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Trinitrobenzenesulfonate Modification of the Lysine Residues in Lactose Repressor Protein†

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ABSTRACT: Modification of the lysine residues in the lactose repressor protein has been carried out with trinitrobenzenesulfonate. Reaction of lysine residues at positions 33, 37, 108, 290, and 327 was observed. Inducer binding was increased by modification with this reagent, while both nonspecific DNA binding and operator DNA binding were diminished, although to differing degrees. The loss in operator DNA binding capacity was complete with modification of ~2 equiv of lysine per monomer. The extent of reaction was affected by the presence of both sugar and DNA ligands; binding activities of the modified protein and reaction pattern of the lysines were perturbed by these ligands. The presence of operator or nonspecific DNA during the reaction protected against specific and nonspecific DNA binding activity loss. This protection presumably occurs by steric restriction of reagent access to lysine residues which are essential for both nonspecific and operator binding interactions. Lysines-33 and -108 were protected from modification in the presence of DNA. These experiments suggest that the charge on the lysine residues is important for protein interaction with DNA and that steric constraints for operator DNA interaction with the protein are more restrictive than for nonspecific DNA binding. In contrast, inducer (isopropyl β-D-thiogalactoside) presence partially protected lysine-290 from modification while significantly enhancing reaction at lysine-327. Conformational alterations consequent to inducer binding are apparently reflected in these altered lysine reactivities.

The lactose repressor protein regulates the expression of the genes coding for the *lac* enzymes in *Escherichia coli* by binding with high affinity to the operator sequence in the DNA (Miller & Reznikoff, 1980). The repressor is a tetramer of identical subunits (monomer M_r ~37 500). In the presence of inducer molecules, the affinity of the repressor protein for the operator sequence of the DNA is diminished, and the excess of nonoperator regions in the *E. coli* genome can compete effectively with operator for binding to repressor-inducer complex. The characteristics of the interaction of repressor with inducer, nonspecific DNA, and operator DNA have been widely examined. The affinities of various nonspecific DNAs and operator-containing DNAs have been measured (Miller & Reznikoff, 1980; Winter & von Hippel, 1981); the salt dependence of binding of these different DNA species varies

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considerably (deHaseth et al., 1977a,b; O'Gorman et al., 1980; Barkley et al., 1981).

The roles of specific amino acid residues in the functioning of the lactose repressor have been examined by using genetic methods (Müller-Hill et al., 1977; Pfahl et al., 1974; Coulondre & Miller, 1977; Miller, 1979; Miller et al., 1979) and chemical modification (Fanning, 1975; Hsieh & Matthews, 1981; O'Gorman & Matthews, 1977; Manly & Matthews, 1979; Burgum & Matthews, 1978; Brown & Matthews, 1979; Alexander et al., 1977). Cysteine residue participation in lactose repressor function has been explored with a variety of reagents (Manly & Matthews, 1979; Burgum & Matthews, 1978; Brown & Matthews, 1979). The role of tyrosine residues has been examined by using iodination and nitration reactions (Fanning, 1975; Hsieh & Matthews, 1981; Alexander et al., 1977); the un-ionized phenol group of these residues appears to be required for maintenance of DNA binding activity in the protein (Hsieh & Matthews, 1981). Genetic studies have indicated the essential nature of several tyrosine residues in DNA binding activity (Coulondre & Miller, 1977; Miller et al., 1979); substitution of these residues with other amino acid side chains results in decreased affinity for DNA. Results from 2,3-butanedione modification of repressor suggest that arginine residues are also involved in DNA binding (P. A. Whitson and K. S. Matthews, unpublished data).

The specific importance of lysine residues in DNA binding activity has been examined by genetic methods; of the 11 lysines in each repressor monomer, those at positions 2, 33, and to a lesser extent 84 and 290 appear to be essential for DNA binding (Pfahl et al., 1974; Coulondre & Miller, 1977; Miller, 1979; Miller et al., 1979, 1978; Schmitz et al., 1978; Miller & Schmeissner, 1979). The proteins with substitutions in these positions are at least partially i^- in phenotype. The importance of the basic amino acids in the interaction of the lactose repressor protein with DNA can be deduced from the requirement for the protein to interact with the polyacidic nucleic acid. Basic groups on the surface of the protein must be available to participate in ion pairs with the phosphate groups of the DNA. The ionic component of the binding reaction provides approximately 50% of the energy of binding of the repressor with operator DNA and essentially all of the binding energy for nonspecific DNA (deHaseth et al., 1977b; Barkley et al., 1981). Effects of lysine modification on repressor binding to specific and nonspecific DNA as well as inducer may provide insight into the participation of these residues in repressor function. In the absence of an X-ray crystallographic structure or a detailed NMR analysis of the intact protein, implication of specific side chains in the various functional roles of the protein rests on the combination of genetic and chemical data. Trinitrobenzenesulfonate reacts with lysine to yield a product that is neutral in charge (Plapp et al., 1971); the advantage of this reagent is the introduction of a spectral probe into the structure of the protein to provide a reporter group for location of the modified residues in the primary structure. The lactose repressor has therefore been modified with trinitrobenzenesulfonate to correlate effects on the binding activities of the repressor protein with the sites of reaction.

