THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 9, Issue of March 25, pp. 5862-5867, 1992
Printed in U.S.A.

An Operator-induced Conformational Change in the C-terminal Domain of the λ Repressor*

(Received for publication, March 27, 1991)

Rina Saha‡, Utpal Banik§, Sumita Bandopadhyay§, Nitai C. Mandal‡, Bhabatarak Bhattacharyya‡¶, and Siddhartha Roy§¶

From the §Department of Biophysics and the ‡Department of Biochemistry, Bose Institute, P 1/12 C.I.T. Scheme VII M, Calcutta, India

4,4′-bis(1-anilino-8-naphthalenesulfonic acid (Bis-ANS), an environment-sensitive fluorescent probe for hydrophobic region of proteins, binds specifically to the C-terminal domain of λ repressor. The binding is characterized by positive cooperativity, the magnitude of which is dependent on protein concentration in the concentration range where dimeric repressor aggregates to a tetramer. In this range, positive cooperativity becomes more pronounced at higher protein concentrations. This suggests a preferential binding of Bis-ANS to the dimeric form of the repressor. Binding of single operator O_R1 to the N-terminal domain of the repressor causes enhancement of fluorescence of the C-terminal domain bound Bis-ANS. The binding of single operator O_R1 also leads to quenching of fluorescence of tryptophan residues, all of which are located in the hinge or the C-terminal domain. Thus two different fluorescent probes indicate an operator-induced conformational change which affects the C-terminal domain. The significance of this conformational change with respect to the function of λ repressor has been discussed.

Gene expression in prokaryotes and eukaryotes is often regulated at the transcriptional level. These regulatory mechanisms are often complex and require interplay of several protein factors, the structural and energetic aspects of which are not well understood. Bacteriophage λ uses a complex genetic switching system that comprises a number of operator sites and several proteins of which the repressor is the key component. The phage uses this regulatory circuit to choose between the lytic and lysogenic modes of its development (Herskowitz and Hagen, 1980). The affinity of the repressor for the three operators O_R1, O_R2, and O_R3 decreases in that order. The repressor binds cooperatively to these operator sequences to block rightward transcription from p_R and also acts as an activator of transcription from an adjacent promoter P_{rm} in the opposite direction. These functions are thought to be mediated by homologous and heterologous protein-protein contacts (Johnson *et al.*, 1979; Hochschild and Ptashne, 1988).

λ repressor consists of 236 amino acids, and its active form is a dimer. The protein is reported to have two functional domains. The N-terminal domain, spanning from 1 to 92 amino acid residues is involved in DNA binding (Sauer *et al.*, 1979) and possibly also in interaction with RNA polymerase (Guerente *et al.*, 1982; Hawley and McClure, 1983), while the C-terminal domain spanning from 132 to 236 residues, is involved in dimerization (Pabo *et al.*, 1979). Recently, it has been proposed that the cooperative binding of λ repressor to operators separated by several turns of DNA involves contact between two bound repressor molecules through its C-terminal domain and looping out of the intervening DNA sequence (Griffith *et al.*, 1986; Hochschild and Ptashne, 1986). Although the structure of the N-terminal fragment and its complex with operator DNA is known, structure of hinge region or the C-terminal domain is not known (Jordan and Pabo, 1988).

We have chosen to study dynamics and conformational change in λ repressor, particularly involving C-terminal domain and the hinge region, during its interaction with the operator DNA by fluorescence spectroscopy. All three tryptophans in the repressor are situated in the C-terminal domain and may be used as fluorescent probes for any operator induced global conformational change in the protein. More convincing evidence, however, may be obtained with a suitable C-terminal domain-specific external probe. Cysteines and lysines are most often used as attachment points for external fluorophores. The cysteine residues of the repressor, although situated in the C-terminal domain, are totally unreactive towards sulfhydryl reagents and hence are unsuitable for covalent labeling with external fluorophores. The amino group specific fluorescent reagents label the N-terminal amino acid first and hence are unsuitable for insertion of covalent fluorescent probes into the C-terminal domain.[1] Thus, a specific noncovalent fluorescent probe for the C-terminal domain is needed to study conformational changes during interaction with DNA, as conventional methods of inserting fluorescent probes into the C-terminal domain are unapplicable.

Bis-ANS,[2] in many ways, is an excellent fluorescent probe for this purpose. Its quantum yield and emission maximum are sensitive to environmental polarity (Rosen and Weber, 1969). It binds tightly to specific sites in many proteins (Wu and Wu, 1978; Lawson and York, 1987). It has affinity for accessible hydrophobic regions (Prasad *et al.*, 1986), which are characteristic of many protein-protein contact sites. In addition, its absorption and emission spectra are well separated from the absorption spectrum of DNA. In this article we have explored Bis-ANS and internal tryptophans as probes

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom all correspondence should be addressed.

[1] U. Banik and S. Roy, unpublished observations.

[2] The abbreviations used are: Bis-ANS, 4,4′-bis(1-anilino-8-naphthalenesulfonic acid); BSA, bovine serum albumin; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; IPTG, β-isopropyl thiogalactoside; FITC, fluorescein-5-isothiocyanate.

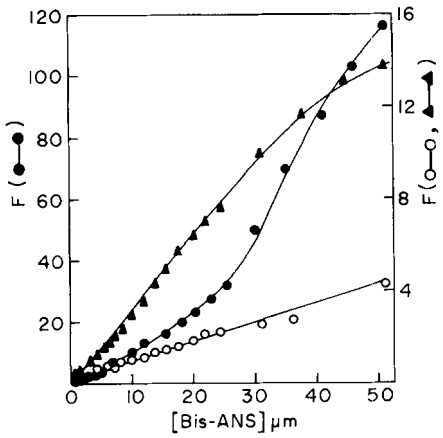

FIG. 1. **Titration of λ repressor with Bis-ANS.** λ repressor concentration was 1 μM (▲) and 25 μM (●) in 0.1 M potassium phosphate buffer, pH 8.0, at 25 °C. Control (○) containing same solution but no λ repressor is also shown. Emission wavelength was at 500 nm, and excitation wavelength was at 455 nm.

