. The transition, as monitored by the emission maximum shift, moves toward higher repressor concentrations with increasing temperatures. This trend is similar to the trend seen in tetramer−dimer dissociation when monitored by fluorescence anisotropy. The range of protein concentrations where the major part of the emission maximum shift occurs is not inconsistent with tetramer−dimer dissociation constants obtained by fluorescence anisotropy experiments, suggesting no significant effect of acrylamide on the protein association−dissociation equilibrium (Banik *et al.*, 1993).

Since self-assembly changes the environment of one tryptophan, its identity may reveal the region of protein that may be involved in self-assembly. NBS has been used extensively to oxidize tryptophan residues in proteins (Ramchandran & Witkop, 1967). The oxidation product has a changed absorption spectrum and is nonfluorescent (Peterman & Laidler, 1980). At low pH, NBS oxidation causes chain cleavage (Spande *et al.*, 1966). At around neutral pH, no significant cleavage occurs, although more NBS is required to oxidize tryptophan residues. Those tryptophan residues which are less accessible to collisional quenchers may also be protected from NBS modification.

Figure 5 shows a plot of F/F_o vs added NBS for the λ-repressor and the corresponding shift of the emission maximum at pH 7.0 at 10 and 0.5 μM protein concentrations. We have carried out the experiment at pH 7.0 because it requires less NBS to oxidize the tryptophans than at pH 8.0. The Lehrer plot of acrylamide quenching at pH 7.0 is very similar to that at pH 8.0 (data not shown), indicating similar tryptophan environments. At 10 μM protein concentrations, as increasing amounts of NBS are added, the fluorescence intensity decreases linearly up to about 50% of the initial value and then gradually flattens out to a final value of about 25%. The emission shifts up to about 335 nm and then remains constant. In contrast, at 0.5 μM protein concentration, the fluorescence intensity decrease is linear well past where the plot levels off at 10 μM protein concentration. This indicates that the leveling off phenomenon and its cause, the reduced reactivity of one tryptophan residue, are protein concentration-dependent. Since the most inaccessible tryptophan in the associated state has an emission maximum of approximately 335 nm, the most straightforward explanation

Table 1: Summary of Tryptophan Fluorescence Quenching Data[a]

	[λ-rep] (μM)	K_{sv1}	K_{sv2}	K_{sv3}	F1	F2	F3
by NLL	0.5	81 ± 12	3.2 ± 0.72		0.31 ± 0.06	0.69 ± 0.06	
	10	50 ± 21	3.12 ± 0.3	1.42 ± 0.17	0.11 ± 0.01	0.63 ± 0.06	0.26 ± 0.07
by Lehrer	0.5	45	<6	NQ	0.4	0.51	0.09
	10	38	<6	NQ	0.25	0.47	0.28

[a] All Stern–Volmer constants are in units of M^{-1}. NLL refers to the nonlinear least-squares method, and Lehrer refers to constants derived from the Lehrer plot. NQ refers to nonquenchable fluorescence.

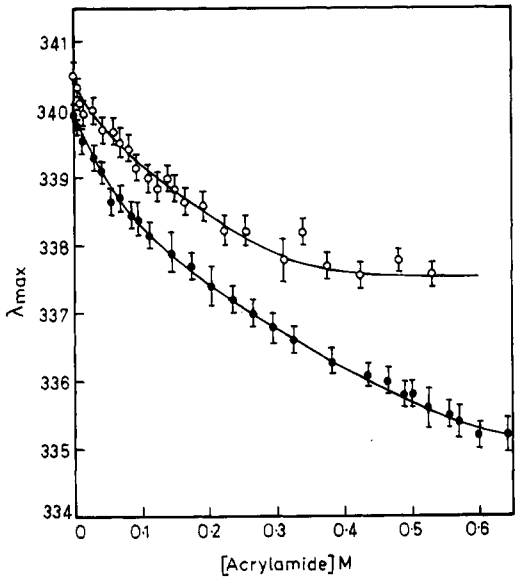

FIGURE 3: Plot of emission maximum versus acrylamide concentration at 10 μM (●) and 0.5 μM (○) λ-repressor. The solution conditions and excitation and emission wavelengths are same as in Figure 1. Each point is an average of four independent determinations.