Materials and Methods

Isolation of Repressor. Repressor was purified from *Escherichia coli* CSH 46 according to the method described by Rosenberg et al. (1977) as modified by O'Gorman et al. (1980). The purified protein was subjected to sodium dodecyl sulfate gel electrophoresis to assess its purity (>95%) (Weber et al., 1972).

Assay of Repressor. Isopropyl β-D-thiogalactoside (IPTG)[1] binding activity was determined by the ammonium sulfate precipitation method or the nitrocellulose filter binding method described by Bourgeois (1971); the buffer used was 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.01 M $MgCl_2$, 0.1 mM DTT, and 0.2 M KCl. Both methods yielded identical results. The operator DNA binding assay was performed on nitrocellulose filters as described previously (Riggs et al., 1968; Hsieh & Matthews, 1981) at a λp*lac* DNA concentration of 2×10^{-11} M; the buffer used for operator assays was 0.01 M Tris-HCl, pH 7.4, 0.01 M KCl, 0.01 M $Mg(OAc)_2$, 0.1 mM DTT, 0.1 mM EDTA, 5% Me_2SO, and 50 μg/mL bovine serum albumin. Differences in initial operator activity of the repressor (30–90%) did not alter the relative activities observed for the modified protein in these experiments and appear to be related to the λp*lac* preparation utilized rather than the protein integrity. Nonspecific DNA binding assays were carried out by using the same DNA concentration as for the operator assay with the addition of 10^{-3} M IPTG to the assay medium; the details of this procedure have been described by O'Gorman et al. (1980). The buffer was identical with that for the operator assay without bovine serum albumin. Equilibrium dissociation constants were determined by the nitrocellulose filter binding method described above by using the ^{32}P-labeled 40 bp operator fragment at 7.8×10^{-12} M. The dissociation rate for the repressor–operator complex was measured by equilibrating the ^{32}P-labeled 40 bp operator fragment (7.8×10^{-12} M) and protein (concentration at 75% saturation from binding curves) for 15 min in buffer at 4 °C, after which an excess of unlabeled operator-containing pLA 322-8 (15-fold over labeled DNA) was added. Triplicate aliquots (0.1 mL) were withdrawn from a total volume of 4 mL and filtered at the desired times; background was determined by adding IPTG to 10^{-3} M.

Core Preparation and Purification. The trypsin-resistant core protein was prepared and purified as described by Matthews (1979). The purity of the core protein was evaluated by sodium dodecyl sulfate gel electrophoresis, amino acid analysis, and poly[d(A-T)] competition with operator DNA binding before use (Matthews, 1979). Untreated *lac* repressor was utilized for comparison.

Isolation of Operator DNA Fragments. The *lac* operator DNA fragments were isolated from plasmid pOE101 (obtained from J. R. Sadler and J. Betz, University of Colorado Medical Center) and purified according to Sadler et al. (1980). Labeled fragment was obtained by treatment with DNA polymerase Klenow fragment in the presence of [α-^{32}P]dATP followed by electrophoresis on a 7% polyacrylamide gel to isolate the oligonucleotide.

Reaction with Trinitrobenzenesulfonate (Habeeb, 1966). Protein (0.5–2 mg/mL) in 2.5% triethanolamine buffer, pH 9.0, was reacted with appropriate concentrations of trinitrobenzenesulfonate at room temperature. Reaction was carried to >90% completion (1.5–2.0 h) as determined spectrophotometrically. The protein was passed through a Sephadex G-25 column (1.4 × 16 cm) equilibrated in ammonium bicarbonate (0.1 M, pH 8.0) to remove any unreacted reagent, and aliquots were taken for binding analysis at pH 7.4. In experiments where the effects of ligands were measured, the inducer (IPTG) concentration was 10^{-3} M; dialysis following the Sephadex G-25 chromatography step ensured removal of

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactoside; ONPF, *o*-nitrophenyl β-D-fucoside; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Me_2SO, dimethyl sulfoxide; bp, base pair.

all bound inducer. Nonspecific DNA (calf thymus) concentration used was 0.1–0.2 mg/mg of repressor; 1/10 volume of DNA was added to the protein solution, and the solution was allowed to equilibrate prior to addition of other ligands and reagent. Where present, operator DNA fragment concentration was 12.5 μg/mL.