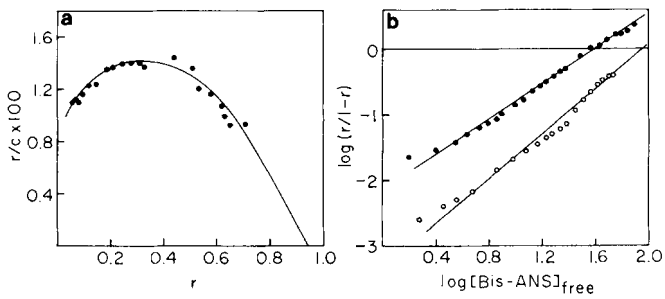

FIG. 2. *a*, Scatchard plot of Bis-ANS binding to λ repressor. Repressor concentration was 1 μM in 0.1 M potassium phosphate buffer, pH 8.0, at 25 °C. Bis-ANS binding to λ repressor was calculated as described under "Methods." *b*, Hill plot of Bis-ANS binding to λ repressor. *Solid circles* (●) are the Hill plot of the same data as in *panel a*. *Open circles* (○) are Hill plot with 25 μM λ repressor. Excitation wavelength was 455 nm, and emission wavelength was 500 nm.

to monitor operator-induced conformational change in λ repressor.

EXPERIMENTAL PROCEDURES[3]

RESULTS

Bis-ANS Binding to λ Repressor—Fig. 1 illustrates the effect of adding increasing concentrations of Bis-ANS to two different repressor concentrations (1 and 25 μM). From the figure it is evident that in both the cases, Bis-ANS binds to the repressor leading to enhanced fluorescence, but the nature of binding is somewhat different. Binding of Bis-ANS at 1 μM repressor concentration is not pronouncedly sigmoidal. On the other hand, binding of Bis-ANS to repressor at 25 μM concentration is pronouncedly sigmoidal, suggesting cooperative interactions.

Fig. 2 (*a* and *b*) shows Scatchard and Hill plots for the binding of Bis-ANS to λ repressor. The plots were obtained from titration of fixed concentration of the repressor with increasing concentrations of Bis-ANS (direct titrations), using fluorescence enhancement values (Q) obtained from a reverse titration plot (Mas and Colman, 1985). The convex upward curve (Fig. 2*a*) characterized by a well pronounced maximum in the Scatchard plot shows positive cooperativity of ligand binding (Cantor and Schimmel, 1980). Only the plot for 1 μM λ repressor is shown. Precipitation occurs at Bis-ANS concentrations beyond that shown in Fig. 2*b*. The intercept on the r-axis gave a value of 0.95/subunit as the number of binding sites. Fig. 2*b* shows Hill plot for 1 and 25 μM repressor. The half-saturating ligand concentration is simply the apparent dissociation constant for the ligand macromolecule interaction. In this case, the apparent K_d values at 1 and 25 μM repressor are 39 and 89 μM, respectively. Apparent K_d at three other repressor concentrations were also determined from the respective Hill plots. The values are given in Table I. The slope of the Hill plot gives the Hill coefficient (n_H) which may be taken as a qualitative indicator of magnitude of cooperative effect. Table I shows the dependence of apparent dissociation constant and Hill coefficient at several repressor concentrations. It is quite evident from the table that both Hill coefficient and apparent dissociation constant increase significantly at higher repressor concentrations. The dimeric repressor is known to associate in this protein concentration range to tetramers (Brack and Pirotta, 1975). Thus, such protein concentration-dependent increase of Hill coefficient and apparent binding constants may be attributed to preferential binding of Bis-ANS to the dimeric form of the repressor. In order to further explore the validity of the above model we have used nonlinear least square fitting of the experimental data to the model in which Bis-ANS binds to a single site on repressor monomer preferentially in the dimeric form. We have attempted to fit the experimental data at three different repressor concentrations, *e.g.* 1, 4, and 12 μM. The quality of fit is good, as judged by the low chi-square values. The Bis-ANS dissociation constant from the repressor dimer at 1, 4, and 12 μM repressor concentrations are 29, 34, and 29 μM, respectively, indicating good agreement (data not shown).

Localization of the Bis-ANS Binding Site—Two domains and the hinge region of the λ repressor differ in protease susceptibility, melting temperature (Pabo *et al.*, 1979), and urea denaturation profile.[1] Thus, we have used controlled protease digestion, partial melting, and partial urea-induced denaturation to localize the Bis-ANS binding site on the intact repressor. Fig. 3 illustrates the effect of trypsin digestion of the repressor on the fluorescence of repressor-Bis-ANS complex. The tryptophan fluorescence decayed rapidly initially and then leveled off at approximately 70% of initial fluorescence intensity. The Bis-ANS fluorescence, however, increased rapidly to 150% of initial value during the first hour and then declined to a value of approximately 95% of initial value. The nature of the two curves suggests two consecutive phases of reaction. During the first phase, which lasted about 3 h, tryptophan fluorescence decreased with a simultaneous increase of Bis-ANS fluorescence, followed by the second phase in which Bis-ANS fluorescence declined without any further significant change in tryptophan fluorescence. The corresponding gel electrophoresis data show that initially trypsin cleaved the repressor producing three fragments fol-

TABLE I

Dependence of apparent dissociation constant (K_d) and Hill coefficient (n_H) on repressor concentration

[Repressor]	K_d	n_H
μM	μM	
0.5	38	1.35
1.0	39	1.33
4.0	52.5	1.38
12.0	55	1.58
25	89	1.7