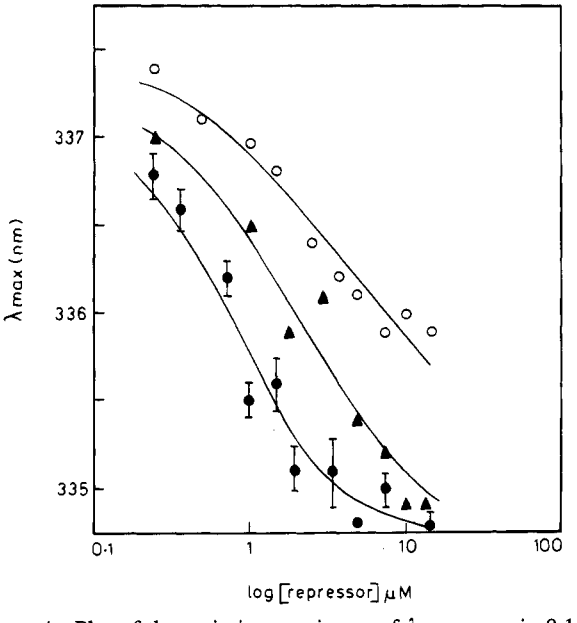

FIGURE 4: Plot of the emission maximum of λ-repressor in 0.1 M phosphate buffer, pH 8.0, containing 0.5 M acrylamide, as a function of λ-repressor concentration at 25 °C (●), 30 °C (▲), and 37 °C (○). The excitation and emission band-passes were 5 and 1.5 nm, respectively.

would be that the tryptophan most inaccessible to acrylamide is also much less accessible to NBS oxidation than the other two tryptophans in the associated state. Such a conclusion is contingent upon the fact that the protein does not unfold

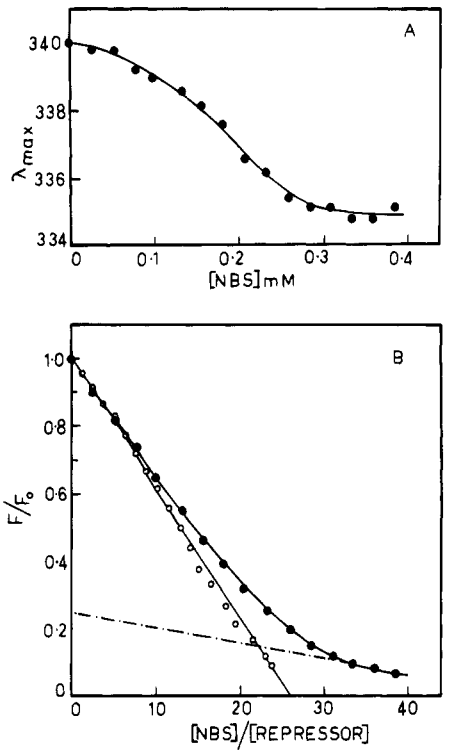

FIGURE 5: NBS modification of λ-repressor in 0.1 M phosphate buffer, pH 7.0. (A) Plot of *N*-bromosuccinimide concentration versus emission maximum at 10 μM repressor concentration. The excitation wavelength was 295 nm. (B) Plot of F/F_0 versus NBS/[Rep] at 10 μM (●) and 0.5 μM (○) repressor concentrations. The excitation and emission wavelengths were 295 and 340 nm, respectively.

upon NBS modification. We have studied far-UV CD, size-exclusion HPLC, sulfhydryl reactivity, and residual tryptophan fluorescence quenching by acrylamide in the NBS-modified repressor. All these parameters suggest that the NBS-modified repressor retains its structural integrity and native fold (data not shown).

