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Lactose Repressor Protein Modified with Dansyl Chloride: Activity Effects and Fluorescence Properties†

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ABSTRACT: Chemical modification using 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride) has been used to explore the importance of lysine residues involved in the binding activities of the lactose repressor and to introduce a fluorescent probe into the protein. Dansyl chloride modification of *lac* repressor resulted in loss of operator DNA binding at low molar ratios of reagent/monomer. Loss of nonspecific DNA binding was observed only at higher molar ratios, while isopropyl β-D-thiogalactoside binding was not affected at any of the reagent levels studied. Lysine residues were the only modified amino acids detected. Protection of lysines-33 and -37 from modification by the presence of nonspecific DNA correlated with maintenance of operator DNA binding activity, and reaction of lysine-37 paralleled operator binding activity loss. Energy transfer between dansyl incorporated in the core region of the repressor protein and tryptophan-201 was observed, with an approximate distance of 23 Å calculated between these two moieties.

The lactose repressor protein regulates the transcription of the *lac* metabolizing enzymes in *Escherichia coli* by specific interaction at the operator site in the genome (Miller & Reznikoff, 1980). This binding is modulated by the presence of sugar ligands bound to the tetrameric repressor protein (M_r ~150 000); inducers decrease the affinity of the protein for operator DNA, while antiinducers stabilize the repressor–operator complex (Miller & Reznikoff, 1980). The interaction of operator DNA with repressor protein is salt dependent, and six to eight ion pairs have been estimated to participate in complex formation (Record et al., 1977; Barkley et al., 1981; Winter & von Hippel, 1981). Thus, positive charges on amino acid side chains in the repressor would be expected to play a role in binding of the protein to the polyacidic backbone of the target DNA. The importance of specific lysine residues has been suggested by genetic (Miller, 1979) and recent chemical studies (Whitson et al., 1984). Two domains within the repressor, the NH_2-terminus (amino acids 1–59) and the core protein (amino acids 60–360), can be isolated by mild proteolytic digestion (Platt et al., 1973; Geisler & Weber, 1977). The NH_2-terminus binds to nonspecific DNA and operator-containing DNA (Jovin et al., 1977; Ogata & Gilbert, 1979), while the core protein binds to inducer and to operator DNA (Platt et al., 1973; Matthews, 1979). The two tryptophans in the repressor monomer are located in the core domain of the protein (Beyreuther et al., 1975); an amber mutation has been isolated which can be suppressed in the appropriate bacterial strain with consequent introduction of a tyrosine at the site normally occupied by Trp-220 (Sommer et al., 1976). With this mutant protein, any energy transfer to other fluorescent moieties must be from Trp-201. In this study, we have reacted the repressor molecule with 5-(dimethyl-

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amino)naphthalene-1-sulfonyl chloride (dansyl chloride)[1] (1) to determine the effects of modification on binding activity, (2) to incorporate a fluorescent probe into the repressor in order to monitor the local environment and any changes in response to binding, and (3) to obtain information regarding the structural arrangement of the protein.

Materials and Methods

Chemicals. Dansyl chloride and IPTG were purchased from Sigma. [^{14}C]IPTG and [*methyl*-^{14}C]dansyl chloride were purchased from Research Products International. [*methyl*-^{3}H]Dansyl chloride was purchased from New England Nuclear. 2-(Chloromercuri)-4-nitrophenol was purchased from Eastman, recrystallized from water before use, and maintained at 4 °C. Calf thymus DNA and poly[d(A-T)] were obtained from Sigma; the DNA solutions were passed repeatedly through a 16-gauge needle (15–20 times) for shearing to uniform size ($\sim 15 \times 10^6$ daltons).

Isolation of Lactose Repressor. Repressor was isolated from *Escherichia coli* CSH 46 according to the methods described by Rosenberg et al. (1977) with modifications (O'Gorman et al., 1980). The isolated repressor was >95% pure by SDS gel electrophoresis (Weber et al., 1972; Laemmli, 1970). Depending on the modification conditions to follow, portions of protein were thawed and dialyzed into appropriate buffer immediately before use. Buffer was flushed with nitrogen to prevent the oxidation of cysteine during dialysis.

Assay of Repressor. IPTG binding activity was determined by the ammonium sulfate precipitation method described by Bourgeois (1971). The operator DNA binding assay was performed by using [^{3}H]thymine-labeled λp*lac*5 DNA with nitrocellulose filtration methods (Hsieh & Matthews, 1981; Riggs et al., 1968). The operator DNA concentration in the binding reaction mix was $(1.1–1.9) \times 10^{-11}$ M. Nonspecific DNA binding assays were performed by using nitrocellulose filters as described previously (O'Gorman et al., 1980). Percent operator or nonspecific DNA binding activity was determined by comparing the amount of modified protein required to retain 50% of the total DNA bound to the same value for unmodified protein. The protein concentration was determined either by the absorbance at 280 nm ($\epsilon_{280nm}^{0.1\%} = 0.59$), by the Folin–Ciocalteau reaction (Chou & Goldstein, 1960), or by Bio-Rad Coomassie blue assays (Bradford, 1976).