[3] "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

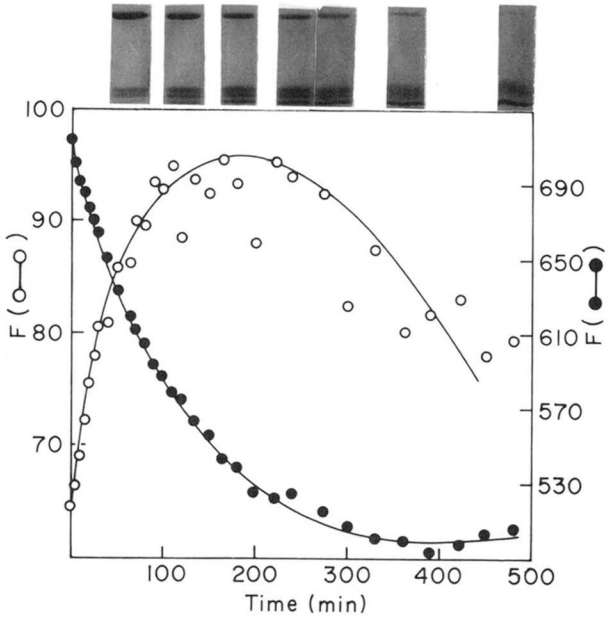

FIG. 3. **Time course of trypsin digestion of λ repressor.** A solution containing 11 μM repressor and 2 μM Bis-ANS was incubated with trypsin in a ratio of 50:1 (w/w) in 0.1 M potassium phosphate buffer, pH 8.0. *Open circles* (○) indicate change in fluorescence intensity of bound Bis-ANS; excitation was at 385 nm, and emission was at 500 nm. *Solid circles* (●) indicate change in tryptophan fluorescence, for which excitation was at 290 nm and emission was at 340 nm. The trypsin digestion was stopped at various times indicated, and the products were analyzed by electrophoresis on a 13.5% sodium dodecyl sulfate-polyacrylamide gel.

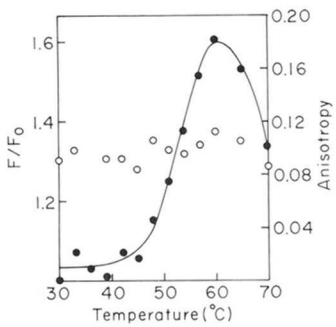

FIG. 4. **Temperature dependence of Bis-ANS binding to λ repressor.** The reaction mixture contained 4 μM repressor and 2 μM Bis-ANS in 0.1 M potassium phosphate, pH 8.0. *Solid circles* (●) indicate change of fluorescence with increase of temperature. *Open circles* (○) indicate change in anisotropy of Bis-ANS as a function of temperature. Excitation was at 385 nm, and emission was at 500 nm.

lowed by the second phase, which led to further degradation of the larger fragment. Most proteases are known to cut in the hinge region around amino acid residues 92–100. This produces a C-terminal/hinge fragment and a smaller N-terminal fragment. Subsequent cuts often occur around amino acid residues 120–132, producing a smaller C-terminal fragment. Thus the initial phase can be interpreted as a cut at around amino acid residues 92–100, followed by a subsequent cut at around amino acid residues 120–132. No major loss of Bis-ANS fluorescence under this conditions suggests that Bis-ANS does not bind exclusively in the hinge region.

Fig. 4 shows the effect of temperature on binding of Bis-ANS to the repressor. Bis-ANS fluorescence increased with increase of temperature. It continued to increase well past the melting temperature of N-terminal domain (51 °C). Bis-ANS fluorescence started to decrease above 60 °C. The anisotropy, however, did not change significantly with temperature. This indicates that Bis-ANS does not bind in the N-terminal domain. 5 M urea causes the N-terminal domain of the λ repressor to denature completely without causing major denaturation of the C-terminal domain.[1] Bis-ANS binding to λ repressor was carried out in several urea concentrations, and the results are shown in Table II. The results show that even at 5 M urea, where the N-terminal domain was completely denatured, significant Bis-ANS binding was retained, suggesting that Bis-ANS binds to the C-terminal domain.

In order to confirm the localization of Bis-ANS binding site we have measured the distance of bound Bis-ANS from the N terminus by fluorescence energy transfer. N-terminal amino group of repressor was labeled with FITC under controlled conditions. A similar reaction pattern has been observed in ribonuclease A (Garel, 1976). Bis-ANS and fluorescein form a suitable donor-acceptor pair for singlet-singlet fluorescence energy transfer, because there is considerable overlap of emission spectra of Bis-ANS and absorption spectra of fluorescein. The results are shown in Table III. The upper limit of the transfer efficiencies and lower limit of the corresponding distances were calculated according to the procedure described under "Methods." The transfer efficiency and corresponding distances are determined at four different Bis-ANS concentrations and is shown in Table III. The average distance between the donor and the acceptor was found to be 56 Å. Electron microscopic study of the repressor by Brack and Pirotta (1978) reported a size of 45 Å × 65 Å for the repressor dimer. Assuming that the N-terminal of the repressor is at one end of such an elongated molecule, a distance of at least 56 Å from the N terminus would place the Bis-ANS binding site on the C-terminal domain.

In order to demonstrate convincingly that the binding of Bis-ANS occurs at the C-terminal domain, we have studied the binding of Bis-ANS to the N- and C-terminal fragments (1–92 and 93–236, respectively). The fragment 93–236 was chosen, because as shown in the previous trypsin digestion pattern, complete digestion of the hinge leads to somewhat weaker Bis-ANS binding, which could be difficult to detect. Fig. 5 shows the titration of whole repressor, N-terminal (1–

TABLE II

Urea concentration dependence of Bis-ANS binding to the λ repressor

Urea concentration	F/F_0[a]
M	
0	5.3
3.4	4.6
5.0	3.5
6.0	2.0
8.5	1.3

[a] F is the fluorescence of Bis-ANS in the presence of λ repressor. F_0 is the fluorescence of Bis-ANS in the absence of λ repressor.

