Sequence-Specific DNA Displaces 6-*p*-Toluidino-2-naphthalenesulfonate Bound to a Hydrophobic Site on the DNA-Binding Domain of *Drosophila* c-myb†

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ABSTRACT: The N-terminal DNA-binding domain of c-myb oncoprotein binds to DNA in a sequence-specific manner. The domain, consisting of three imperfect tandem repeats, has tryptophan residues at very regular intervals and this is believed to be of some significance in the DNA-binding activity of the protein. We have found that the hydrophobic-site-specific probe 6-*p*-toluidino-2-naphthalenesulfonate (TNS) binds to the bacterially expressed DNA-binding domain of *Drosophila* c-myb protein (R123). TNS has a single binding site on this protein with an apparent dissociation constant in the range of (5–8) × 10^{-7} M. When the TNS–protein complex was treated with an oligomeric DNA duplex having a cognate myb-binding site, the TNS was displaced from the complex. Nonspecific DNA duplex oligomers were ineffective, indicating that TNS displacement was a sequence-specific process. We examined further some features of the TNS-binding site on the protein, taking advantage of the fluorescence properties of the protein and the bound TNS. Our data indicate that the TNS binding occurs in a peripheral site on the protein in a manner that allows the bound TNS to be solvent accessible. Furthermore, there are indications that tyrosine(s) and tryptophans of the protein mediate resonance energy transfer to the bound TNS. From fluorescence-quenching data of the protein and protein–TNS complex, we could assess that both solvent-accessible and internal tryptophans are in the vicinity of the bound TNS. Our data are consistent with the proposal that the bound TNS occupies a part of the DNA-binding site of the protein and is made up of contributions from at least two of the three typical repeating units of *Dm*-myb protein.

The cellular and viral myb proteins are nuclear proteins that bind to DNA in a sequence-specific manner (Biedenkapp et al., 1988; Nishina et al., 1990; Sakura et al., 1989; Howe et al., 1990; Oehler et al., 1990). The DNA-binding activity is mediated by an evolutionarily highly conserved amino-terminal region which consists of three imperfect repeats (R1-R2-R3) of approximately 50–53 residues each, characterized by tryptophans repeating every 18/19 residues in each stretch (Klempenauer et al., 1987; Ibanez et al., 1988; Howe et al., 1990). It is believed that the tryptophan repeats play some important role in the DNA–protein interaction and recognition process. There is evidence that two stretches (R2-R3) are critical for the DNA-binding activity in chicken v-myb and mouse c-myb and the precise role of R1 repeat is not clear. Recently, Ogata et al. (1992) have determined the three-dimensional structure of a single repeating unit (R3) using a synthetic peptide and nuclear magnetic resonance (NMR)[1] spectroscopy. Their results show that the R3 peptide has structural features such as a helix-turn-helix motif found in many other DNA-binding proteins (Freemont et al., 1991). Two of the three tryptophans present in the R3 domain form part of the α-helices, and all the tryptophans contribute to form a hydrophobic core. In another study, the R2-R3 region of chicken c-myb protein has been investigated (Frampton et al., 1991) and the presence of hydrophobic regions in both the domains has been suggested.

In our recent studies using the bacterially expressed DNA-binding domain of *Drosophila* c-myb (R123), we observed that when a specific DNA sequence was bound to the protein, the tryptophan fluorescence was quenched substantially. This indicated that the specific DNA may bind to the protein in the vicinity of several tryptophans. We present evidence in this report that there is a quasihydrophobic site on the protein to which the hydrophobic probe TNS binds and that an oligonucleotide duplex bearing a cognate myb-binding site displaces the bound TNS from the protein specifically. Furthermore, the TNS-binding site was found to be in the vicinity of tyrosine and several tryptophans. We reason that the specific DNA binds to the protein more strongly than TNS and thus displaces TNS when it is added to the soltuion containing protein–TNS complex.

MATERIALS AND METHODS

Reagents. Ethylene glycol was from Fluka. DMSO, PEI, IPTG, molecular weight markers, acrylamide, SP-Sephadex, Dowex 1X8, TNS, MES, HEPES, and ammonium sulfate were purchased from Sigma. DE-52 anion-exchange resin was from Whatmann, and bacteriological media components were from Difco. All other reagents were of analytical grade. TNS was dissolved in *N*,*N*-dimethylformamide and the concentration of the stock solution was determined to be 170 μM by absorption measurement using a molar extinction coefficient of 1.89 × 10^{-4} at 317 nm (McClure & Edelman, 1967).