The identity of the least accessible tryptophan in the associated state was judged from peptide mapping. The denatured and blocked λ-repressor was exhaustively digested with trypsin, and the digest was analyzed by reversed-phase HPLC. Figure 6A shows the A_{220} profile of a trypsin digest of unmodified λ-repressor. About 17 peaks can be seen, consistent with the theoretically expected 17 tripeptides or higher peptides. When the same chromatogram was monitored at 295 nm (see Figure 6B), only three peaks are seen. At 295 nm, only tryptophan-containing peptides absorb, and only three peaks are expected (we will refer to them as peaks a, b, and c in the order of elution). Analysis of the tryptic digest of NBS-modified λ-repressor is shown in Figure 6C. Although there are few other changes, peaks a and c are clearly missing, and peak b is at the same position as before.

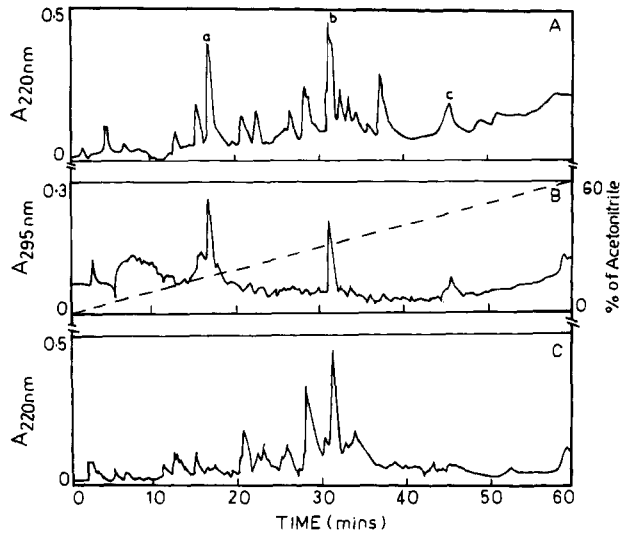

FIGURE 6: Elution profiles of tryptic digests of native and NBS-modified λ-repressor on a μ-Bondapak C-18 reversed-phase column. (A) Native λ-repressor monitored at 220 nm. (B) Native λ-repressor monitored at 295 nm. (C) NBS-modified λ-repressor monitored at 220 nm. The gradient used was 0−60% acetonitrile in 0.1% TFA in 0−60 min.

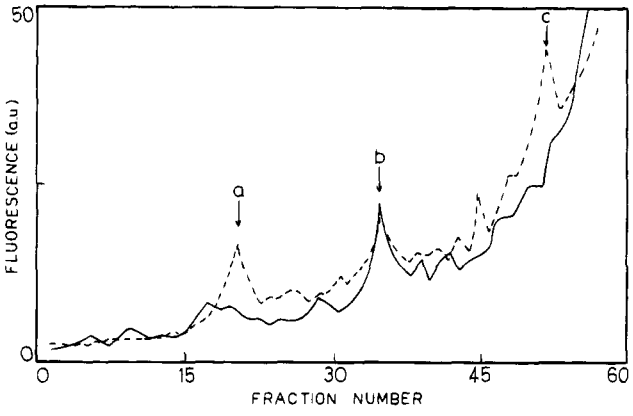

FIGURE 7: Fluorescence profile of the tryptic digest of native (- - -) and NBS-modified (—) λ-repressor. Arrows indicate the expected elution profile of peaks a, b, and c. The elution conditions are same as in Figure 6. The eluate was collected in 1 mL fractions and diluted by adding 0.1 M potassium phosphate buffer, pH 8.0, and the fluorescence was measured at 340 nm (excitation 295 nm).

This indicates that the tryptophan in the peptide corresponding to peak b remains unmodified after NBS modification.