The extent of reaction was routinely determined by measuring the absorbance at 367 nm ($\epsilon = 1.1 \times 10^4$ M^{-1} cm^{-1}; Plapp et al., 1971). Alternatively, unreacted lysines were measured by reacting with fluorodinitrobenzene or ninhydrin as described below; the three methods yielded similar results. Modified protein (1 mL) was mixed with 0.25 mL of 1 M sodium carbonate buffer, pH 8.8, and 0.1 mL of 10% fluorodinitrobenzene in ethanol. The reaction proceeded for 1.5 h in the dark at 40 °C with shaking. The precipitate generated was centrifuged and washed twice with water, twice with ethanol, and once with diethyl ether. The precipiate was then hydrolyzed in 6 N HCl in vacuo for 20 h at 110 °C. The concentration of ϵ-(dinitrophenyl)lysine was determined by elution on the 5-cm column of a Beckman 120C amino acid analyzer. The color value for ϵ-(dinitrophenyl)lysine was determined by using a standard.

Reacted protein (0.25 mL) was mixed with 0.5 mL of ninhydrin solution (20 mg/mL in 75% methylCellosolve, 25% 4 N sodium acetate, and 0.4 mg/mL $SnCl_2$). The mixture was boiled for 15 min and cooled, and 50% ethanol (1 mL) was added. Following centrifugation to remove any precipitate, the absorbance at 570 nm was determined and compared to a standard curve generated by using unmodified protein.

Determination of Extent of Reaction of Cysteine Residues. Following reaction of repressor protein with trinitrobenzenesulfonate, the protein was dialyzed against 1% triethanolamine, pH 9.0; to 1 mL of protein were added 0.1 mL of iodoacetamide (1 M in water) and 0.9 g of urea. The mixture was incubated 2 h at 45 °C and subsequently dialyzed against water for 12 h. The protein was precipitated with 10% trichloroacetic acid, the suspension centrifuged, and the precipitate resuspended in 6 N HCl. The solution was evacuated and the protein hydrolyzed for 20 h, 110 °C, in vacuo. Amino acid analysis to determine (carboxymethyl)cysteine content was carried out on the long column of a Beckman 120C amino acid analyzer (Spackman et al., 1958). The number of cysteines modified was also determined by titration of denatured protein with 2-(chloromercuri)-4-nitrophenol as described previously (Manly & Matthews, 1979).

Reaction of protein previously modified with *N*-ethylmaleimide (Brown & Matthews, 1979) to protect the cysteines was also carried out. The repressor (1 mg/mL) was reacted in 0.24 M potassium phosphate, pH 7.0, and 5% glycerol, with *N*-ethylmaleimide (0.015 M), for 2.5 h at ambient temperature. Following this reaction, the protein was modified with trinitrobenzenesulfonate (2.5×10^{-4} M) as described above.

Peptide Mapping of Modified Protein. Protein modified with trinitrobenzenesulfonate was dialyzed into 0.1 M NH_4HCO_3, pH 8.0, or passed through a preequilibrated Sephadex G-25 column, for trypsin digestion (1% by weight/addition). Proteolysis was carried out at 37 °C for 12–15 h with a second addition of trypsin 3 h after the initial addition. Following lyophilization and centrifugation to remove insoluble and undigested material, the soluble peptides were separated by reverse-phase high-performance liquid chromatography (HPLC) on a Brownlee R-300 column (C-18) by using either a Laboratory Data Control HPLC system or a Gilson Model 302 HPLC system. A gradient from 0 to 60% acetonitrile in 50 mM ammonium acetate, pH 7.0, was used to elute the peptides. Absorbance at 400 nm was monitored to detect the trinitrophenylated peptides. Peaks were collected manually, lyophilized, and subjected to isocratic separation to further purify the peptides for amino acid analysis. Lyophilized peptides were resuspended in 6 N HCl and hydrolyzed for 24 h at 110 °C in vacuo. Analysis was carried out on a Beckman 121 MB amino acid analyzer at the Protein Sequencing Center, University of Texas, in Austin, TX.

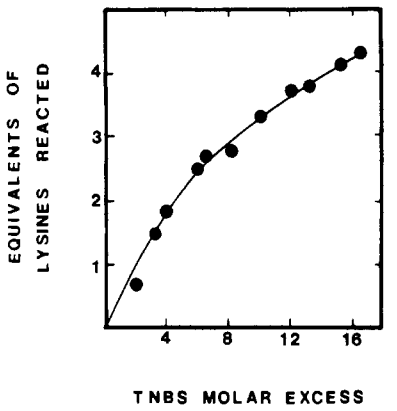

FIGURE 1: Extent of reaction of lysines with varying molar excess of trinitrobenzenesulfonate. Repressor protein (0.6–2.0 mg/mL) in 2.5% triethanolamine, pH 9.0, was reacted with varying molar excesses of trinitrobenzenesulfonate. The reaction was allowed to continue at ambient temperature until spectroscopic measurements indicated that the reaction was >90% complete (1.5–2 h). In some cases, lysine solution (0.1 M, 5 μL) was added to take up any remaining reagent; this procedure did not affect the results obtained. The extent of reaction was monitored by absorbance at 367 nm following isolation of the reacted protein on a Sephadex G-25 column (1.4 × 16 cm) equilibrated with 0.1 M NH_4HCO_3, pH 8.0.