Protein Preparation. The *Dm*-myb R123 protein was expressed in the *Escherichia coli* strain BL21(DE3) carrying the overexpression plasmid for the protein (Anup Madan et al., submitted for publication). An overnight-grown culture of the cells in LB medium containing 100 μg/mL of ampicillin was diluted and grown till A_{600} reached 0.7. At this stage, the cells were induced by addition of IPTG to a final

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[1] Abbreviations: TNS, 6-*p*-toluidino-2-naphthalenesulfonate; DMSO, dimethylsulfoxide; DMF, *N*,*N*-dimethylformamide; CD, circular dichroism; UV, ultraviolet; NMR, nuclear magnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; PEI, polyethylenimine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MES, *N*-morpholinoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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concentration of 25 μM and were further grown for 3 h. The cells were then harvested by centrifugation at 5000 rpm for 10 min at 10 °C using a GSA rotor. They were resuspended in 20 mL of the lysis buffer (8 M urea, 20 mM Tris·HCl, pH 8.0, 2 mM EDTA, 1 mM PMSF) per liter of the culture and lysed by sonication (5 × 1 min at 40% maximum intensity) using a Branson Sonifier. The cell debris was removed by centrifugation at 10 000 rpm (10 min) using a Sorvall SS34 rotor. The nucleic acids and phospholipids were removed from the supernatant by precipitating with 0.03% PEI, and the precipitate was removed by centrifugation at 10 000 rpm for 10 min at 4 °C using an SS34 rotor. The protein was precipitated by adding saturated ammonium sulfate to a final concentration of 50% and pelleted at 15 000 rpm for 10 min using an SS34 rotor. The pellet was suspended in 20 mL of lysis buffer/L of the original cell culture and loaded on a DE-52 column (5 × 45 cm) pre-equilibrated and developed with lysis buffer. The active fractions containing the *Dm*-myb R123 protein were collected, and pH was adjusted to 6.5 by adding MES. The solution was then loaded on an SP-Sephadex column (2.5 × 15 cm) pre-equilibrated with buffer A (8 M urea, 20 mM MES, pH 6.5, 0.15 M NaCl). The column was washed extensively with buffer A and developed with a linear gradient of NaCl (0.15–1.0 M NaCl) in buffer A. The recombinant protein eluted at ca. 0.83 M NaCl. After the protein was folded by gradually diluting the urea to a final concentration of 0.2 M with 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 10 mM DTT. The protein was concentrated by ultrafilteration using an Amicon YM-10 membrane and equilibrated to storage buffer (20 mM Tris·HCl, pH 8.0) by dialysis. The purity of the protein was checked by 15% SDS–PAGE and was found to be >95%.

Fluorescence Methods. Fluorescence measurements were done on a Shimadzu RF540 spectrofluorimeter with 15 min of preincubation of sample at room temperature for equilibration. Spectra were collected with 1-nm wavelength increments and were corrected for solvent contributions.

TNS was used as a probe for hydrophobic sites on the R123 protein. (Strickland & Bhattacharya, 1984; Strickland et al., 1984; Steiner et al., 1985, 1987; Lin et al., 1991). TNS binding to the protein was analyzed according to the following two equations (Scatchard, 1949; Klotz & Hunston, 1971):

$$\frac{\nu}{[\mathrm{TNS}]} = \frac{1}{K_d}(N - \nu) \tag{1}$$

$$\frac{1}{\nu} = \frac{K_d}{N[\mathrm{TNS}]} + \frac{1}{N} \tag{2}$$

where K_d is the dissociation constant of the R123–TNS complex, ν is the moles of the ligand bound per mole of the protein, N is the number of binding sites, and [TNS] is the concentration of TNS. Here, it is assumed that the multiple binding sites have same fluorescence characteristics. This assumption is validated by the linearity of the Scatchard and Klotz plots (Figure 1B).

Displacement of the bound TNS on the protein was examined using self-complementary oligonucleotides d-GGTACGCGTACC for nonspecific binding and d-ACCGT TAACGGT for specific binding to the protein. The nucleotides underlined define the specific myb-recognition sequence. The oligonucleotides were synthesized on an Applied Biosystems ABI 381A DNA synthesizer and were purified by reverse-phase HPLC (Eadie et al., 1987).

Fluorescence-quenching experiments (Lakowicz, 1983; see Eftink, 1991 for a recent review) designed to probe the solvent accessibilities of TNS and the tryptophan residues were carried out with the neutral quencher acrylamide. The data were analyzed using the following equations (Lehrer, 1971; Eftink, 1991):

$$\frac{F_o}{F} = (1 + K_{sv})[\mathrm{Q}] \tag{3}$$

$$\frac{F_o}{F_o - F} = \frac{1}{f_a k_q \tau_o [\mathrm{Q}]} + \frac{1}{f_a} \tag{4}$$

Here, F_o and F are the fluorescence intensities of the protein in the absence and presence of quencher. K_{sv} is the collisional Stern–Volmer constant, f_a is the fraction of quenchable fluorescence, k_q is the rate constant for quenching, and τ_o is the fluorescence lifetime of the fluorophores in the absence of the quencher.

UV Difference Spectroscopy. UV difference spectra used to estimate the solvent accessibility of tryptophan residues were recorded with two solvent perturbants, 20% DMSO (Sigma) and 20% ethylene glycol (Fluka). All measurements were done at 20 °C with matched tandem cells of 0.5-cm path length per compartment.