TABLE III

Fluorescence energy transfer and distance between FITC and Bis-ANS

F_{D+A} is the fluorescence intensity in the presence of donor and acceptor. F_A is the fluorescence intensity of the acceptor. r is the fractional saturation on Bis-ANS. E is the energy transfer efficiency. R is the calculated distance. R_{av} is the average distance.

[Bis-ANS]	F_{D+A}/F_A	r	E	R	R_{av}
μM					
5	1.08	0.056	0.396	47.8	
10	1.056	0.123	0.147	59.8	55.8
15	1.088	0.205	0.119	62.3	
20	1.258	0.277	0.256	53.3	

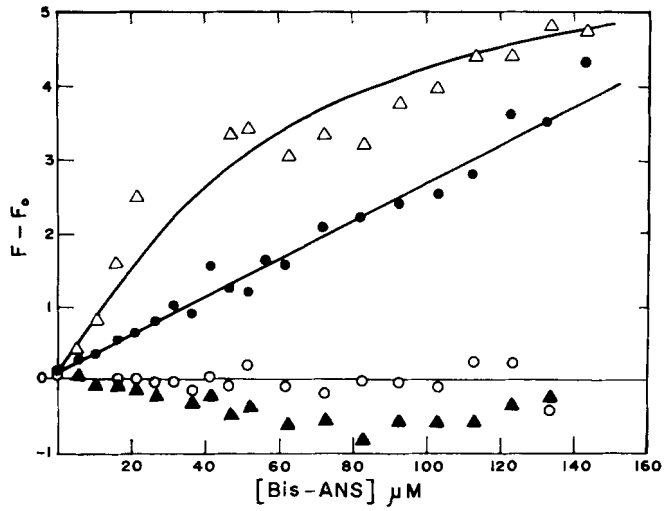

FIG. 5. **Titration of λ repressor (△), N-terminal domain (○), C-terminal domain (●), and N-terminal domain/operator complex (▲) with Bis-ANS.** Protein concentration in all the experiments were 1 μM, and the operator concentration was 0.5 μM. The excitation wavelength was 455 nm, and the emission wavelength was 500 nm. The fluorescence value in each experiment (F) was subtracted from fluorescence value of corresponding Bis-ANS concentration in buffer only (F_0). Other solution conditions are as in Fig. 1.

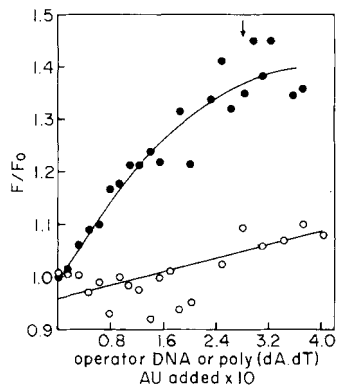

FIG. 6. **Binding of λ repressor and Bis-ANS complex with operator DNA and poly(dA·dT).** *Solid circles* (●) represent titration of 2 μM repressor and 1 μM Bis-ANS complex with operator DNA in 0.1 M potassium phosphate buffer, pH 8.0, at 25 °C. *Open circles* (○) represent titration of the same complex with poly(dA·dT). Excitation was at 385 nm, and emission was at 500 nm. *Arrow*, point where the ratio of operator to repressor dimer is 1.

92), and C-terminal domains (93–236) with Bis-ANS. Bis-ANS clearly binds to the C-terminal domain, and virtually no binding can be detected with the N-terminal domain. The N-terminal domain/operator complex also does not show any evidence of Bis-ANS binding. These results clearly demonstrate that Bis-ANS does not bind to the N-terminal domain and the C-terminal domain is at least partly involved in Bis-ANS binding.

Operator-induced Conformational Change—Localization of Bis-ANS binding on the C-terminal domain (and possibly partly on the hinge region) allowed us to use it as a probe for any global conformational change in the repressor molecule as DNA binds to the N-terminal domain. Fig. 6 shows increase of Bis-ANS fluorescence during titration of λ repressor/Bis-ANS complex with operator DNA. The specificity of the effect is demonstrated since the fluorescence increase shows saturation at operator to repressor dimer ratio of about 1. Since, the operator-N-terminal domain complex does not bind Bis-ANS, this increase is unlikely to be due to binding of Bis-ANS to the protein-DNA interface. Titration with poly(dA·dT) also causes increase of Bis-ANS fluorescence, the magnitude of which is much less compared with that obtained with operator. This could be due to weaker binding of poly(dA·dT) to repressor.

In order to reinforce the above conclusion, we have used tryptophans of the λ repressor as a probe for global conformational change. All three tryptophan residues (129, 142, and 230) of the λ repressor are situated in either the hinge or the C-terminal domain. As shown in Fig. 7, the binding of single operator O_R1 leads to quenching of tryptophan fluorescence. The titration shows saturation behavior, with very little quenching occurring after repressor dimer to operator ratio 1:1. The titration with operator also leads to a 0.6-nm red shift of the emission maxima (data not shown). The value of A_{295} after the titration was only 0.05, indicating that the quenching was not due to inner filter effect. Poly(dA·dT) at similar conditions have no detectable effect.

DISCUSSION

Sigmoidal binding of the ligand to a multi-subunit protein can occur for various reasons. If cooperativity, however, is protein concentration-dependent then it is likely that a ligand preferentially binds to one of several oligomeric forms in equilibrium with each other. Such instances are well known in cases of enzymes like phosphofructokinase and glutamate dehydrogenase (Frieden and Colman, 1967). Binding of Bis-ANS to the λ repressor is sigmoidal in the protein concentration range 0.5–25 μM, and the binding becomes less sigmoidal at lower protein concentrations. λ repressor dimer is known to associate to tetramers in this protein concentration range (Brack and Pirotta, 1975). Thus protein concentration-dependent cooperativity may be explained by preferential binding of Bis-ANS to dimeric form of the repressor. Simulation studies also agree with this conclusion.

