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Formation of Mixed Disulfide Adducts at Cysteine-281 of the Lactose Repressor Protein Affects Operator and Inducer Binding Parameters†

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ABSTRACT: The lactose repressor protein has been modified with three sulfhydryl-specific reagents which form mixed disulfide adducts. Methyl methanethiosulfonate (MMTS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) completely reacted with all three cysteine residues, whereas only partial reaction was observed with didansylcystine. Cysteines-107 and -140 reacted stoichiometrically with MMTS and DTNB, while Cys-281 was modified only at higher molar ratios. Didansylcystine reacted primarily with cysteines-107 and -140. Affinity of MMTS-modified repressor for 40 base pair operator DNA was decreased 30-fold compared to unmodified repressor, and this decrease correlated with modification of cysteine-281. DTNB-modified repressor bound operator DNA with a 50-fold weaker affinity than unmodified repressor. Modification of the *lac* repressor with didansylcystine decreased operator binding only 4-fold, and nonspecific DNA binding increased 3-fold compared to unmodified repressor. No change in the inducer equilibrium binding constant was observed following modification with any of these reagents. In contrast, inducer association and dissociation rate constants were decreased ~50-fold for repressor completely modified with MMTS or DTNB, while didansylcystine had minimal effect on inducer binding kinetics. Correlation between modification of Cys-281 and the observed decrease in rate constants indicates that this region of the protein regulates the accessibility of the sugar binding site. The parallel between the increase in the K_d for repressor binding to operator, the altered rate constant for inducer binding, and modification of cysteine-281 suggests that this region of the protein is crucially involved in the function of the repressor protein.

Synthesis of lactose metabolic enzymes in *Escherichia coli* is regulated by the interaction of the lactose repressor with operator DNA (Miller & Reznikoff, 1980). The affinity of the repressor for operator is modulated by the binding of small sugar molecules. These inducers elicit a conformational change in the repressor protein to a form with decreased affinity for operator DNA (Lin & Riggs, 1975). Because the affinity of the protein for nonspecific DNA is not affected by inducer binding, the remainder of the genomic DNA competes ef-

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fectively with the operator region for binding to the repressor–inducer complex (Lin & Riggs, 1975) and thereby facilitates the induction process.

Although the amino acid sequence of the repressor (150 000-dalton tetramer) has been determined (Beyreuther et al., 1975; Farabaugh, 1978), the protein has not been crystallized. In the absence of X-ray crystallographic data, alternate methods for determination of the structure–function relationships in this protein have been utilized. Among these, chemical modification studies have provided significant information regarding the roles of several amino acids in repressor function (Fanning, 1975; Yang et al., 1977; Burgum & Matthews, 1978; Brown & Matthews, 1979a,b; Manly & Matthews, 1979; Hsieh & Matthews, 1981; Whitson et al., 1984). Genetic and proteolytic studies have associated inducer binding with the tetrameric core region (amino acids 60–360; Platt et al., 1973); this region is also thought to be involved in subunit–subunit interactions (Pfahl et al., 1974; Miller et al., 1975; Schmitz et al., 1976; Miller, 1979). DNA binding has been associated with the amino-terminal region (amino acids 1–60) of the protein (Pfahl, 1972; Pfahl et al., 1974; Jovin et al., 1977). However, determinants for operator binding in the core region have been suggested by chemical modification (Burgum & Matthews, 1978; Manly & Matthews, 1979) and direct binding studies (Matthews, 1979; Manly et al., 1984).

The lactose repressor contains three cysteine residues located at positions 107, 140, and 281 (Beyreuther et al., 1975; Farabaugh, 1978). Previous experiments with maleimide probes as well as other sulfhydryl-specific reagents demonstrated that two of the three cysteines could be reacted with minimal loss of operator binding activity but complete modification of all three cysteine residues was accompanied by diminished operator binding capacity, presumably due to steric constraints (Brown & Matthews, 1979a). Methyl methanethiosulfonate (MMTS)[1] is a reversible blocking agent which is highly specific for free sulfhydryls (Smith et al., 1975) and links a small, non-hydrogen-bonding methanethiol group to the protein by a mixed disulfide bond. The methanethiol group can be easily displaced by addition of sulfhydryl compounds (Smith et al., 1975). Didansylcystine (DDC) (Oh & Churchich, 1974) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959) also form mixed disulfide bonds with cysteine residues. These reagents have been utilized to examine the reaction patterns and effects of modification for specific cysteine residues in the lactose repressor protein.

Materials and Methods

Reagents. Methyl methanethiosulfonate (MMTS) was obtained from Aldrich Chemical Co. The purity was checked by NMR spectroscopy: δ 2.65 (3 H, s), 3.22 (3 H, s). 2-(Chloromercuri)-4-nitrophenol (MNP) was obtained from Eastman and recrystallized from methanol–water before use. MNP (1 mM stock solution) was dissolved in 0.02 M NaOH and stored at 4 °C. 2-(Bromoacetamido)-4-nitrophenol (BNP) was obtained from Sigma. Directly before use, BNP was dissolved in methanol at a concentration of 0.04 M. Isopropyl β-D-thiogalactoside (IPTG), fluorescein mercuric acetate (FMA), and DTNB were purchased from Sigma. Didansylcystine and IAEDANS were obtained from Molecular Probes, Inc.