The fraction of the tryptophans accessible to the solvent was calculated by using the equation:

$$F_{\mathrm{Trp}} = \frac{(\Delta\epsilon_d/\epsilon_a)_p}{(\Delta\epsilon_d/\epsilon_a)_u} P \tag{5}$$

where $\Delta\epsilon_d$ is the maximum in the UV difference spectrum, and ϵ_a is the absorption maximum of the protein. The subscripts p and u refer to completely folded and unfolded (8 M urea) states of the protein, respectively. The factor P corrects for the effect of urea on the difference spectral intensities of the chromophore (Herskovits, 1962a,b; Williams et al., 1965) and was calculated from

$$P = \frac{(\Delta\epsilon_d/\epsilon_a)_{rp}}{(\Delta\epsilon_d/\epsilon_a)_{ru}} \tag{6}$$

where the subscripts rp and ru refer to a model reference compound in the absence and presence of 8 M urea, respectively. The model compound used in the present study was a mixture of tryptophan, tyrosine, and phenylalanine in the proportion 9:3:2, which is the proportion of these residues in the R123 protein.

RESULTS

Binding of TNS to R123 and Evidence for a Single Binding Site. The compound TNS has very little or no fluorescence in aqueous solutions but intensely fluoresces upon its binding to hydrophobic sites of proteins (Strickland & Bhattacharya, 1984; Strickland et al., 1984; Steiner et al., 1985, 1987; Lin et al., 1991). We have therefore used TNS as a possible probe to detect hydrophobic sites on the topography of the DNA-binding domain of *Dm*-myb R123. Figure 1A records the fluorescence spectra of a fixed amount of protein treated with varying amounts of TNS. The observed fluorescence of TNS is seen to increase proportionately with increasing input of TNS until saturation is reached. A similar binding characteristic was also seen when protein was incrementally added to an excess amount of TNS. When TNS was added to the protein in the presence of 8 M urea, no TNS-specific fluorescence could be seen. This indicates that the fluorescence results from TNS binding to the protein in its native state