Figure 7 shows the tryptophan fluorescence of collected fractions of eluant from the C-18 reversed-phase column. The tryptic digest of unmodified λ-repressor shows three clear fluorescence peaks at the expected elution positions of the three tryptophan-containing peptides (peaks a, b, and c). In the tryptic digest of the NBS-modified λ-repressor, the fluorescence intensity corresponding to peaks a and c is much reduced and peak b is unaffected. This clearly indicates that the tryptophan that is protected from NBS oxidation is contained in the peptide which corresponds to peak b.

Fractions containing peptide b were collected and subjected to amino acid analysis (Heinrikson & Meredith, 1984). The result is shown in Table 2. The amino acid analysis corresponds to the expected amino acid composition of tryptic peptide 225−236. The identity of this peak is also supported by three cycles of sequencing. The first cycle showed the expected amino acid valine although there were two other contaminating peaks, including leucine. The

Table 2: Amino Acid Analysis of Peak b

amino acid	expected composition	obtained composition[a]
Glu	3.0	2.5
Ser	1.0	0.8
Gly	1.0	1.2
Thr	1.0	1.1
Ala	1.0	—[b]
Pro	1.0	1.0
Val	1.0	1.0
Ile	1.0	0.7
Leu		1.0[c]
Phe	1.0	0.75

[a] Other amino acid values were Arg, 0.22; and Lys, 0.3; and were neglected. [b] Alanine could not be determined because of the presence of a large peak at the same position in the blank as well as in the analysis. [c] Probably contamination; sources unknown.

second and third cycles were much cleaner and as expected were isoleucine and alanine. We have also labeled the sulfhydryl groups of repressor with PM instead of NEM and subjected it to trypsin digestion and peptide mapping. Out of the three tryptophan-containing peptides, only Trp 142-containing peptide contains a sulfhydryl group. Since PM is more hydrophobic than NEM, only the W142-containing peptide is expected to elute later. Such an analyis showed that the elution of peak c is dealyed by 6 min upon modification with PM while the elution times of peaks a and b remained unchanged. This identifies peak c as peptide 136−183. The identities of the peaks are also supported by analysis of the elution order from the reverse-phase column. Browne *et al.* (1982) have shown that elution of peptide from the μ-Bondapak C-18 column in 0.1% TFA and an acetonitrile gradient can be predicted from the amino acid composition with a high degree of accuracy. The predicted elution order of the tryptophan-containing tryptic peptides of the λ-repressor is 129−134, 225−236, and 136−183 (Sauer & Anderegg, 1978), which agrees well with amino acid analysis, PM labeling, and sequencing of the peak B peptide.

In order to reinforce the conclusions drawn from the acrylamide quenching experiments and peptide mapping, we have constructed a F235C λ-repressor mutant by a PCR-directed mutagenesis procedure (Horton & Pease, 1991). Two oligonucleotides, one overlapping with the internal *Hind*III site and another near the C-terminus and bearing the desired mutation and creating a new restriction site in the noncoding region, were used as PCR primers. After PCR, the fragment was cut with appropriate restriction enzymes and cloned into the original plasmid. Plasmid carrying this mutant cI gene was able to confer immunity to infection by phage λ. The isolated protein shows a far-UV CD spectrum identical to that of the wild-type repressor, and the dimer−monomer dissociation constant is similar to that of the wild-type repressor, suggesting no significant loss of biological activity [the details of the mutagenesis procedure and properties of the mutant will be described elsewhere (Bandyopadhyay *et al.*, unpublished observation)]. The sulfhydryls in the native λ-repressor (180, 215, and 219) are unreactive to DTNB even at 6 M urea concentration (Banik *et al.*, 1992). A similar reactivity pattern is seen in the mutant protein, with the exception of C235 which is reactive (data not shown). This mutant protein was labeled at C235 by acrylodan (AC). The emission spectrum of the AC-labeled repressor is shown in Figure 8. The emission maximum is at 510 nm at 0.5 μM,