Results

Modification of Repressor with Trinitrobenzenesulfonate. The lactose repressor protein was modified with increasing molar ratios of trinitrobenzenesulfonate over protein monomer. The equivalents of lysines modified determined by several methods gave similar results (ninhydrin reaction, fluorodinitrobenzene reaction followed by amino acid analysis, and quantitation of the trinitrophenyl moiety incorporated into the protein by spectrophotometry). The extent of trinitrobenzenesulfonate modification of the repressor protein measured at the plateau of the time course for the reaction increases with reagent concentration (Figure 1). Incorporation at higher molar excesses of reagent leveled off at ~4.5 equiv of modified lysine per monomer. Thus, not all lysines in the native repressor were available for reaction with this reagent. Cysteine modification is a potential side reaction with trinitrobenzenesulfonate, and the extent of cysteine reaction was carefully monitored since oxidation of cysteine causes loss of operator DNA binding capacity (Manly & Matthews, 1979). When the free sulfhydryl content was measured by titration with 2-(chloromercuri)-4-nitrophenol, it was determined that less than 5% of the cysteines were affected by the modification. When the trinitrophenylated protein was reacted with iodoacetamide under denaturing conditions, any unreacted sulfhydryl groups would be converted to (carboxymethyl)cysteine; the concentration of this amino acid was determined by acid hydrolysis and subsequent amino acid analysis. By this method, less than 15% of the three cysteines per monomer were affected by reaction with trinitrobenzenesulfonate. In addition, prior blocking of the cysteine residues at positions 107 and 140 with *N*-ethylmaleimide (Brown & Matthews, 1979) did not alter the reaction of the protein with trinitrobenzenesulfonate or the activities of the modified protein. Modified protein

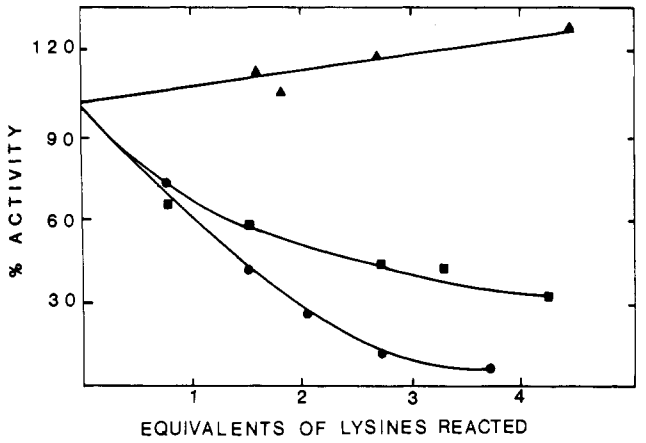

FIGURE 2: Effect of trinitrobenzenesulfonate reaction on the activities of the repressor protein. Reaction of the protein was carried out as described in Figure 1 by using various molar excesses of reagent in 2.5% triethanolamine buffer, pH 9.0. Reaction was taken to >90% completion. Following isolation of the modified protein by Sephadex G-25 chromatography, the extent of lysine modification was determined by absorbance at 367 nm, and the functional activities of the protein were measured by assay at pH 7.4 as described under Materials and Methods. (▲) Inducer binding activity; (■) nonspecific DNA binding activity; (●) operator DNA binding activity.

(10-fold molar excess of reagent) eluted as tetramer from Sephadex G-150 and appeared as a single band of higher mobility than unmodified repressor on native polyacrylamide gels.

Effects of Modification on Binding Activities. The inducer, nonspecific DNA, and operator DNA binding activities of the modified protein were determined at pH 7.4 following reaction at pH 9. Repressor control samples that were treated identically to reacted samples were included in every experiment to ensure no effect of experimental protocol on the integrity of the protein. A plot of binding activities vs. the equivalents of lysine reacted is shown in Figure 2. An increase in the inducer binding activity of the protein is observed as the extent of modification increases. In contrast, each of the DNA binding activities of the protein decreases as the number of reacted lysines increases. The loss of operator DNA binding capacity is greater relative to nonspecific DNA binding at corresponding levels of modification.

Effect of Ligands on Reaction of Repressor with Trinitrobenzenesulfonate. The presence of ligands affects the extent of reaction of the repressor protein with trinitrobenzenesulfonate observed at the plateau of the time course. Inducer increases the degree of modification observed with this reagent; at a 10-fold molar excess of reagent, the ratio of equivalents of modified lysine in the presence of IPTG relative to reaction with no ligand present is 1.1 ± 0.06. In contrast, under similar conditions the presence of nonspecific DNA significantly decreases the relative amount of trinitrophenylation; the ratio of incorporation with DNA present vs. free repressor is 0.71 ± 0.08. These differences are reflected in the activities of the corresponding modified repressor proteins. Inducer presence during reaction results in a slightly greater decrease in operator DNA binding activity relative to unliganded protein, while DNA presence protects against loss of DNA binding capacity (Table I and Figure 3). The apparent dissociation constant for a 40 bp operator fragment (i.e., the protein concentration at half-saturation) increases by ~10^4 when repressor or repressor–inducer is modified with a 10-fold molar excess of reagent over monomer; under the same conditions, only a 10-fold increase is observed when reaction occurs in the presence of nonspecific DNA (Table I). By use of protein concentrations sufficient to obtain ~75% binding of