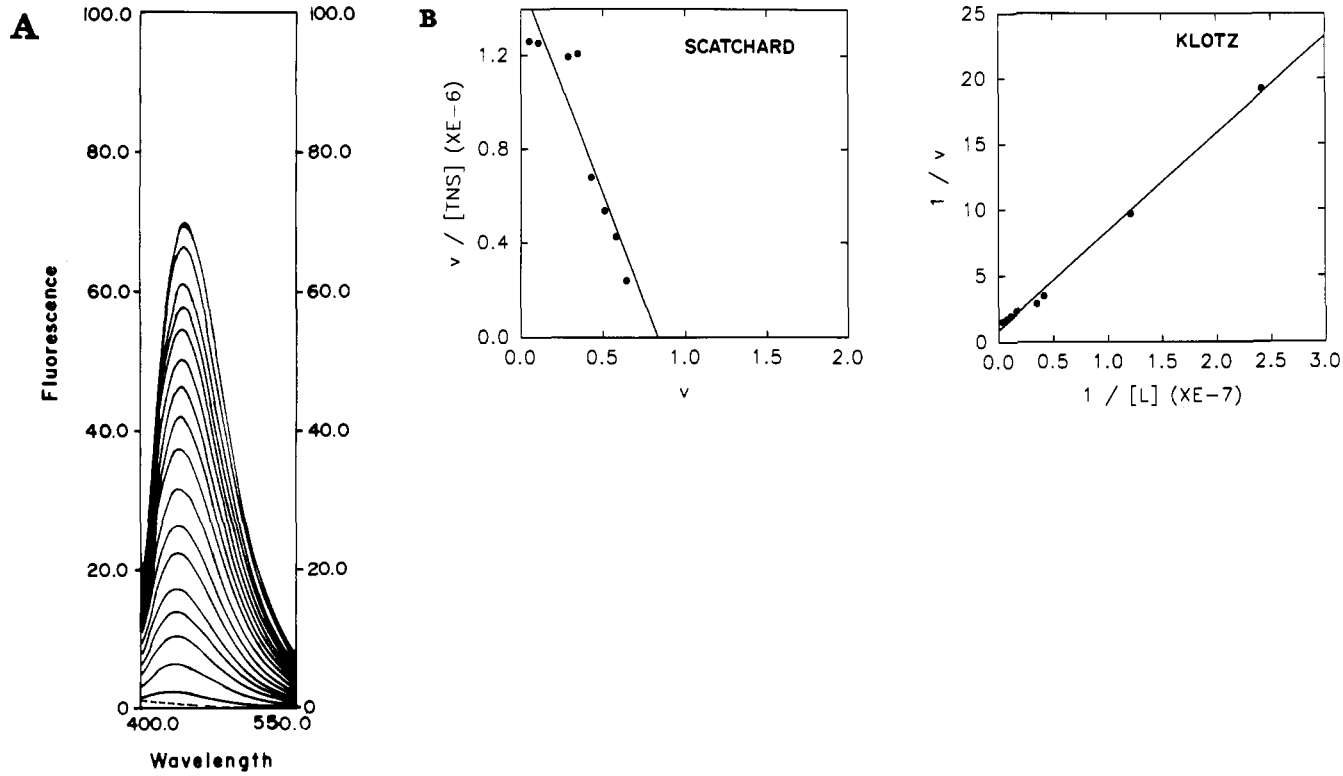

FIGURE 1: (A, left) Fluorescence spectra (λ_{ex} = 326 nm) of 7.0 μM *Dm*-myb R123 protein in the presence of different concentrations of TNS (—) and in absence of TNS (– –) in 10 mM HEPES buffer, pH 7.0. TNS was added in aliquots from stock solution of 170 μM. The emission spectra were collected after incubating the samples with TNS for 30 min. The TNS concentration increases in steps of 1 μM from the bottom curve to the top curve, the starting concentration being 1 μM and the ending being 18 μM. (B, middle and right) Scatchard plot and Klotz plot for the determination of the number of binding sites (N) of the TNS on the protein and the dissociation constant (K_d) of the TNS–protein complex using the data shown in Figure 1A. The data was analyzed according to the Scatchard and Klotz equations. From the slope and intercept of both the plots, the number of binding sites of TNS and dissociation constant were determined. Using these values of N and K_d, new free-ligand concentrations were calculated which, in turn, were used to recalculate (by means of least-square fit) new values of K_d and N. This iteration procedure was repeated until the difference between the successive values of K_d was less than 1%.

because the binding is abolished in the presence of urea due to protein unfolding.

We carried out two separate experiments to examine if the binding of TNS to the R123 protein was reversible. First, a TNS-saturated R123 sample was adjusted to 8 M urea. The sample was then dialyzed against the buffer containing 8 M urea to remove free TNS followed by dialysis against buffer without urea. The last step removes urea and allows the protein to refold to its native state. After these steps the protein sample failed to show any residual TNS-specific fluorescence. When fresh TNS was added to this dialyzed and refolded sample, TNS-specific fluorescence reappeared, confirming that R123 protein had indeed folded back to its native conformation. Similarly, in a second experiment, the anion-exchange resin Dowex-1X8 was added to the R123 protein sample saturated with TNS. Upon incubation at room temperature, the TNS fluorescence steadily decreased and reached a basal level in a few minutes. This experiment again shows that the bound TNS can be removed by shifting the free TNS equilibrium in favor of the Dowex–TNS complex. In a control experiment it was verified that the R123 protein itself did not bind to Dowex and TNS fluorescence could be observed by removing the Dowex and adding fresh aliquots of TNS to the sample. From these experiments we conclude that the TNS binding to R123 protein is reversible.

The TNS fluorescence values obtained at different levels of TNS were analyzed using the Scatchard and Klotz binding equations to estimate the number of TNS-binding sites (N) and the relevant dissociation constant K_d). From these plots shown in Figure 1B, we found that *Dm*-myb R123 had N = 0.84 and $K_d = 5.5 \times 10^{-7}$ M by Scatchard analysis while N = 1.10 and $K_d = 8.0 \times 10^{-7}$ M was determined from Klotz analysis. Taken together, we conclude that there is a single TNS-binding site of moderate affinity on the myb R123 protein.

Sequence-Specific DNA Displaces the Bound TNS. Experiments were carried out to see whether the TNS binding was affected by specific DNA binding to the protein. These results, presented in Figure 2A, show that indeed, when specific target DNA is added to TNS–R123 complex, the TNS-specific fluorescence decreases and at least a stoichiometric amount of specific DNA was needed to ablate essentially all the TNS fluorescence of a preformed complex. A nonspecific DNA oligomer, in contrast, had only a marginal effect on the fluorescence of the TNS–R123 complex when added in the same concentration range (Figure 2B). These results show clearly that myb-specific DNA essentially ablates the TNS-specific fluorescence of the complex whereas a nonspecific DNA oligomer fails to do so under similar conditions. We rationalize the decrease of fluorescence to a displacement of TNS by specific DNA.

We have determined in separate experiments that the specific DNA binds to the protein with an affinity constant of about $\sim 10^{10}$, while the nonspecific DNA has an affinity of $\sim 10^5$. Since the TNS binding constant is $\sim 10^7$, its displacement by specific but not by nonspecific DNA can be simply rationalized in terms of a selective displacement of TNS from its binding site by specific DNA.