Isolation of Core Protein. The core repressor protein was purified according to the method described previously (Matthews, 1979; Hsieh & Matthews, 1981). The isolated core protein exhibited inducer-sensitive operator DNA binding, which was minimally affected by the addition of poly[d(A-T)]. The concentration of the core protein was determined by the absorbance at 280 nm ($\epsilon_{280nm}^{0.1\%} = 0.51$).

Amino Acid Analysis. Protein samples were hydrolyzed in 6 N HCl for 20 h at 110 °C followed by removal of the HCl under vacuum (Spackman et al., 1958). Analysis was carried out on a Beckman 120C amino acid analyzer, on a Glenco amino acid analyzer equipped with a 0.32 × 30 cm column of DC-4A resin and Pico-Buffer System II (from Pierce), or on a Beckman 121 MB amino acid analyzer equipped with a Spectra-Physics Model 4100 data acquisition system at the Protein Sequencing Center, Department of Zoology, The University of Texas at Austin.

Reaction with Dansyl Chloride. Dansyl chloride solution of 0.2 M was made fresh in dimethylformamide. Appropriate portions were added to repressor (1 mg/mL) in 0.24 M potassium phosphate, pH 8.0, and 5% glucose; the solution was adjusted to 10% dimethylformamide. Final solution pH was 8.4. The reaction was carried out at room temperature followed by addition of 0.1 volume of 1 M Tris-HCl, pH 7.8. Hydrolyzed reagent was removed by Sephadex G-25 chromatography or by extensive dialysis until no fluorescence was observed in the outside buffer under ultraviolet light.

Determination of Extent of Dansyl Incorporation. Appropriate amounts (~0.01 mCi) of either [^{3}H]- or [^{14}C]dansyl chloride were added to the dansyl chloride stock solution (0.02 M) used for reaction with protein. Following modification, the hydrolyzed reagent was removed by extensive dialysis. The radioactivity retained in the dialysis bag with repressor was determined and the resulting value converted to moles of DNS by comparison to a portion of the known stock solution counted under the same conditions. The values for DNS bound per monomer were obtained by dividing the amount of DNS bound by the amount of monomer present. In addition, the bound DNS concentration was determined by the fluorescence in 8 M urea using a standard curve generated by using known concentrations of DNS-lysine.

Measurement of Absorbance and Fluorescence Spectra. Absorbance spectra were measured on a Cary 118 spectrophotometer using 1-cm path-length quartz cuvettes. Fluorescence excitation and emission spectra were obtained by using an SLM 400 fluorometer and were recorded on a Hewlett Packard 9825A calculator. The fluorescence lifetime was measured on an SLM Model 4800 fluorometer equipped with a phase modulator at the Department of Medicine, Baylor College of Medicine.

Peptide Mapping by High-Pressure Liquid Chromatography. Repressor protein in 0.1 M ammonium bicarbonate, pH 7.8, was digested with trypsin and lyophilized. The peptides were dissolved into elution buffer, 0.05 M ammonium acetate, pH 7.0. Peptides were separated by using a Laboratory Data Control HPLC system on a Brownlee aquapore RP-300 (4.6 mm × 25 cm) column equipped with an RP-300 guard column (4.6 mm × 3 cm). The sample injected ranged from 0.2 to 2.0 mg in 25–150 μL. The elution gradient applied was from 20% to 60% acetonitrile (40–60 mL each) in the same buffer. The dansyl-containing peptides were observed by using a 365-nm filter; each peptide was collected manually and the solution lyophilized. Depending on the purity of the peptide, a second chromatography on the column with isocratic elution was performed prior to amino acid analysis.

Isolation of Mutant A220 Repressor. Mutant A220 repressor was isolated from strain A220 (obtained from Dr. P. Lu, University of Pennsylvania) according to the methods described by Sommer et al. (1976).