Table I: Half-Saturation Concentrations for Binding to Operator Fragment[a]

	$[\text{protein}]_{\text{half-saturation}}$ (M)
repressor	3.6×10^{-11}
repressor modified with TNBS[b]	2.2×10^{-7}
repressor–IPTG modified with TNBS[b,c]	4.5×10^{-7}
repressor–DNA modified with TNBS[b,d]	4.4×10^{-10}

[a] Protein samples at various concentrations were mixed with ^{32}P-labeled 40 bp operator DNA fragments (7.8×10^{-12} M) to generate a binding curve at pH 7.4 as described under Materials and Methods. The protein concentration at half-saturation was then determined. [b] Modification was carried out at a 10-fold molar excess of reagent over monomer in 2.5% TEA, pH 9.0, for 2 h (>95% completion of reaction) as described under Materials and Methods. [c] IPTG concentration for reaction was 10^{-3} M. [d] DNA concentration for reaction was 0.1 mg/mg of protein.

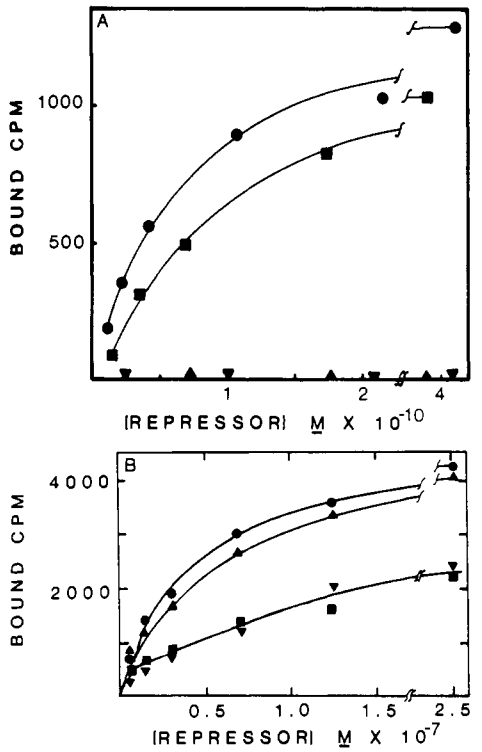

FIGURE 3: Effect of the presence of ligands during reaction with trinitrobenzenesulfonate on the DNA binding capacity of the protein. Repressor protein (2.0 mg/mL) in 2.5% triethanolamine, pH 9.0, was reacted with a 10-fold molar excess of reagent over monomer. The reaction proceeded for 1.5 h at ambient temperature to >95% completion; the protein was then passed through a Sephadex G-25 column (1.4 × 16 cm) and dialyzed against several changes of 2.5% triethanolamine, pH 9.0, to remove any remaining inducer. Modification was carried out in parallel with IPTG (1×10^{-3} M) or calf thymus DNA (0.1 mg/mg of protein) present. The operator and nonspecific DNA binding activities at pH 7.4 (λp*lac* DNA concentration, 2×10^{-11} M) and extent of reaction were determined as described under Materials and Methods. (A) Operator DNA binding. The background cpm in the presence of inducer have been subtracted from each binding curve. (●) Repressor (unmodified); (▼) repressor modified with trinitrobenzenesulfonate; (▲) repressor modified in the presence of inducer; (■) repressor modified in the presence of calf thymus DNA. (B) Nonspecific DNA binding. (●) Repressor (unmodified); (▼) repressor modified with trinitrobenzenesulfonate; (▲) repressor modified in the presence of calf thymus DNA; (■) repressor modified in the presence of inducer (1×10^{-3} M).

operator DNA, dissociation rate constants were measured by addition of a 15-fold excess of unlabeled operator-containing DNA; the rates observed for repressor modified alone or with inducer present were similar to those of unreacted protein. This result indicates that the apparent K_d measured is due to