λ repressor contains two domains, N-terminal and C-terminal, connected by a hinge region. The hinge region may have a folded structure (Gimble and Sauer, 1986). Protease cleavage under controlled limiting conditions produces cuts in the hinge region (Pabo *et al.*, 1979). When Bis-ANS-repressor complex is digested with trypsin, the Bis-ANS fluorescence increases indicating increased binding or increased

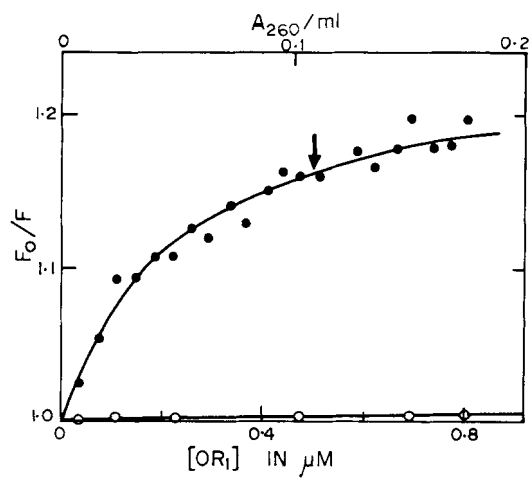

FIG. 7. **O_R1 titration of λ repressor.** λ repressor at a concentration of 1 μM in 0.1 M potassium phosphate, pH 8.0, was titrated with O_R1 (●) or poly(dA·dT) (○). The *arrow* indicates the point where the repressor dimer to O_R1 is 1:1. The excitation wavelength was 295 nm, and the emission wavelength was 340 nm.

quantum yield of the bound Bis-ANS. This indicates that Bis-ANS binding site is probably not located exclusively in the hinge region. Increase of fluorescence of bound Bis-ANS upon trypsin cleavage could occur because either the 93–236 fragment favors dimeric form (Pabo *et al.* 1979) or the hinge (93–132) may have increased interaction with the Bis-ANS-C-terminal domain complex in the fragment (93–236). Such hinge-C-terminal domain interaction has been postulated for the analogous C2 repressor from bacteriophage P22 (DeAnda *et al.*, 1983). As shown under "Results," under conditions where N-terminal domain has denatured without significant denaturation of C-terminal domain, significant Bis-ANS binding is observed. This suggests that Bis-ANS binding site is at least partly on the C-terminal domain of the λ repressor. Fluorescence energy transfer studies also indicate that the Bis-ANS binding site is at least 56 Å away from the N terminus, again suggesting the C-terminal domain is involved in the Bis-ANS binding. Binding studies with isolated N- and C-terminal fragments also clearly indicate that Bis-ANS binding occurs on the C-terminal domain and N-terminal domain is not involved (although the hinge region may be partly involved). Preferential binding of Bis-ANS to the dimeric form of the repressor and the location of binding site at the C-terminal domain raises an interesting possibility that the Bis-ANS binding site is at or near the dimer-dimer interface. It is known that Bis-ANS favors hydrophobic sites and many subunit interfaces are hydrophobic in nature. Thus Bis-ANS could act as an inhibitor of cooperativity in λ repressor. It also raises the possibility of identifying dimer-dimer contact sites by affinity labeling with a Bis-ANS analog.

It is well known that the λ repressor binds operators through the N-terminal domain (Jordan and Pabo, 1988). When Bis-ANS-repressor complex was titrated with a 17-base pair O_R1 fragment the fluorescence increased approximately by 40%, showing a sharp end point at operator to repressor dimer ratio of 1. This increase in fluorescence could be due to increased binding of Bis-ANS to the operator-repressor complex or increased quantum yield of the bound Bis-ANS or shifting of the tetramer-dimer equilibrium towards dimer to which Bis-ANS binds preferentially. The latter possibility can be excluded since the fluorescence increase remained virtually identical at higher protein concentrations (data not shown). This implies that upon DNA binding to N-terminal domain a conformational change occurs which is transmitted to the C-terminal domain. This conformational change either lowers the dissociation constant or increase the quantum yield of Bis-ANS/repressor complex. Titration with poly(dA·dT) also causes some increase in fluorescence of bound Bis-ANS, although the magnitude of the increase is much smaller. This could be due to weaker affinity of the λ repressor for poly(dA·dT) and incomplete binding. Such conformational change has been noted before for poly(dA·dT) bound *lac* repressor (York *et al.*, 1978). A second fluorescent probe (internal tryptophans) was used to verify the operator-induced conformational change. All the tryptophans in the λ repressor are distal to the operator binding site. The existence of an operator-induced global conformational change is reinforced by quenching of tryptophan fluorescence and red shift of emission maxima upon operator binding.

As pointed out by Hochschild and Ptashne (1988), the C-terminal domain must be able to rotate freely while the N-terminal domain remains bound and anchored to the operator DNA. Without such freedom of rotation, contact between C-terminal domains of two dimers bound to adjacent and non-adjacent operator sites becomes impossible. We speculate that the conformation of C-terminal domain and the hinge of the repressor is substantially different when bound to specific operator or even poly(dA·dT). This conformation may be more flexible, and it may be the active conformation for cooperative interactions as well as one-dimensional diffusion along DNA (York *et al.*, 1978). We suspect that the evolutionary necessity to have a more compact structure for the free repressor was to prevent the more flexible active conformation to be degraded faster by cellular proteases. We note that Gimble and Sauer (1986) have reported that several mutant repressors bearing mutations in the hinge region which presumably disrupts the hinge structure (making the repressor structure more open) are much more susceptible to proteolytic degradation.

Acknowledgments—We thank Professor Mark Ptashne for the plasmid pEA305. We thank Professor Jack S. Cohen for synthetic oligonucleotides O_R1.