suggesting a highly polar environment for the AC-C235 in the dimeric repressor (Haugland, 1992). Addition of wild-type λ-repressor causes fluorescence enhancement and a concomitant emission maximum shift to the blue. The lower inset shows the enhancement of fluorescence intensity and the emission maxima shift as a function of unlabeled λ-repressor added. Both the values tend to level off beyond 15 μM protein concentration, approaching values of 504 nm and 1.7-fold for emission maximum and fluorescence enhancement, respectively. The range of protein concentrations where these changes occur is consistent with the range of protein concentrations where self-assembly has been reported to occur (Banik *et al.*, 1993; Laue *et al.*, 1993; Brack & Pirotta, 1975). The upper inset shows Tempol quenching of acrylodan fluorescence at 0.5 and 18.5 μM protein concentration. At 0.5 μM, the K_{sv} is 6 mM^{-1}, which is reduced significantly to 3.57 mM^{-1} at 18.5 μM repressor concentration, suggesting decreased accessibility of acrylodan-C235 upon protein association.

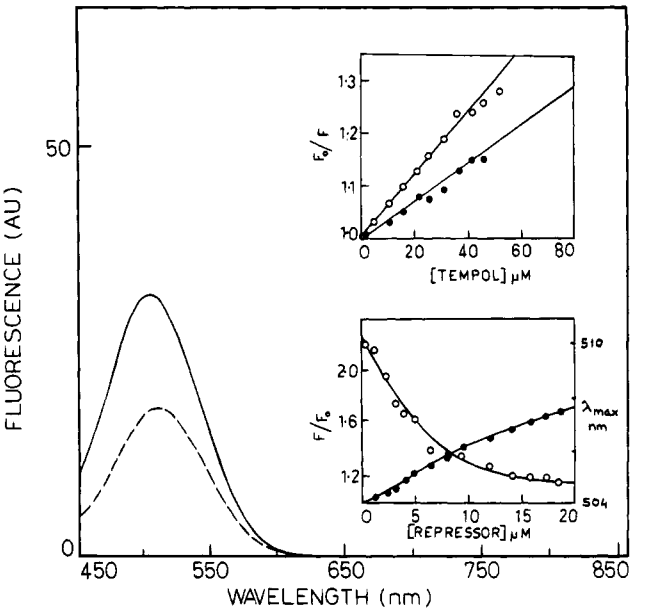

FIGURE 8: Emission spectrum of 0.5 μM AC-labeled F235C λ-repressor in 0.1 phosphate buffer, pH 8.0 at 25 °C, as a function of added wild-type λ-repressor. (- - -) No addition; (—) 18.5 μM. The excitation wavelength was 390 nm. The lower inset shows the shift of the emission maximum (○) and fluorescence enhancement (●) as a function of added wild-type λ-repressor. The upper inset shows the Stern–Volmer plot of Tempol quenching of AC fluorescence at 0.5 μM AC-labeled F235C λ-repressor, in the presence of 18 μM wild-type λ-repressor (●) and in its absence (○). The excitation wavelength was 390 nm, and the emission wavelength was 510 nm. The buffer conditions were the same as above.

DISCUSSION

Identification of protein–protein contact regions of regulatory proteins is of interest, because of its functional significance. In the absence of a crystal structure, two approaches may yield direct information. One is to identify and study mutants that are defective in protein association; the other is to use spectroscopic techniques. Combining information from both these techniques may yield a clearer picture. Protein association in λ-repressor occurs in the low micromolar range, making it ideal for fluorescence spectroscopic investigation. We have used both internal and external fluorophores to probe the nature of residues involved in protein association.

The tryptophans of the λ-repressor are accessible to acrylamide to different degrees. The spectrum of one tryptophan shifts almost 10 nm to the blue upon protein association. This shift is accompanied by a drastic change in the accessibility of this tryptophan toward collisional quenchers. Such dramatic changes of the emission maximum and accessibility are characteristics of the shift from the exposed state to subunit interfaces. We have shown that NBS causes rapid oxidation of two tryptophans in the associated state, while leaving one residue mostly unaffected. The emission maximum of this latter residue strongly suggests that this is the most inaccessible and blue-shifted residue in the associated state.