The Proximity of the Bound TNS to Tyrosines and Tryptophans. The myb DNA-binding domain R123 is unique

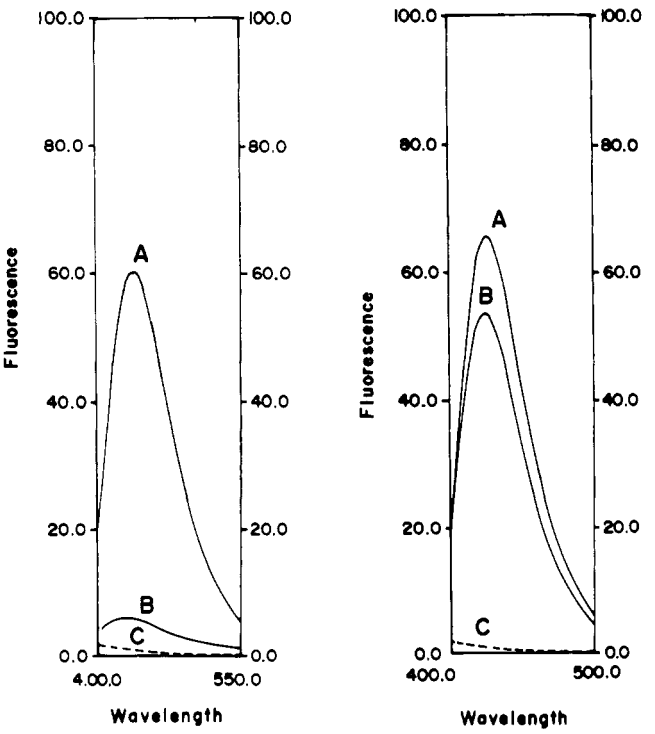

FIGURE 2: (A, left) DNA binding activity of the conserved stretch of *Drosophila melanogaster* c-myb (R123) expressed in *E. coli* monitored by changes in TNS fluorescence. The protein–TNS complex was formed by incubating 7.0 μM protein and 25 μM TNS in 10 mM HEPES, pH 7.0, for 30 min at room temperature. To the complex the oligonucleotide (d-ACCGTTAACGGT) containing a specific target sequence was added an equimolar amount of protein, and samples were incubated for 60 min at room temperature. The samples were excited at 326 nm. Curve A shows the emission spectrum of the protein-TNS complex while curve B shows the spectrum of the protein-TNS complexed with specific DNA. Curve C shows the fluorescence spectrum of TNS alone. The protein and the DNA alone didn't show any fluorescence under these conditions. (B, right) Changes in the TNS-specific fluorescence of the TNS-R123 complex in presence of DNA oligomer containing a nonspecific target. The protein–TNS complex was formed by incubating 7.0 μM protein and 25 μM TNS in 10 mM HEPES, pH 7.0, for 30 min at room temperature. To the complex the oligonucleotide (d-GGTACGCG-TACC) containing a nonspecific target was added an equimolar amount of protein, and samples were incubated for 60 min at room temperature. The samples were excited at 326 nm. Curve A shows the emission spectrum of the protein–TNS complex while curve B shows the spectrum of the protein–TNS complexed in presence of nonspecific DNA. Curve C shows the fluorescence spectrum of TNS alone. The protein and the DNA alone did not show any fluorescence under these conditions.

in having nine tryptophans distributed in an orderly manner in three tandem repeating units. We therefore looked into the possibility that the bound TNS could interact with some of these tryptophans and other aromatic amino acids. That TNS could indeed interact with some of the tyrosine and tryptophan residues was borne out from the following results.

We monitored the simultaneous changes in fluorescence emission of both protein and protein–TNS complex with excitation at 279 nm when a fixed amount of protein was titrated with increasing amounts of TNS. The results are presented in Figure 3A. Three features are apparent from this figure. First, with the increased TNS binding to the R123, the protein fluorescence (λ_{em} 340 nm) itself decreases and, concomitantly, TNS-specific fluorescence (λ_{em} 435 nm) increases. Second, the protein-specific fluorescence is not abolished completely but reaches a limiting lower value even in the presence of saturating amounts of TNS. An essentially similar qualitative result was obtained when tryptophan-