REFERENCES

Amann, E., Brosius, J. & Ptashne, M. (1983) *Gene (Amst.)* **864,** 167–178
Brack, C. & Pirotta, V. (1975) *J. Mol. Biol.* **96,** 139–152
Cantor, C. & Schimmel, P. R. (1980) *Biophysical Chemistry*, Part II, Freeman, San Francisco
Dahlquist, F. W. (1978) *Methods Enzymol.* **48,** 270–279
DeAnda, J., Poteete, A. R. & Sauer, R. T. (1983) *J. Biol. Chem.* **258,** 10536–10542
Farris, F. J., Weber, G., Chiang, C. C. & Paul, I. C. (1978) *J. Am. Chem. Soc.* **100,** 4469–4474
Frieden, C. & Colman, R. F. (1967) *J. Biol. Chem.* **242,** 1705–1715
Garel, R. (1976) *Eur. J. Biochem.* **70,** 179–189
Gimble, F. S. & Sauer, R. T. (1986) *J. Mol. Biol.* **192,** 39–47
Griffith, J., Hochschild, A. & Ptashne, M. (1986) *Nature* **322,** 750–752
Guerente, L., Nye, J. S., Hochschild, A. & Ptashne, M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2236–2239
Hawley, D. K. & McClure, W. R. (1983) *Cell* **32,** 327–333
Herskowitz, I. & Hagen, D. (1980) *Annu. Rev. Genet.* **14,** 399–445
Hochschild, A. & Ptashne, M. (1986) *Cell* **44,** 925–933
Hochschild, A. & Ptashne, M. (1988) *Nature* **336,** 353–357
Johnson, A. D., Meyer, B. J. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 5061–5065
Johnson, A. D., Pabo, C. O. & Sauer, R. T. (1980) *Methods Enzymol.* **65,** 839–856
Jordan, S. R. & Pabo, C. O. (1988) *Science* **242,** 893–899
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lawson, R. C. & York, S. S. (1987) *Biochemistry* **26,** 4867–4875
Mas, M. T. & Colman, R. F. (1985) *Biochemistry* **24,** 1634–1646
Pabo, C. O. & Lewis, M. (1982) *Nature* **298,** 443–447
Pabo, C. O., Sauer, R. T., Sturtevant, J. M. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1608–1612
Prasad, A. R. S., Ludena, R. F. & Horowitz, P. M. (1986) *Biochemistry* **25,** 739–742
Riggs, A. D., Suzuki, H. & Bourgeois, S. (1970) *J. Mol. Biol.* **48,** 67–83
Rosen, C. G. & Weber, G. (1969) *Biochemistry* **8,** 3915–3920
Sauer, R. T., Pabo, C. O., Meyer, B. J., Ptashne, M. & Backman, K. C. (1979) *Nature* **279,** 396–400
Stec, W. J., Zon, G. & Uznanski, B. (1985) *J. Chromatogr.* **326,** 263–280
Ward, L. D. & Timasheff, S. N. (1988) *Biochemistry* **27,** 1508–1514
Wu, C. W. & Stryer, L. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1104–1108
Wu, F. Y. H. & Wu, C. W. (1978) *Biochemistry* **17,** 138–144
York, S. S., Lawson, R. C. & Worah, D. M. (1978) *Biochemistry* **17,** 4480–4486

Supplementary Material to

An Operator Induced Conformational Change in the C-terminal Domain of the Lambda Repressor

by

Rina Saha, Utpal Banik, Sumita Bandopadhyay, Nitai C. Mandal, B.Bhattacharyya and Siddhartha Roy

Materials: Bis-ANS was obtained from Molecular Probes, Inc (Eugene, OR, USA). 2-mercaptoethanol, SDS, Calf thymus DNA, BSA (fraction V), Bromophenol blue, PMSF, Commassie brilliant blue G and R, polyethyleneimine, trypsin, poly(dA-dT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). TEMED was obtained from Koch Light Laboratories Ltd., England. Bactotryptone, bactoagar and yeast extract were purchased from Difco Laboratories, Michigan, USA. CM sephadex A-50 was purchased from Pharmacia United (Sweden). All other chemicals were of analytical grade. Plasmid pEA305 was a gift from Prof.M.Ptashne. Crude 17 base pair O_R1 was a gift from Dr.J.S.Cohen and was further purified according to Stec et al., (1985) in a LKB HPLC using a reverse phase C-18 column.

Methods

Repressor isolation: Lambda repressor was isolated from E.coli RR1 15 lac2 carrying a plasmid pEA305 which contains wild type cI gene under the control of tac promoter (Amann et.al, 1983). The tac promoter is tryptophan-lac fusion promoter which is repressed by lac repressor and inducible with IPTG. When fully induced lambda repressor constitutes about 30% of the total cellular proteins. The cells containing the above plasmid were grown upto A_{595} = 0.6, then IPTG at a final concentration of 1 mM was added and grown for further 2 hours. Repressor was purified according to the method I of Johnson et al., (1980) involving polyethyleneimine precipitation followed by chromatography on CM-Sephadex A-50 and Hydroxylapatite column. Assay of lambda repressor was done by DNA filter binding procedure of Riggs et al., (1970). Aliquots of repressor were mixed with assay buffer containing about 0.8 µg of [^{3}H]-λ-DNA in a total volume of 0.25 ml. After incubation for 10 min at 20°C, 0.1 ml was slowly filtered on a millipore filter previously soaked in wash buffer, followed by washing with 0.5 ml of the same wash buffer maintained at that temperature. The bound radioactivity was quantitated by scintillation counting. Purified repressor was analyzed by electrophoresis on 13.5% SDS polyacrylamide gel and gave a single band (Laemmli, 1970) of MW of approximately 26,000 dalton. Protein was estimated by absorbance measurement at 280 nm using a Shimadzu UV-160 spectrophotometer. $E^{1\%}_{280}$ value used was 11.3. The repressor concentration was always calculated in terms of monomer unless otherwise stated. From 6 grams of starting cell about 15 milligrams of pure repressor was obtained.