Results

Reaction of Repressor with Dansyl Chloride. Reaction of repressor with dansyl chloride was complete within 2 min, and the extent of modification was not changed up to 1 h of reaction time at pH 8.4. The effects of dansyl chloride modification on the various binding activities of the repressor are shown in Figure 1. IPTG binding affinity was minimally affected by modification, while partial loss of nonspecific DNA binding activity was observed over the same range of reagent concentrations. In contrast, for operator DNA binding, ~50% of the activity was lost at a 5-fold molar ratio of reagent, and essentially no activity remained at a 32-fold molar ratio. The extent of dansyl incorporation per repressor monomer is shown

[1] Abbreviations: dansyl or DNS, 5-(dimethylamino)naphthalene-1-sulfonyl; IPTG, isopropyl β-D-thiogalactoside; ONPF, *o*-nitrophenyl β-D-fucoside; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

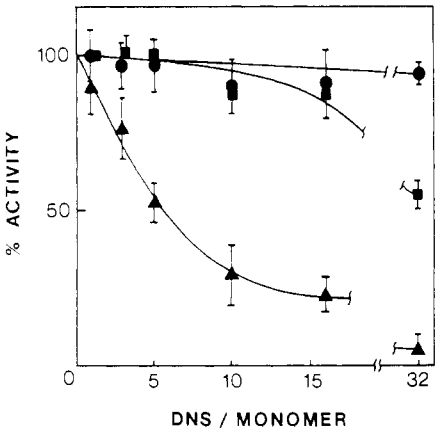

FIGURE 1: Binding characteristics of DNS-modified repressor. The binding activity of protein modified at increasing molar ratios of dansyl chloride was monitored. Reaction (15 min at pH 8.4) and subsequent assays were carried out as described under Materials and Methods. (●) IPTG (inducer) binding activity; (■) nonspecific DNA binding activity; (▲) operator DNA binding activity. Values represent mean ± standard deviation of two to six measurements on different samples.

Table I: Dansyl Equivalents Incorporated

molar ratio of reagent to repressor monomer	equiv of dansyl incorpd per monomer		
	radiolabel[a]	fluorescence[b]	HPLC[c]
5	0.2	0.5	0.3
10	0.8	1.0	1.2
16	1.6	1.5	1.9
32	4.5		3.6

[a] [^{3}H]Dansyl chloride was reacted with repressor and the extent of radiolabel incorporated determined as described under Materials and Methods. [b] Fluorescence of the dansylated repressor in 8 M urea was compared to a standard curve generated by using dansyllysine. [c] The areas under each of the HPLC peaks detected at 365 nm were summed to yield a total extent of incorporation.

in Table I. The only modified amino acid which could be identified following acid hydrolysis and thin-layer chromatography using commercially available dansyl-labeled amino acids as standards was ε-dansyllysine. Modification in the presence of inducer (IPTG) or antiinducer (ONPF) did not cause any change in the extent of incorporation or alter effects on binding activities. However, reaction in the presence of nonspecific DNA protected the protein from the loss of operator DNA binding activity. At a 16-fold molar ratio, >70% of the operator binding was preserved in the presence of nonspecific DNA (calf thymus DNA) as compared to ~20% binding activity without nonspecific DNA in the reaction mix (Figure 2). The total extent of dansyl incorporation was decreased by ~15%. No reaction of reagent was observed with the calf thymus DNA.

Identification of Modified Peptides. High-pressure liquid chromatography of the trypsin-digested dansylated repressor showed five major peaks and several minor peaks when monitored at 365 nm (Figure 3). By separation of the NH_2-terminus and the core protein prior to exhaustive trypsin digestion and chromatography, peaks could be assigned to NH_2-terminal (peaks 1 and 5) and core peptides [peaks 2–4 and small amounts (~20%) of a core peptide in peak 5]. Amino acid analysis of peptides isolated by subsequent isocratic elution allowed assignment of several of the peptides to sites in the primary structure. The NH_2-terminal peptides contained lysines-33 (peak 5) and -37 (peak 1), while two of the peaks assigned to core protein contained lysines-108 and -324/327. The remaining peaks could not be assigned. Modification of lysine-37 (peak 1) most closely paralleled loss

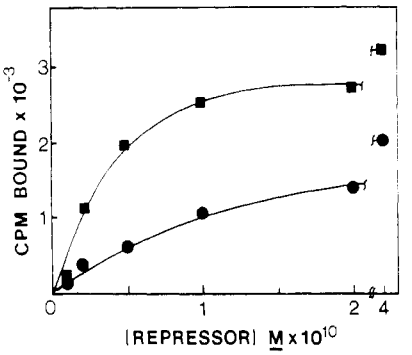

FIGURE 2: Protection of operator DNA activity by the presence of nonspecific DNA in the reaction mix. Repressor (1 mg/mL) was modified with a 16-fold molar ratio of dansyl chloride and subsequently assayed for operator DNA binding activity as described under Materials and Methods. The amount of radiolabeled DNA bound in the presence of inducer has been subtracted to produce these binding curves. Operator DNA (λ*plac*) concentration was 1.5×10^{-11} M. (●) Repressor modified with dansyl chloride; (■) repressor modified with dansyl chloride in the presence of calf thymus DNA (0.16 mg/mg of repressor).

in operator activity (Figure 4). Both lysine-33 (~35%) and lysine-37 (~50%) in the NH_2-terminus were significantly protected from reaction by the presence of nonspecific DNA in the reaction mix as indicated by decreased peak areas in the HPLC elution profile (Figure 5). Only small changes in the peak area of modified peptides were observed in the presence of inducer. The majority of dansyl incorporation at a 16-fold molar ratio (>75%) was located in the core region. Calculation of total modification by summing peak areas corresponded well with dansyl incorporation values measured directly at various reagent concentrations (Table I).