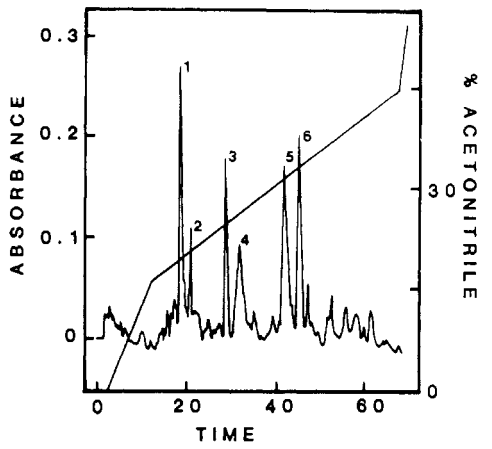

FIGURE 4: High-performance liquid chromatography of trypsin-digested, modified repressor protein. Repressor was modified with a 10-fold molar excess of trinitrobenzenesulfonate per monomer for 1.5 h at room temperature in 2.5% triethanolamine, pH 9.0 (>95% completion of reaction). Peptides were isolated as described under Materials and Methods and were eluted by using a 0–60% acetonitrile gradient in 50 mM ammonium acetate, pH 7.0. Absorbance of the eluant was monitored at 400 nm. Peptides were identified following isocratic elution by amino acid analysis.

a small amount of unreacted repressor remaining (0.01%). In contrast, the apparent K_d measured following reaction in the presence of nonspecific DNA appears to reflect a decreased affinity of this protein for the operator DNA rather than the presence of unmodified repressor.

Protection of both specific and nonspecific DNA binding activities by DNA presence during reaction is observed; the protection observed was similar whether calf thymus DNA, poly[d(A-T)], or operator DNA was present. The addition of inducer did not alter the decreased reactivity observed in the presence of nonspecific DNA, nor did it affect the protection of DNA binding activities observed under these reaction conditions. To ascertain that the presence of DNA did not decrease the reaction of lysine residues with trinitrobenzenesulfonate in general, the reaction of bovine serum albumin with the reagent was measured in the presence and absence of nonspecific DNA; no effects of the DNA were observed on the reaction with bovine serum albumin. Reactions also were carried out with high salt present (1 M NaCl) to dissociate any repressor–DNA complexes. While these conditions increased the extent of reaction with repressor and with bovine serum albumin, no effect on the reaction was noted on the addition of nonspecific DNA to either protein solution.

Reaction of the Core Protein with Trinitrobenzenesulfonate. The core protein of the repressor was modified with trinitrobenzenesulfonate. An increased reactivity for the core–inducer complex similar to that observed for the intact protein was found. In contrast, no effect of nonspecific DNA on the reaction with the core protein was found. The K_d for interaction of operator DNA with the core protein was increased upon trinitrophenylation by ~5-fold at a 10-fold excess of reagent and ~20-fold at a 60-fold excess of reagent.

Identification of the Sites of Reaction with Trinitrobenzenesulfonate. High-performance liquid chromatography of the trypsin-digested modified proteins yielded peptide maps consisting of six major peaks; the pattern observed at 10-fold molar excess of reagent is shown in Figure 4. Since absorbance was monitored at 400 nm, only modified peptides were visible. Each peak was collected and analyzed by HPLC using isocratic elution; the peptides ranged from 60 to 99% pure with the exception of peak 2, which eluted as three different peaks. Amino acid analysis of the peptides collected allowed the

Table II: Trinitrophenylated Peptides from *lac* Repressor

peak	peptide	lysine	RMS deviation from expected peptide[a]	purity from isocratic elution[b] (%)
1	36–51	37	0.77	61
2	ND[c]	ND[c]		<40
3	102–118	108	0.61	74
4	264–294	290	0.92	99
5	327–340	327	0.49	71
6	23–35	33	0.51	80

[a] RMS = x^2/(number of different amino acids − 1) where $x^2 = (x_i - x_a)^2 + (x_i - x_b)^2 + \ldots$; x_a = the number of a-type amino acids in the expected peptide, and x_i = the number of corresponding amino acids in the experimental peptide. [b] Each peak collected from the original gradient was lyophilized and subjected to HPLC a second time with elution at a constant percent acetonitrile. The percent area of the peak analyzed is presented in the table. [c] Amino acid analysis of the three peaks obtained from isocratic elution of peak 2 did not correlate well with any repressor peptides.

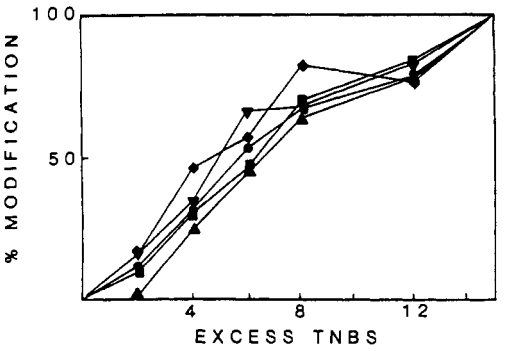

FIGURE 5: Extent of modification of individual lysines in the lactose repressor protein. Protein in 2.5% triethanolamine, pH 9.0, was reacted with the indicated molar ratios of reagent over repressor monomer for 1.5 h; reaction was >90% complete by spectroscopic determination for each reagent concentration used. Peptides were isolated as described in Figure 4 and under Materials and Methods. The area for each peak in the HPLC elution pattern was normalized within each data set relative to the amount of protein applied to the column, and the area determined was compared to the corresponding area for 15-fold molar excess. (♦) Peak 1 (amino acids 36–51; lysine-37), (■) peak 3 (amino acids 102–118; lysine-108), (▼) peak 4 (amino acids 264–294; lysine-290), (●) peak 5 (amino acids 327–340; lysine-327), and (▲) peak 6 (amino acids 23–35; lysine-33).

identification of five of the lysines modified by trinitrobenzenesulfonate (Table II). Comparison of each peak area relative to the corresponding peak at a 15-fold excess of reagent indicated that similar degrees of reaction occurred at each of the modifiable lysines at various excesses of reagent (Figure 5). All maps were carried out on protein samples produced under conditions which gave >90% complete reaction at that reagent concentration by spectrophotometric assessment.