Purification of N- and C-terminal domains: N- and C-terminal domain fragments of lambda repressor (1-92 and 93-236 respectively) were isolated after papain digestion according to Pabo et al., (1979) with slight modification. Kinetics of papain digestion was calibrated by quenching the reaction at different times with Koshland reagent III and running it on SDS-polyacrylamide gel. The obtained pattern was very similar to that of Pabo et al., (1979). The N-terminal domain was purified on a SP-sephadex C-25. After initial washthrough the N-terminal domain was eluted with a gradient of 1 mM to 100 mM KCl. Appropriate fractions were pooled, concentrated by lyophilization and dialyzed in a 2000 MW cutoff dialysis tubing against 0.1 M potassium phosphate buffer, pH 8.0. The purified material showed a single band on SDS-polyacrylamide gel electrophoresis corresponding to MW of approximately 10 Kilodaltons and gave fluorescence emission maximum at 303 nm, which is characteristic of non-tryptophan containing proteins (There are no tryptophans in the N-terminal domain). For C-terminal domain (93-236) purification, the lambda repressor was digested with papain for appropriate time and the mixture was then put on a SP-sephadex C-25 column and flow through collected. The flow through was then further purified over a Sephadex G-75 column equilibrated with 0.1 M phosphate buffer, pH 8.0. The identity of the fragment was established by comparing the mobility of the fragments on SDS-PAGE with that of a papain digest which contains all three C-terminal fragments. When analyzed by SDS-polyacrylamide gel electrophoresis it showed a single band with mobility equal to that of band 'a' in Pabo et al., (1979). Protein concentration of the fragments were determined by calculated extinction coefficients based on known tyrosine and tryptophan content of the fragments.

Determination of binding constant: Bis-ANS at a fixed concentration was titrated with increasing concentrations of repressor and the fluorescence was measured (reverse titration). Straight line obtained by plotting volume corrected 1/F vs. 1/[Repressor] was extrapolated to infinite repressor concentration to obtain F_{max}. In a second titration, repressor at a fixed concentration was titrated with increasing Bis-ANS concentration (direct titration) and the concentration of repressor complex at each point was determined by the method of Mas & Colman (1985) using fluorescence enhancement value (Q) (derived from F_{max} value obtained from previous titration). Apparent binding constants were obtained from fluorescence data by Scatchard and Hill analysis (Dahlquist, 1978).

Excitation wavelength of 455 nm and emission wavelength of 500 nm were used in all binding studies (direct and reverse titrations) to avoid inner filter effect. Excitation wavelength of 385 nm was used in all other studies to maximize sensitivity where there were no significant inner filter effect.

Simulation of preferential binding of Bis-ANS to repressor dimer: The Model

Let R_2 represent the repressor dimer, R_4 represent the repressor tetramer and K_4 be the dimer-tetramer association constant. Let K_2 be the Bis-ANS binding constant to the repressor dimer, K_4 be the Bis-ANS binding constant to the repressor tetramer and binding stoichiometry be one per repressor monomer. Let P_0 be the total protein concentration in terms of repressor monomer and [B] represent the Bis-ANS conentration. We make one additional assumption that total Bis-ANS concentration $[B]_t$ is equal to the free Bis-ANS concentration $[B]_f$. This assumption is justified since significant binding takes place only at Bis-ANS concentraitons which are much higher than the protein concentrations.

$$2R_2 \overset{K_4}{\rightleftharpoons} R_4$$

$$R_2 + B \overset{K_2}{\rightleftharpoons} R_2.B$$

$$R_2.B + B \overset{K_2}{\rightleftharpoons} R_2.B_2$$

$$R4 + B \overset{K_4}{\rightleftharpoons} R4.B$$

$$R_4.B + B \overset{K_4}{\rightleftharpoons} R_4.B_2$$

$$R_4.B_2 + B \overset{K_4}{\rightleftharpoons} R_4.B_3$$

$$R_4.B_3 + B \overset{K_4}{\rightleftharpoons} R_4.B_4$$

$$P_0 = 2.[R_2] + 2.[R_2.B] + 2.[R_2.B_2] + 4.[R_4] + 4.[R_4.B] + 4.[R_4.B_2] + 4.[R_4.B_3] + 4.[R_4.B_4]$$

Transforming all the complex concentrations in terms of $[B]_f$ and association constants we get

$$P_0 = 2.[R_2].X + 4.K_4.[R_2]^2.Y$$

Where $X = 1 + K_2.[B]_f + (K_2.[B]_f)^2$

and $Y = 1 + K_4.[B]_f + (K_4.[B]_f)^2 + (K_4.[B]_f)^3 + (K_4.[B]_f)^4$

or $4.K_4.[R_2]^2.Y + 2.[R_2].X - P_0 = 0$

Solving for $[R_2]$

$$[R_2] = Z = \frac{-X + \sqrt{X^2 + 4.P_0.K_4.Y}}{4.K_4.Y}$$

Total fluorescence value F is given by the following equation

$$F = C.[B]_f + C.Q.[R_2.B] + 2.C.Q.[R_2.B_2] + C.Q.[R_4.B] + 2.C.Q.[R_4.B_2] + 3.C.Q.[R_4.B_3] + 4.C.Q.[R_4.B_4]$$

Assuming $[B]_f = [B]_t$ and transforming all the complex concentrations in terms of $[B]_f$ and association constants

$$F = C.[B]_f + [R_2].C.Q.G + [R_2]^2.K_4.C.Q.H$$

where $G = K_2.[B]_f + 2.(K_2.[B]_f)^2$

and $H = K_4.[B]_f + 2.(K_4.[B]_f)^2 + 3.(K_4.[B]_f)^3 + 4.(K_4.[B]_f)^4$

or $F/C.[B]_f = 1 + [R_2].Q.G/[B]_f + [R_2]^2.K_4.Q.H/[B]_f$

Since $C.[B]_f$ is the total fluorescence without protein (F_0)

$$(F/F_0)-1 = [R_2].Q.G/[B]_f + [R_2]^2.K_4.Q.H/[B]_f$$

or $\{(F/F_0)-1\}.[B]_f/Q = [R_2].G + K_4.[R_2]^2.H$

dividing by P_0, we get

$$r = Z.G/P_0 + K_4.Z^2.H/P_0$$

where r is the fractional site occupation per monomer (Mas & Colman, 1985).