Spectral Studies of Dansyl Repressor. The dansylated repressor showed an additional absorption maximum at 340 nm. Both repressor and dansyl repressor exhibited similar tryptophan emission spectra with a maximum at 342 nm when excited at 285 nm (Figure 6). The quantum yield of the peak corresponding to tryptophan emission decreased as the extent of modification increased. An additional emission peak attributable to dansyl emission, with a maximum at 510 nm, could be observed in dansyl-modified repressor; the quantum yield for this peak increased as the extent of modification increased. Excitation at 340 nm yielded the dansyl spectra shown in the inset in Figure 6. A high quantum yield was observed with excitation at 340 nm as compared to excitation at 285 nm using the same spectrofluorometer settings. Addition of IPTG caused a blue shift of the tryptophan emission maximum from 342 to 336 nm in both native and dansyl-modified repressor (Figure 6). Minimal wavelength shifts in response to inducer were observed in dansyl spectra whether excitation was at 285 or 340 nm (Figure 6, inset).

Evidence for Energy Transfer. Evidence for energy transfer between tryptophan residues and incorporated DNS was provided by (1) the decrease in the tryptophan quantum yield, (2) a corresponding decrease of tryptophan lifetime, (3) an increase in the region of the dansyl repressor excitation spectrum corresponding to tryptophan absorbance, and (4) the concomitant increase of the dansyl repressor emission spectrum when excited at 285 nm. Transfer efficiencies (E) were calculated by using the three methods shown in Table II for repressor reacted at two molar ratios of dansyl chloride. The first method gives values significantly larger than those obtained by the other two means of determining E. Method C is the most accurate, as it monitors the acceptor molecule rather than the donor and is thus not subject to any additional

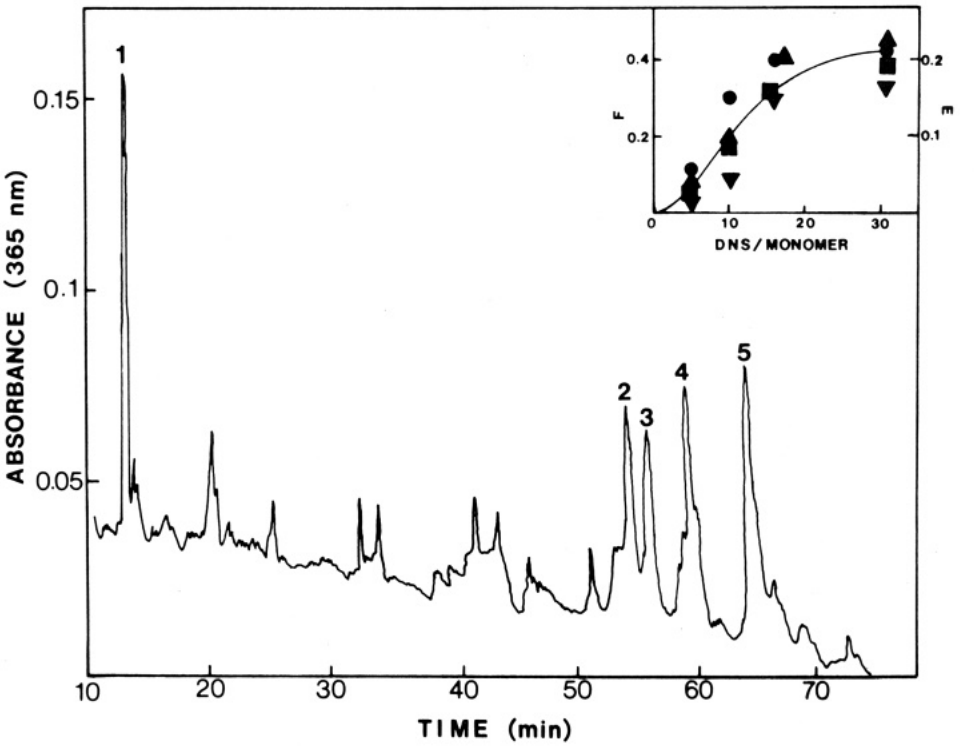

FIGURE 3: Elution profile of trypsin-digested DNS-modified repressor from high-pressure liquid chromatography. HPLC was performed as described under Materials and Methods. The absorbance of the effluent was monitored at 365 nm. The column was equilibrated with 0.05 M ammonium acetate, pH 7.0; following sample injection, a gradient from 20% to 60% acetonitrile was utilized for elution at a rate of 2.0 mL/min. (Inset) Fractional modification of each peptide determined relative to the amount of peak 1 reacted at a 32-fold molar ratio of DNS/monomer: (▼) peak 3; (▲) peak 4; (■) peak 5. Modification of the lysines in these three peaks correlated with the efficiency of energy transfer (●).