Reaction of trinitrobenzenesulfonate in the presence of calf thymus DNA or IPTG altered the peptide maps of the products. The presence of calf thymus DNA protected lysines-33 and -108 (and partially 37) from modification and slightly enhanced reaction at lysine-290, while IPTG significantly enhanced the modification of lysine-327 and protected lysine-290 (Figure 6). Some variability in the degree of enhancement/protection in different experiments was observed, but enhancement or protection was consistent. A 10-fold excess of trinitrobenzenesulfonate per monomer resulted in the modification of ~30% fewer lysines per monomer in the presence of calf thymus DNA. This difference in modification corresponds directly with the loss in peak area observed when the reaction was executed in the presence of calf thymus DNA (76% of control). In the presence of IPTG, there was an increase in peak area observed in the peptide map (121%),

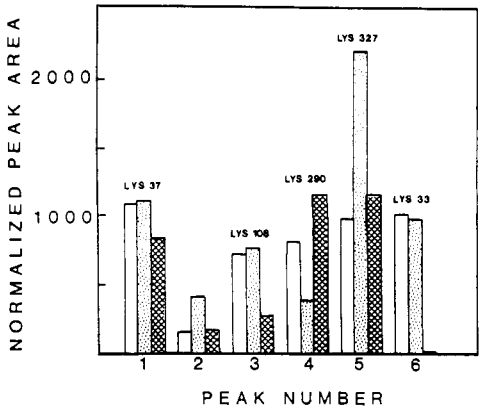

FIGURE 6: Effects of ligands on extent of trinitrobenzenesulfonate modification at individual lysines. Reaction with a 10-fold molar excess of reagent was carried out in 2.5% triethanolamine for 1.5 h (>95% completion). Area from HPLC elution profile was normalized for each peak relative to the amount of protein injected onto the HPLC column. (Rectangle) A 10-fold molar excess of TNBS was used to modify repressor; (dotted rectangle) repressor modified in the presence of IPTG (10^{-3} M); (crosshatched rectangle) repressor modified in the presence of calf thymus DNA (0.1 mg of calf thymus DNA/mg of repressor protein).

consistent with the increased modification observed in the presence of inducer.

Discussion

Reaction of the protein with trinitrobenzenesulfonate resulted in the incorporation of the trinitrophenyl group into the protein structure only at lysine residues. Alkaline conditions were required for significant reaction of the ϵ-amino group of the lysines. Previous studies have indicated that reaction at pH 9 followed by assay at pH 7.4 does not affect repressor protein activity (Yang et al., 1977). While reaction occurs with the pH 9 conformation of the protein, the assays assess the effect of modified lysines on the protein conformation and interactions at pH 7.4. Structural changes consequent to pH increase may be fixed by reaction of lysines in the protein and therefore do not reverse upon lowering pH; any such alterations that affect activity would be indistinguishable in this experimental protocol from direct effects of modification. However, reactions of most reagents with lysines are minimal at lower pH ranges; thus, assessment of lysine role(s) in repressor function by chemical modification necessitates work at high pH. This technique provides an alternative approach to genetic methods for detecting participation of specific residues in binding activities of this regulatory protein. While necessarily limited in the ways indicated, these data are of use in understanding the modes of repressor regulatory action.

Determination of the extent of reaction by direct spectrophotometry or by measurement of unreacted lysines yielded similar values for extent of modification. To further assure that reaction with lysines was exclusive, the reactivity of the cysteine residues was explored. Fewer then 15% of the cysteine residues were affected by reaction with trinitrobenzenesulfonate; in addition, if the protein was modified with *N*-ethylmaleimide to block the cysteine residues prior to reaction with trinitrobenzenesulfonate, no change in the activity or reactivity patterns was observed. Thus, all of the label that is incorporated occurs at lysine sites in the primary sequence of the protein.

The binding activities of the modified protein were affected by the reaction; inducer binding activity was increased, nonspecific DNA binding activity decreased, and operator binding activity more dramatically decreased. For nonspecific DNA binding, 50% loss in activity occurs when approximately 2 equiv of (trinitrophenyl)lysine are present per protein monomer, while for operator DNA binding, 50% loss corresponds to introduction of slightly more than 1 equiv per monomer.