A computer program in BASIC was written where values of K_2 and K_4 was varied systematically over a given range (at given values of P_0, Q and K_4) to generate a series of theoretical r values. Corresponding experimental r values were obtained from experimental F and F_0 at several $[B]_f$ values. Chi-square values were used to judge the goodness of fit. The set of K_2 and K_4 that gave the minimum Chi-square value was chosen as the best fit.

In order to restrict the search time, we have restricted the range of K_4 between 1 and 10,000 and range of K_2 between 10,000 and 100,000 (preferntial binding to the dimeric form).

Trypsin digestion and gel electrophoresis: Solution containing 11 µM repressor and 2 µM Bis-ANS was incubated with trypsin in a ratio of 1:50 (w/w) in 0.1 M potassium phosphate buffer, pH 8.0 at 25°C in a fluorescence cuvette. Fluorescence intensity of bound Bis-ANS was monitored at different times after the addition of trypsin. At different times, a certain amount of aliquot was withdrawn and the reaction was quenched by adding PMSF to a final concentration of 1 mM. These samples were run on 13.5% SDS-Polyacrylamide gel according to Laemmli (1970).

Temperature and urea concentration dependence of Bis-ANS binding to the repressor: A solution containing 4 µM repressor and 2 µM Bis-ANS in 0.1 M potassium phosphate, pH 8.0 was placed in a capped fluorescence cuvette for temperature dependent studies. The fluorometer chamber was heated externally by a circulating water bath and temperature of the chamber was directly measured by a thermometer placed in the chamber. Fluorescence polarization of Bis-ANS was also measured as a function of temperature. Anisotropy was calculated according to the following equation:

$$A = \frac{I_{\parallel} - G I_{\perp}}{I_{\parallel} + 2G I_{\perp}}$$

Urea concentration dependence was carried out in 0.1 M potassium phosphate buffer, pH 8.0, containing different concentrations of urea. Fluorescence spectra of buffers containing the same concentrations of urea, but no protein or Bis-ANS was subtracted from each spectra. Ratio of fluorescence of 3 µM Bis-ANS and 4 µM repressor to 3 µM Bis-ANS at a given urea concentration was taken as a measure of Bis-ANS binding to the repressor.

Titration of lambda repressor with operator DNA and poly(dA-dT): Poly (dA-dT) solution was dialyzed against 0.1 M potassium phosphate buffer, pH 8.0. Lambda repressor at a concentration of 1 µM in 0.1 M potassium phosphate, pH 8.0 was titrated with O_R1 or poly (dA-dT) and the tryptophan fluorescence was monitored at 340 nm. The excitation wavelength was 295 nm. To study the effect of DNA binding on Bis-ANS/repressor complex, 2 µM repressor and 1 µM Bis-ANS was titrated with increasing concentrations of operator DNA and poly (dA-dT) separately at 25°C. Change in fluorescence intensity of bound Bis-ANS was measured at 500 nm. The excitation wavelength was 385 nm. Signal averaging was used to improve signal to noise ratio. The N-terminal, C-terminal domain fragments and N-terminal domain/operator complex at a protein concentration of 1 µM was titrated with increasing concentrations of Bis-ANS and fluorescence value was determined at 500 nm. The excitation wavelengths were 455 nm.

Fluorescence energy transfer and distance calculation: Lambda repressor was specifically labeled at the N-terminal amino acid with FITC. Location of the label was obtained by comparing the cleavage pattern of limited proteolysis with papain and trypsin (Banik, U. & Roy, S. unpublished observation). The distance between bound Bis-ANS (the energy donor) and FITC (the acceptor) was determined with the help of the following equation

$$R = R_0 \{E^{-1} - 1\}^{1/6}$$

where R = Apparent distance between donor and acceptor,

E = Energy transfer efficiency

R_0 = Distance where transfer efficiency is 50 %

Energy transfer efficiency can be calculated using the following equation:

$$F_{D+A}/F_A = 1 + (\varepsilon_D.C_D.E)/\varepsilon_A.C_A$$

where F_{D+A} is the fluorescence intensity of the donor in presence of the accepter. F_A is the fluorescence of the acceptor. ε_D is the extinction coefficient of the donor. ε_A is the extinction coefficient of acceptor (Cantor and Schimmel, 1980).

R_0 is given by

$$R_0 = (J K^2 Q n^{-4})^{1/6}.(9.79 \times 10^3) \text{ cm.}$$

where J is a measure of spectral overlap (the overlap integral), Q is the quantum yield of the donor, n is the refractive index and K is the orientation factor. Extinction coefficients of the acceptor at different wavelengths was calculated from the absorbance spectra of FITC labeled lambda repressor and the known extinction coefficient of FITC of 72,000 at 495 nm (Garel, 1976). Extinction coefficient of donor Bis-ANS was taken as 16,790 at 385 nm (Farris et al., 1978). Ratio of C_D/C_A is the fractional saturation and is obtained from Scatchard plot of Bis-ANS binding to unlabeled repressor at same concentrations. Fluorescence due to direct excitation of Bis-ANS was corrected for by subtracting the fluorescence value of an equal concentration of free Bis-ANS. No correction for direct excitation of bound Bis-ANS was done and hence all the energy transfer values reported here are an upper limit, while the corresponding distances are to be regarded as lower limits. Overlap integral was calculated according to method of Wu & Stryer (1972). Value of K^2 was taken for random orientation, which is 2/3. Quantum yield of Bis-ANS was calculated according to the method of Ward and Timasheff (1988) using Quinine sulfate in 1 N H_2SO_4 as standard.