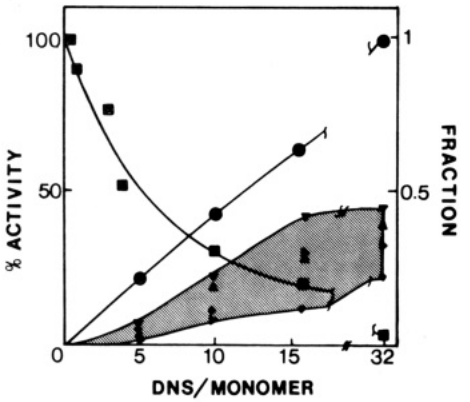

FIGURE 4: Comparison of modification of HPLC peaks with operator activity loss. The operator activity (■) was determined as described in Figure 1. Fraction corresponds to the area of the peak observed on HPLC runs at the indicated molar ratios of dansyl chloride relative to the peak 1 area at 32-fold reagent: (●) peak 1; (♦) peak 2; (▶) peak 3; (▼) peak 4; (▲) peak 5. The shaded area represents peaks 2–5 whose reaction does not parallel the decrease in activity. The modification of peak 1 most closely correlated with the loss in operator DNA binding activity; this peak contained the NH_2-terminal peptide with lysine-37.

influences on donor fluorescence.

Characteristics of Mutant A220 Repressor and Dansyl Chloride Modification. The presence of two tryptophan and multiple dansyllysine residues in each repressor monomer complicates distance calculations based on energy transfer; in order to identify which tryptophan was involved in this process, we utilized a tryptophan nonsense mutant suppressed to yield a tyrosine at the tryptophan-220 site (mutant A220). The A220 repressor possesses affinity for operator DNA similar to the wild-type repressor but decreased inducer binding activity (~20-fold; Sommer et al., 1976). The emission spectra

Table II: Transfer Efficiency of DNS-Modified Proteins

	x-fold molar ratio of [DNS]/[monomer]	% transfer efficiency using method A[a]	B[b]	C[c]	R^d (Å)
repressor	10	41.3	22.2	15.0	23.4
	16	57.0	35.2	21.3	
A220 repressor[e]	10	39.6	20.0	15.9	23.2
	16	52.8	30.6	21.7	
core protein	10	40.9	26.3	20.4	22.7
	16	55.5	39.0	23.2	

[a] $E = 1 - Q_{DA}/Q_D$. [b] $E = 1 - \tau_{DA}/\tau_D$. [c] $E = F_{DA_{285}}{}^{510}f_{A_{340}}I_{340}A_{340}/F_{A_{340}}{}^{510}f_{D_{285}}I_{285}A_{285}$. For details of the calculation, see Tu et al. (1978). [d] $R = R_0(E^{-1} - 1)^{1/6}$, where R_0 is the Forster distance (Forster, 1966) at which 50% energy transfer occurs and was calculated according to Tu et al. (1978) with $\kappa^2 = {}^2/_3$, $n = 1.354$, $Q_D = 0.21$, and $J_v = 5.1 \times 10^{-15}$ cm^3 M^{-1}; $R_0 = 23.9$ Å. [e] A220 repressor is a mutant repressor with a tyrosine inserted at tryptophan-220.

Table III: Characteristics of A220 Repressor and Wild-Type Repressor

	wild-type repressor	A220 repressor
$\epsilon^{0.1\%}_{280nm}$	0.59 ± 0.02	0.46 ± 0.02[a]
emission max (nm)	342	338
emission max (+IPTG) (nm)	336	338
lifetime[b] (ns)	7.0	5.3

[a] Obtained from Sommer et al. (1976). [b] Measured with a phase-modulated fluorometer. The excitation wavelength was 285 nm. The filter used for emission was a Corning 7-60 filter.

at various molar excesses of dansyl chloride are shown in Figure 7. The tryptophan emission maximum was blue shifted relative to wild-type repressor, with a maximum at 338 nm (vs. 342 nm). Neither the tryptophan nor the dansyl emission spectra were IPTG sensitive (Sommer et al., 1976); thus, Trp-201 appears not to be involved in or affected by inducer binding. A comparison of wild-type repressor and A220 re-