The reaction of trinitrobenzenesulfonate with the repressor was altered by ligands. The presence of inducer increased the extent of reaction ~10% when a 10-fold molar ratio of reagent over monomer was used. Reaction under these conditions resulted in slightly increased operator DNA activity loss, with no effect on loss of nonspecific DNA binding relative to unliganded protein. In the presence of nonspecific DNA under the same conditions, a 30% decrease in extent of reaction was observed, with significantly less diminution of both DNA binding activities. Similar protection was observed for calf thymus DNA, poly[d(A-T)], and operator DNA fragments. Inducer did not affect the decreased reactivity nor activity protection observed in the presence of nonspecific DNA. These effects were specific for the intact lactose repressor protein, as the presence of DNA did not affect the reaction of trinitrobenzenesulfonate with bovine serum albumin or core protein under similar conditions. If the nonspecific DNA was dissociated by high salt in the reaction mix, the extent of reaction increased to that measured for repressor alone, and no protection by lysine trinitrophenylation or DNA binding activities was observed.

Core protein isolated by mild trypsin treatment was reacted with the reagent. This core protein binds to operator DNA in an inducer-sensitive fashion (Matthews, 1979). An increased extent of reaction in the presence of inducer and increased IPTG affinity for the product were also noted for the core protein; the residues whose reactivity was altered by inducer were in the core region of the molecule (lysines-290 and -327). Trinitrophenylation of the core protein results in an increase in the dissociation constant of the core for operator DNA; therefore, residues in this region of the protein appear to be essential for operator DNA binding. The presence of nonspecific DNA does not affect the extent of reaction or the loss in operator DNA binding activity observed for the core protein.

The lysine residues implicated in DNA binding by genetic methods are at positions 2, 33, 84, and 290 (Pfahl et al., 1974; Coulondre & Miller, 1977; Miller, 1979; Miller et al., 1979, 1978; Schmitz et al., 1978; Miller & Schmeissner, 1979), a range spanning almost the entire sequence of the monomer and including both NH_2-terminus and core lysine residues. From the HPLC mapping of the trypsin-digested protein, lysine-33, lysine-108, and to some extent lysine-37 were protected from modification in the presence of calf thymus DNA. Lysine-33 involvement in DNA binding is indicated by both genetic and chemical methods. The participation of the NH_2-terminal region in both operator-specific and nonspecific DNA binding is accepted in the literature, but more recent results have demonstrated that the core domain of the protein is also essential for the interaction of the repressor protein with the specific operator DNA sequence (Matthews, 1979). The oxidation of Cys-107 can be correlated with loss of operator DNA binding capacity (Manly & Matthews, 1979). The protection from modification of lysine-108 in the presence of DNA and the accompanying activity effects support a role for this portion of the core domain in maintenance of operator binding activity. The slight increase in reaction at lysine-290 indicates either an alteration in this region of the molecule in response to DNA binding or possibly a protein–DNA interface which concentrates reagent; mutants that affect operator DNA binding have been mapped to this region of the repressor (Pfahl, 1981). The absence of reaction at both lysine-2 and

the α-NH_2 group is consistent with other results from this laboratory which indicate that this region of the protein is not accessible for reaction (K. S. Matthews et al., unpublished data).

The presence of inducer (IPTG) partially protected lysine-290 from modification by trinitrobenzenesulfonate, while reaction with lysine-327 was enhanced 2-fold. The conformational change concomitant with IPTG binding apparently renders lysine-327 more accessible and lysine-290 less accessible to the reagent. Lysine residues whose reactivity is altered by inducer binding are solely in the core region of the molecule with none in the NH_2-terminus. The core region of the repressor molecule has been implicated in inducer binding by genetic studies (Miller, 1979) and binding measurements using domains produced by proteolytic digestion (Platt et al., 1973), and the conformational change accompanying inducer binding is largely confined to this domain (Matthews, 1979; Friedman & Matthews, 1978; Boschelli et al., 1981).

The results of these experiments are consistent with the known roles of the two domains of the repressor in its binding activities. Only reactivity of residues in the core domain is affected by the binding inducer, while modification of residues in both core and NH_2-terminal domains is altered by the presence of operator or nonspecific DNA. The accompanying effects on activity demonstrate that the determinants for operator and nonspecific DNA binding can be protected by their presence during reaction. Introduction of the trinitrophenyl group yields a neutral product with altered steric properties relative to the parent side chain. The effects on DNA binding activities indicate that the charge on lysine residues 33, 108, and possibly 37 is required for maintaining electrostatic components essential to both nonspecific and operator DNA binding. Whether these components are involved in direct contact with the DNA or serve a critical role in maintaining the active DNA binding structure cannot be discerned by these experiments. As might be anticipated on the basis of the greater specificity and nonionic binding components involved in the interaction, the steric constraints on maintaining operator DNA binding appear more severe than for nonspecific DNA binding.

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Registry No. Lysine, 56-87-1; trinitrobenzenesulfonic acid, 2508-19-2.

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