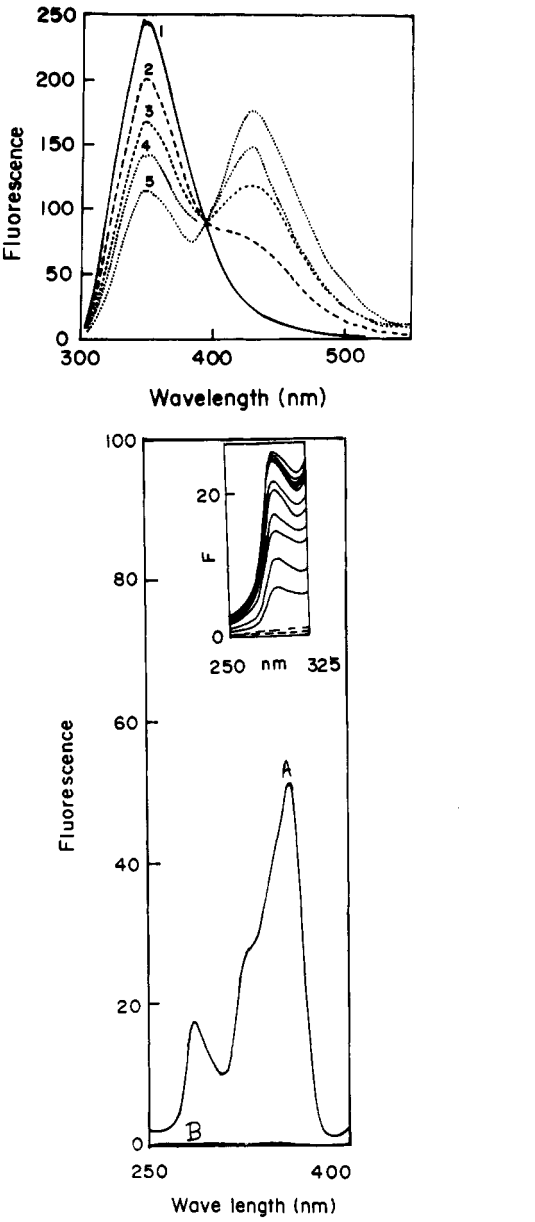

FIGURE 3: (A, top) Fluorescence spectra of 7.0 μM *Dm*-myb R123 protein in the presence of (1) 0, (2) 1, (3) 2, (4) 3.0, and (5) 5.0 μM TNS in 10 mM HEPES buffer, pH 7.0. The samples were excited at 279 nm. The band pass for the excitation and emission monochromator was 10 nm. The spectrum shows an isoemissive point at 396 nm. (B, bottom) Fluorescence excitation spectrum (λ_{em} = 435 nm) of 50 μM *Dm*-myb R123 protein–TNS complex (A) and TNS (B) in 10 mM HEPES buffer, pH 7.0. The changes in the intensity of the novel band (280 nm) in presence of different concentrations of TNS (—) is shown in the inset. For comparison, excitation spectra of protein (– –) and TNS (– –) have also been included in the inset.

specific excitation at 295 nm was used to monitor both the protein and TNS fluorescence. These results indicate that both tyrosine and tryptophan residues may flank the TNS binding site on the protein and mediate possible energy transfer to the bound TNS. Thirdly, an isoemissive point at 396 nm testifies to the presence of myb protein in its free and TNS-bound states.

The issue of the resonance energy transfer between some of the protein fluorophores and the bound TNS was further examined by recording the excitation spectra (λ_{em} 435 nm) of R123 protein with varying amounts of TNS. The results are displayed in Figure 3B. A prominent novel band around 280 nm was observed which was found to be absent in the

Table 1: Stability of the R123–TNS Complex in the Presence of Acrylamide

acrylamide concn (mM)	K_d (M) (determined by Scatchard plot)
0	$(5.5 \pm 1.0) \times 10^{-7}$
0.5	$(1.3 \pm 1.0) \times 10^{-7}$
1.0	$(3.5 \pm 1.0) \times 10^{-7}$

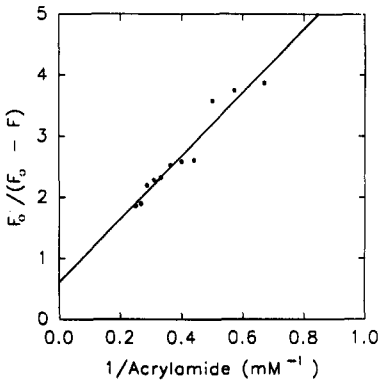

FIGURE 4: Lehrer plot of the acrylamide quenching of the *Dm*-myb R123–TNS complex. The complex was formed by incubating 6.0 μM protein and 25 μM TNS for 30 min. The samples were excited at 326 nm and the quenching of the fluorescence was observed by monitoring changes at 435 nm with increasing concentrations of acrylamide.

absorption spectrum of the TNS and the excitation spectra of the TNS recorded in at least three organic solvents (benzene, toluene, DMF). The intensity of this novel band increases proportionately with increasing TNS concentration until a saturation is reached (inset, Figure 3B). This is a clear indication that resonance energy transfer is taking place between the tryptophan and tyrosine residues of R123 and the bound TNS.

Bound TNS Is Solvent-Accessible. To probe if the bound TNS entered into the deep interior of the protein and thereby insulated itself from the solvent, we took the help of a neutral fluorescence quencher, acrylamide. In preliminary studies we first ensured that acrylamide did not displace or affect the TNS binding to the protein by measuring the dissociation constant of the TNS–protein complex in the presence of acrylamide. The data obtained from the Scatchard plots in the absence and presence of a series of acrylamide concentrations showed K_d values ($K_d \approx 10^{-7}$ M, Table 1) to be of the same order of magnitude and assured us that acrylamide indeed did not interfere in TNS binding to the R123 protein. The quenching effect of various concentrations of acrylamide on the fluorescence of the TNS–R123 complex at 435 nm was then monitored. These data on fluorescence quenching of bound TNS are presented in Figure 4 in the form of a Lehrer plot. The bound TNS is seen amenable to substantial quenching at the acrylamide concentrations tried and the plot indicates that essentially all the TNS fluorescence can be projected as quenchable at high concentration of acrylamide. This result is revealing, in that although the bound TNS is insulated enough from the aqueous environment to be fluorescent, this insulation is not complete. Acrylamide from aqueous solution can still access the bound TNS in its excited state. The efficiency of the TNS fluorescence quenching represented by the Stern–Volmer constant (K_{sv}) has been estimated from the data in Figure 4 to be 3×10^{-5} M^{-1}.