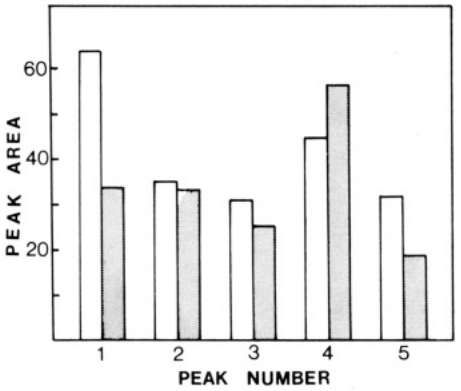

FIGURE 5: Peak areas from HPLC elution profile in the presence and absence of nonspecific DNA. HPLC was carried out as described under Materials and Methods. The areas for each peak (see Figure 3) were measured for modification with a 16-fold molar ratio of DNS/monomer for repressor and repressor–calf thymus DNA. The quantities of protein applied to the HPLC column were identical for each run. Open bars, repressor modified with dansyl chloride; stippled bars, repressor modified with dansyl chloride in the presence of calf thymus DNA (0.16 mg/mg of repressor). Reaction with peptides containing lysine-33 (peak 5) and lysine-37 (peak 1) was diminished by the presence of nonspecific DNA.

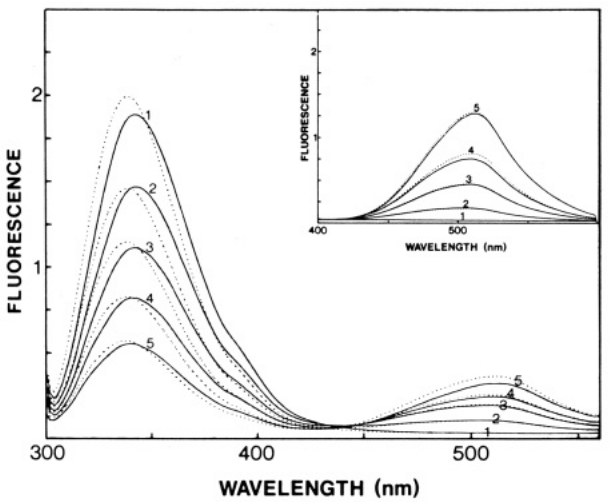

FIGURE 6: Emission spectra of native and DNS-modified repressor. Fluorescence spectra were measured in 0.048 M potassium phosphate, pH 8.0, 5% glucose, and 0.1 mM dithiothreitol using a protein concentration of 0.1 mg/mL. Spectra were determined on an SLM 400 fluorometer using a 4-nm band-pass and an excitation wavelength of 285 nm. Solid lines are spectra of repressor and DNS-modified repressors. The dotted lines are spectra of repressor and DNS-modified repressors in the presence of IPTG (10^{-3} M). Inset: Emission spectra of native and DNS-modified repressor. Excitation wavelength was 340 nm. Solid lines, repressor or DNS-modified repressor; dotted lines, repressor or DNS-modified repressor in the presence of IPTG. (1) Repressor; (2) 5-fold DNS-modified repressor; (3) 10-fold DNS-modified repressor; (4) 16-fold DNS-modified repressor; (5) 32-fold DNS-modified repressor.

pressor is shown in Table III. A decrease of both quantum yield and the lifetime of tryptophan for the A220 repressor was observed upon modification by dansyl chloride; the efficiency of energy transfer calculated from these parameters is shown in Table II. The values obtained for the A220 repressor compare favorably to those for the wild-type repressor. Peptide maps of the modified A220 protein were similar to the pattern observed for wild-type repressor.

Modification of Tryptic Core Protein with Dansyl Chloride. To determine the placement of dansyllysines participating in energy transfer, the core protein was isolated following trypsin digestion and reacted with dansyl chloride. Modification resulted in a decrease of both quantum yield and the lifetime

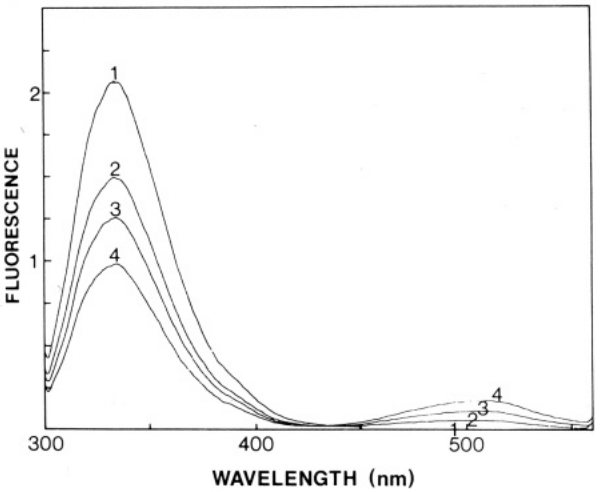

FIGURE 7: Emission spectra of A220 repressor and DNS-modified A220 repressor. Spectra were measured on protein (0.1 mg/mL) in 0.048 M potassium phosphate, pH 8.0, 5% glucose, and 0.1 mM dithiothreitol. Excitation wavelength was 285 nm. Fluorescence was measured on an SLM 400 fluorometer using a 4-nm band-pass. Dotted line spectra (not visible under solid line) were in the presence of 10^{-3} M IPTG. (1) A220 repressor; (2) 5-fold DNS-modified A220 repressor; (3) 10-fold DNS-modified A220 repressor; (4) 16-fold DNS-modified A220 repressor.