Peptide mapping, amino acid analysis, and sequencing have shown that the most blue-shifted and inaccessible tryptophan is W230. The emission spectrum of this tryptophan is significantly changed upon protein association. This indicates a significant change of the environment of W230 upon protein association. In addition, AC-labeled F235C λ-repressor (labeled at residue 235) shows a blue shift in the emission spectrum accompanied by decreased accessibility upon protein association, suggesting involvement of the tail region of the λ-repressor in the self-assembly.

Although studies from several groups agree that self-assembly of λ-repressor occurs in the range of 0.5–40 μM protein concentration (Banik *et al.*, 1993; Laue *et al.*, 1993; Brack & Pirotta, 1975), the degree of coupling between the dimer to tetramer transition and the tetramer to octamer transition appears to be different in different studies. These studies were, however, done under different buffer conditions. In the study where strongly coupled tetramer and octamer formation was observed, solution conditions included millimolar concentrations of Mg^{2+} and Ca^{2+} ions (Senear *et al.*, 1993; Laue *et al.*, 1993). In 0.1 M phosphate buffer, pH 8.0, we had, however, observed tetramer as the predominant species in the 10–20 μM protein concentration range (Banik *et al.*, 1993). Further studies on the assembly of λ-repressor under both these buffer conditions by light scattering, large-zone gel filtration, and fluorescence anisotropy suggest that while under the conditions of Laue *et al.*, (1993) the two steps are strongly coupled, under our conditions the two steps are uncoupled with significant octamer formation occurring above 20 μM (Bandyopadhyay *et al.*, unpublished observation). Thus, we believe the change in W230 and C235 environment that occurs upon protein association is a result of dimer–dimer association to tetramer.

A noncooperative mutant of λ-repressor has been isolated and mapped at position 228 (serine to asparagine) (Hochschild & Ptashne, 1989). A recent report suggests that it may be defective in dimerization as well as in higher order contacts (Beckett *et al.*, 1993). Other cI^- phenotype mutants have also been mapped in the C-terminal tail part of the C-terminal domain (Lieb *et al.*, 1986). Recent work by Whipple *et al.* (1994) has identified an additional noncooperative mutant, T234K. These data, together with the change of environment of W230 and C235 upon tetramer formation, reinforce the idea of involvement of the C-terminal tail part of the C-terminal domain in self-assembly and, perhaps, in cooperative contact.

The question, whether self-assembly and cooperative contacts are identical, partially overlapping, or mutually

exclusive, requires further exploration. Burz and Ackers (1994) have reported mutants that do not self-assemble but show native-like binding cooperativity (notably S228N) and *vice versa*, suggesting that protein−protein contact in self-assembly and cooperativity may not be identical. Several of their mutants, however, are defective in both self-assembly and cooperativity (notably S228R), indicating that protein surfaces involved in cooperative and self-assembly contacts may be partially overlapping. Isolation of a noncooperative mutant at position 147 (Beckett *et al.*, 1993) suggests that dimer−dimer contact may involve other parts of the C-terminal domain as well, although the lack of a crystal structure does not allow us to estimate its proximity to the C-terminal tail part. It is noteworthy that dimer−dimer contact of the lac-repressor and the homologous 434-repressor may also be mediated through the C-terminal region (Nichols *et al.*, 1993; Brenowitz *et al.*, 1991; Carlson & Koudelka, 1993).

In summary, we have shown that tryptophan 230 is highly solvent-exposed in the dimeric state, but becomes significantly hidden in the associated state. A fluorescent probe attached to Cys 235 of a mutant F235C repressor also shows reduced accessibility upon protein association. These data, along with other mutant data, suggest the involvement of the C-terminal tail region of the λ-repressor in self-assembly.

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