Solvent Accessibility of the Tryptophan Residues of the R123 Protein. As noted from Figure 3A, some of the protein fluorescence was lost upon binding of TNS to R123, suggesting

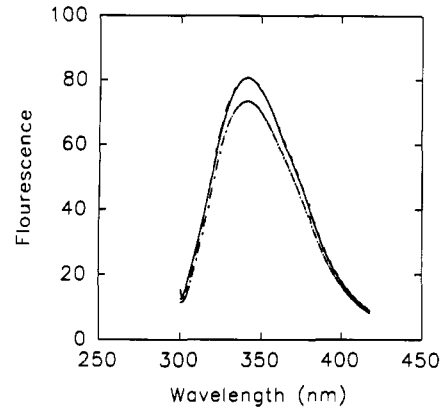

FIGURE 5: Fluorescence emission spectra of R123 protein obtained by exciting at 279 nm (– • –) and 295 nm (—). The emission spectra obtained by exciting at 279 nm, normalized relative to emission at 360 nm, is also shown (– –).

that the bound TNS was in the vicinity of some of the protein fluorophores to cause this static quenching. We therefore attempted to identify such fluorophores from fluorescence-quenching studies. It was necessary first to dissect out individual contributions of tyrosines and tryptophans to the observed fluorescence of the R123 protein. This was accomplished by recording the fluorescence spectra of R123 protein using 279 nm (tyrosine and tryptophan) and 295 nm (tryptophan) specific excitations. The two spectra were remarkably similar (Figure 5). The emission spectra obtained by exciting at 279 and 295 nm were superimposable exactly on normalizing them relative to emission at 360 nm, where the fluorescence originates from the tryptophans exclusively. This indicates that all the R123 fluorescence is from tryptophans and that tyrosines contributed very little if any to the overall fluorescence.

The fractional contribution to the overall protein fluorescence by solvent-accessible tryptophans was then estimated from the Lehrer plot (Figure 6A) using acrylamide as the collisional quencher. From these experiments it was determined that only 33% of the total fluorescence was quenchable and the remaining fluorescence was contributed by the buried, solvent-inaccessible tryptophans. The Stern–Volmer constant for tryptophan-fluorescence quenching was found out to be 1.2×10^{-5} M^{-1}.

The fraction of solvent-accessible tryptophans could also be determined from difference spectra of protein samples recorded from the aqueous and perturbant solvents containing 20% DMSO or 20% ethylene glycol (Figure 6B). The fraction of tryptophan residues accessible to the solvents were found (see Materials and Methods) to be 0.3 for both DMSO and ethylene glycol. We conclude that this fraction of solvent-accessible tryptophans accounts for 33% of the total protein fluorescence.

Nature of the TNS-Binding Site. From the study of the fluorescence characteristics of *Dm*-myb R123 protein and its TNS complex, it has been possible to deduce some features of the TNS-binding site on the R123 protein. Upon binding to R123, TNS quenches the protein fluorescence to the extent of 52%. This magnitude of quenching cannot be accounted for by the loss of fluorescence from the solvent-accessible tryptophans alone since their maximal contribution is only 33% to the overall protein fluorescence. When the protein–TNS complex was subjected to quenching, acrylamide could quench protein specific fluorescence further by only 8%. It can be deduced, therefore, that the quenching of overall protein fluorescence due to the TNS binding can be rationalized as