of tryptophan fluorescence for the core protein. The calculated efficiency of energy transfer is shown in Table II. The similarity in the core transfer efficiencies to those for intact repressor coupled with HPLC peptide patterns similar to those for core domain isolated from modified intact repressor demonstrates that the dansyllysine involved in energy transfer with tryptophan is in the core domain.

Distance Calculation. If only one dansyl incorporated is involved in energy transfer and the reaction is stoichiometric at the lysine involved, the approximate distance between the tryptophan(s) and dansyl can be calculated (see Table II). Comparison of the efficiency of energy transfer calculated by method C with modification of each lysine residue detected by HPLC indicated three peaks whose extent of reaction paralleled efficiency (Figure 3, inset); two of these peaks contained exclusively single core peptides, while peak 5 was a mixture of core (~20%) and NH_2-terminal (~80%) peptides. Since energy transfer was similar for core protein and intact repressor, the modification of NH_2-terminus lysine residues does not result in energy transfer with tryptophan. The dansyl incorporation at each of the remaining two core peptides was similar at a 16-fold excess of reagent (approximately 0.4 dansyl at each site). When the efficiency of energy transfer on the basis of this correlation is corrected, the distance (R) between the dansyl moiety and tryptophan involved in the transfer process was calculated for wild-type and A220 repressors and the core protein (Table II), with substantial agreement obtained among these values. The multiple assumptions involved in the distance calculation (i.e., that orientation is random, that only one dansyllysine is involved, etc.) preclude placing undue significance on this figure. However, the core placement of the modified lysine which serves as acceptor and the identification of Trp-201 as donor are unaffected by these assumptions; these data provide useful information regarding the relative arrangements of the protein domains.

DISCUSSION

The positively charged ϵ-amino groups of lysines in the acidic repressor protein ($pK = 5.6$) potentially dissipate charge repulsion between the polyacidic DNA and the protein and may

be involved in formation of ion pairs between these macromolecules. Genetic studies have suggested that of the 11 lysine residues in the protein, those at positions 2, 33, 84, and 290 may be important for operator binding capacity (Miller et al., 1979; Miller & Schmeissner, 1979; Miller, 1979), while chemical studies have indicated that modification of lysines-33, -108, and (to some extent) -37 affects operator activity (Whitson et al., 1984). In this study, we have modified the lysine residues of the lactose repressor with dansyl chloride. The modification resulted in loss of operator DNA binding and partial loss of nonspecific DNA binding with retention of inducer binding activity.

The maintenance of IPTG binding after dansyl incorporation indicated that the lysines modified in the repressor were not essential for sugar binding, and the absence of inducer effects on the dansyl fluorescence spectra demonstrated that the environment of the incorporated dansyl was not affected by the conformational change elicited by inducer. Further, no difference was observed on either dansyl incorporation or binding activities if inducer was included in the reaction mix. Nonspecific DNA binding was only slightly affected at low levels of modification (<2 mol of dansyl incorporated/mol of monomer); a 50% loss was observed with 4.5 dansyls incorporated per monomer. This high extent of modification may alter the protein conformation instead of reflecting the essential role of the reacted lysine residues. In contrast, operator DNA binding was significantly affected by modification; at low molar ratios of reagent (<0.5 mol of dansyl incorporated/mol of monomer), 50% of this binding capacity was lost. Protection of operator DNA binding capacity from dansyl chloride inactivation was noted when nonspecific DNA was present in the reaction mix; concurrently, a small decrease was observed in the extent of dansyl incorporated. Reaction at lysines-37 and -33 was diminished by the presence of DNA with protection of operator binding, and the modification of lysine-37 paralleled the decrease in operator binding observed in these studies. These residues are in the region of the NH_2-terminus implicated in operator DNA interaction by genetic (Miller, 1979; Pfahl et al., 1974) and chemical (Whitson, et al., 1984) methods and by analogy to other DNA binding proteins (Anderson et al., 1982; Sauer et al., 1982; Matthews et al., 1982; Weber et al., 1982).