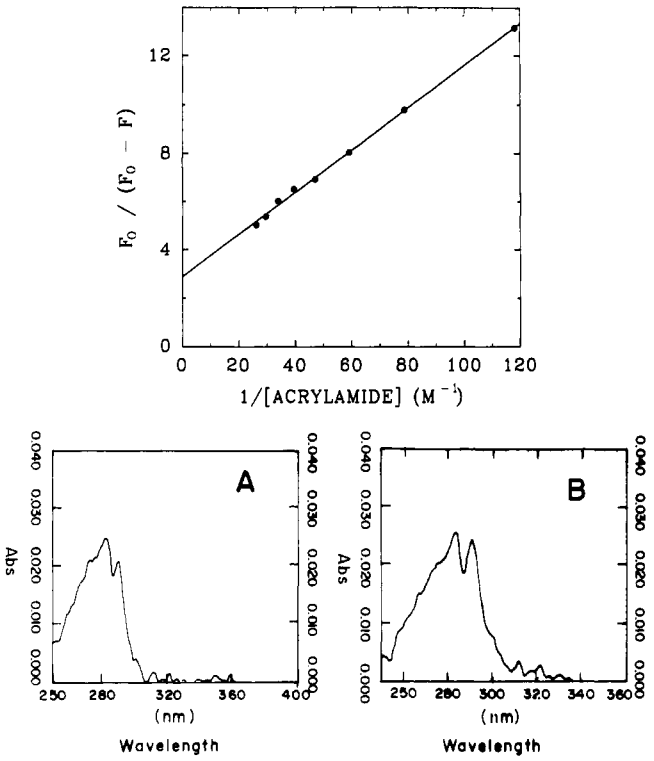

FIGURE 6: (A, top) Lehrer plots of quenching of the fluorescence emission of 50 μM native *Dm*-myb R123 protein by acrylamide. F_0 and F are fluorescence values at 342 nm in the absence and presence of the quencher. The value of f_a (fractional accessibility to the quencher) from the intercept of the plot was found to be 0.3. (B, bottom) UV difference spectrum (panel A) between 10 μM *Dm*-myb R123 protein and the protein in the presence of 20% ethylene glycol in 10 mM Tris·HCl buffer, pH 7.0. The UV difference spectrum between 10 μM Dm-myb R123 protein and the protein in presence of 20% DMSO in 10 mM Tris·HCl buffer, pH 7.0 is shown in panel B. The samples were incubated with the perturbants for 60 min before taking measurements. The fraction of the tryptophans accessible was calculated as described in Materials and Methods.

sum of two contributions: 25% (33% – 8%) of quenching takes place with the solvent-accessible tryptophans and the remaining 27% of the observed quenching is attributable to tryptophans secluded from the solvent. The TNS-binding site appears to be flanked by at least four tryptophans, contributed evenly by both buried and solvent-accessible tryptophans. Furthermore, at least one tyrosine residue is expected near the TNS-binding site since the excitation at 279 nm is effective in resonance energy transfer to the bound TNS.

DISCUSSION

TNS has been used extensively in protein chemistry as a locator of hydrophobic binding sites in view of its differential fluorescence properties. In the case of the *Dm*-myb DNA-binding domain, we could demonstrate only a single binding site with moderate affinity having a dissociation constant in the range of (5–8) × 10^{-7} M. This result is striking because recent work on one of the myb repeats, R3, by NMR (Ogata et al., 1992) showed that all the conserved tryptophans and some other residues contributed to the formation of a hydrophobic core. From the same and a related study (Sarai et al., 1993), it has been proposed that each of the three repeat units of R123 fold autonomously, retaining their individual features of secondary structure including a hydrophobic core. Our data on the overall structure of *Drosophila* R123 as revealed by CD (data not shown) are very similar to that of the *mouse* protein (Ogata et al., 1992). The observation that only a single TNS-binding site is present on *Dm*-myb R123 precludes the possibility that TNS was binding to the hydrophobic cores of individual repeats. On the other hand, it suggests that the single TNS-binding site could result from contributions made by one or more of the three individual repeats.

Our results allow us to classify all the tryptophans of R123 into at least three classes: (a) solvent exposed, (b) buried in the interior, and (c) accessible to TNS. From acrylamide-quenching experiments, in combination with data from solvent perturbation difference spectroscopy, we have shown that the fraction of the tryptophans in the *Dm*-myb R123 accessible to solvent is 0.3. This translates to an equivalent of three tryptophans being solvent exposed. TNS binding to R123 protein abolishes nearly 75% of the tryptophan fluorescence emanated from the solvent-exposed group. Similarly, 27% of overall protein fluorescence and ca. 40% of fluorescence emanated from the buried tryptophans is also quenched upon TNS binding. From this analysis it becomes clear that about four to five of the nine tryptophans are interacting with the bound TNS. The TNS-binding site has to satisfy a geometric requirement in that about four to five tryptophans and at least one tyrosine could interact with TNS in its protein-bound state. The TNS-binding site is therefore likely to be contributed to by at least two of the myb repeats. The protein-bound TNS was found to be solvent-accessible, as most of its fluorescence seen by excitation at 326 nm was quenchable with acrylamide. These results suggest that the TNS-binding site is a part of peripheral topography of the DNA-binding domain. The Stern–Volmer constants for the tryptophan and TNS-fluorescence quenching by acrylamide are seen to be different. This is not unexpected because the two fluorophores are different, and we cannot rule out the possibility that there might be subtle differences in their respective local environments. The observation that the binding of TNS and the sequence-specific target DNA are mutually exclusive under the experimental conditions studied focuses on the importance of TNS-binding site. The displacement of a bound TNS by its cognate target sequence can occur by at least two ways. One, that the target DNA needs the same site occupied by TNS and, two, that the displacement of TNS results due to secondary conformational changes induced on R123 protein by specific DNA binding. Our present data cannot distinguish between these two possibilities. We favor the first possibility, however, for the following reasons. Both TNS and specific-target DNA bind to the protein and each quench half the protein fluorescence. We could not record any compelling evidence in favor of a significant protein conformational change as a result of DNA binding. For instance, CD spectra have shown that basically the overall structure of R123 undergoes a marginal change, if any, upon specific-DNA binding. Similar results have also been reported in the case of mouse R123 (Sarai et al., 1993). At least in this respect, the binding of TNS and specific-target DNA to R123 protein appears to be similar. An independent motivation for the TNS to occupy a DNA binding site may be due to both an anionic (SO_3H) and a hydrophobic group co-existing in the same molecule that can mimic at least in part some features of a nucleotide.

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