The differential nature of operator and nonspecific DNA binding must be considered in viewing these data. Repressor–operator DNA interaction consists of more than 50% nonelectrostatic interaction and theoretically involves 6–8 salt linkages (Winter & von Hippel, 1981; Record et al., 1977; Barkley et al., 1981), whereas repressor–nonspecific DNA binding involves only electrostatic energy components (11–12 salt linkages) (de Haseth et al., 1977; Revzin & von Hippel, 1977). Binding of repressor to nonspecific and operator DNA apparently occurs in a different fashion, but an overlap of amino acid residues involved in these two binding processes would be expected. Protection from modification in the presence of calf thymus DNA and the concomitant protection from activity loss suggest that one or both lysine residues (Lys-33 and Lys-37) are required for operator binding interactions. The correlation of lysine-37 reaction with diminished operator DNA binding further implicates this residue. Interestingly, the protection of operator binding activity by nonspecific DNA occurs under conditions where minimal effects are observed on the nonspecific DNA binding activity, although the protection itself suggests proximity of the nonspecific DNA to the lysines involved. Whether protection involves direct contact between these repressor lysines and nonspecific DNA and whether they are directly involved in operator contact or simply perform a role in maintaining structural integrity are not discernible from these studies; however, the absence of any effect on other binding activities at low molar ratios of reagent suggests that direct contact between the affected lysines and operator DNA is probable.

Several lines of evidence indicate resonance energy transfer occurs between the tryptophan and dansyl incorporated in the protein. Accurate distance measurements based on transfer efficiencies are complicated by several factors, including the presence of two tryptophans at positions 201 and 220 in the primary sequence of the repressor and multiple sites of dansyl incorporation. To clarify which groups were involved in the energy transfer process, we determined the efficiency of energy transfer in dansyl-modified A220 repressor (in which tryptophan-220 has been replaced with tyrosine) and in the isolated core domain; these values are comparable to those obtained for the wild-type repressor. These data and the similarity in modified peptide patterns among these proteins lead to the conclusion that the dansyl invoived in the energy transfer process is in the core region and that the tryptophan is Trp-201. The core placement of the lysine residue(s) involved in energy transfer is not surprising given the apparent molecular arrangement of the repressor protein with the NH_2-termini on the ends of an elongated tetrameric core domain (Charlier et al., 1980; Pilz et al., 1980; McKay et al., 1982). Whether the transfer is intra- or intersubunit cannot be discerned from the data. By assuming that only one of the dansyl groups in the core domain is involved in energy transfer and correcting to stoichiometric incorporation, a distance of ~23 Å between Trp-201 and the dansyllysine can be calculated if random orientation of this donor/acceptor pair is assigned.

In summary, we have specifically modified lysine residue in repressor with dansyl chloride. Protection experiments and the correlation of operator DNA binding activity loss with modification sites demonstrate the requirement for lysine residues 33/37 in operator DNA binding. While an essential role in maintaining the structural integrity of the protein cannot be ruled out, both genetic and chemical data suggest a direct, possibly electrostatic, role for these lysines in binding to DNA. Energy transfer between tryptophan-201 and dansyl incorporated into the core region of the protein was observed and used to calculate an approximate distance of 23 Å between these two residues.

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Registry No. Trp, 73-22-3; lysine, 56-87-1; dansyl chloride, 605-65-2.

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Measurement of DNA–Protein Equilibria Using Gel Chromatography: Application to the *Hin*fI Restriction Endonuclease†

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ABSTRACT: A method is described for measuring equilibrium constants of DNA–protein interactions using gel chromatography. This technique has been used to study the sequence-specific interaction of the *Hin*fI restriction endonuclease with DNA. *Hin*fI has a monomeric molecular weight of 31 000 and exists as a dimer in its active form. The protein binds to supercoiled DNA molecules containing its recognition site with an apparent free energy of −13.9 kcal/mol of sites. This interaction is highly salt sensitive and causes a release of 3.4 ion pairs. The affinity of the nuclease for its recognition site is largely independent of both pH (6.5–8.5) and temperature (7–35 °C) and was not affected by variations in the degenerate middle position of the site. Linear DNA fragments containing the *Hin*fI recognition site were bound as tightly as supercoiled molecules. Binding to nonspecific DNA sites or to methylated DNA sites was approximately 6 orders of magnitude weaker. In general, enzyme activity and binding affinity paralleled each other.

Type II restriction endonucleases provide good model systems for the study of sequence-specific recognition of DNA by proteins. One of the first steps in examining such interactions is the accurate determination of the equilibrium binding constants with specific and nonspecific DNA sites.

The most widely used method employs nitrocellulose filter binding (Riggs et al., 1970). This method is based on the observation that DNA alone will pass through a nitrocellulose filter while protein–DNA complexes are retained. A variety of other techniques have been described in addition to filter binding. Ackers et al. (1983) have developed methods for the

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