Isolation of Repressor. Lactose repressor protein was isolated from *Escherichia coli* CSH 46 as described previously with a purity ≥98% (Rosenberg et al., 1977; O'Gorman et al., 1980a). Samples of frozen repressor were thawed for use and dialyzed into 0.1 M Tris-HCl, pH 7.6. All buffers were deaerated with nitrogen before use to prevent cysteine oxidation, and dithiothreitol was omitted from the buffer. Protein concentrations were determined by using an extinction coefficient of $A_{280nm}^{0.1\%} = 0.6$ or by Bio-Rad Coomassie blue protein assay (Bradford, 1976).

Assay of Activity. The inducer binding activity was assayed by titrating the protein with IPTG and monitoring changes in tryptophan fluorescence emission (O'Gorman et al., 1980b). Repressor was diluted to 10^{-7} M monomer in buffer solutions which had been deaerated extensively with nitrogen; the resultant solution was placed in a cuvette which was sealed with a serum stopper. This procedure minimized photooxidation of the tryptophan during the titration process. Nitrogen was introduced into the cuvette to give a slight positive pressure. Addition of ligand solution was made with a Hamilton syringe through the serum stopper. IPTG binding activity was also assayed by the ammonium sulfate precipitation and nitrocellulose filtration methods (Bourgeois, 1971).

Operator DNA fragments (40 bp) were isolated from plasmid pOE101 by using restriction endonuclease *Eco*RI (Boehringer Mannheim; Lillis et al., 1982). DNA concentrations were determined by the ethidium bromide fluorescence assay of Le Pecq and Paoletti (1966) with calf thymus DNA as a standard. The 40 base pair operator fragment was radiolabeled with [^{32}P]dαATP and [^{32}P]dαTTP using DNA polymerase large fragment (Klenow) and utilized to determine the dissociation constants of modified and unreacted repressors for operator by nitrocellulose filter assays (Riggs et al., 1968). The incubation buffer used in the operator binding assays contained 0.01 M Tris-HCl, pH 7.5, 0.15 M KCl, 10^{-4} M EDTA, 5% Me_2SO, and 50 μg/mL BSA.

Reaction of Repressor with Methyl Methanethiosulfonate. Appropriate molar ratios of MMTS to monomer were added to repressor previously dialyzed into nitrogen-purged 0.1 M Tris-HCl, pH 7.6, and reaction was allowed to proceed for 0.5 h at 4 °C. Following modification, unreacted MMTS was removed by dialysis against several volumes of 0.1 M Tris-HCl, pH 7.6 or 9.0.

Reaction of Repressor with Didansylcystine. Solid didansylcystine was added to repressor previously dialyzed against 0.1 M Tris-HCl, pH 7.5, and reacted for 24 h at 4 °C in the dark. Following reaction, modified protein was extensively dialyzed against 0.1 M Tris-HCl, pH 7.5. The extent of reaction of DDC with repressor was monitored spectrally via dansyl absorbance at 340 nm (ϵ = 4300 M^{-1} cm^{-1}).

Reaction of Repressor with 5,5′-Dithiobis(2-nitrobenzoic acid). Repressor previously dialyzed against 0.1 M Tris-HCl, pH 7.5, was reacted for 1 h at 25 °C with varying molar ratios of DTNB up to 10-fold over repressor monomer. Reagent was added as a solid. The reaction was followed by monitoring the absorbance change at 405 nm. Following modification, unreacted reagent was removed by dialysis against several volumes of 0.1 M Tris-HCl, pH 7.5 or 9.0.

2-(Chloromercuri)-4-nitrophenol Titration. Following modification, protein samples (~0.5 mg/mL) were dialyzed against 0.1 M Tris-HCl, pH 7.0. Protein solution (0.5 mL)

[1] Abbreviations: BNP, 2-(bromoacetamido)-4-nitrophenol; DDC, didansylcystine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FMA, fluorescein mercuric acetate; HPLC, high-pressure liquid chromatography; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; IPTG, isopropyl β-D-thiogalactoside; MMTS, methyl methanethiosulfonate; MNP, 2-(chloromercuri)-4-nitrophenol; NBS, *N*-bromosuccinimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; bp, base pair(s); Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; DMF, dimethylformamide; DTT, dithiothreitol; DNS, dansyl (IAEDANS).

was added to 0.1 mL of 1 M Tris-HCl, pH 7.0, and 50 mM EDTA. Solid urea was added to 8 M and the final volume adjusted to 1 mL. Aliquots (2 μL) of MNP solution (1 mM in 0.1 M NaOH) were added to the protein sample followed by mixing and measurement of absorbance at 410 nm. Free sulfhydryl content was determined from the titration curve (Manly & Matthews, 1979).

2-(Bromoacetamido)-4-nitrophenol Mapping. Samples of modified repressor (3–7 mg) were dialyzed against 0.1 M Tris-HCl, pH 9.0. A 200-fold molar ratio of reagent was added to protein solutions by using a freshly prepared stock of 0.04 M BNP in methanol. Following addition of reagent, samples were made to 8 M in urea and allowed to react for 1.5 h at 37 °C. Unreacted reagent was separated from protein by using a Sephadex G-25 column equilibrated in 8 M urea and 0.1 M ammonium bicarbonate. The protein was precipitated by extensive dialysis against 0.1 M ammonium bicarbonate. The precipitate was pelleted by centrifugation at 11 000 rpm for 15 min, resuspended in 0.1 M ammonium bicarbonate, and warmed to 37 °C. Aliquots of chymotrypsin and trypsin (1% by weight) were added to the protein samples and allowed to react for 2.5 h; a second protease addition was repeated after 2.5 h. Following digestion, samples were lyophilized. Lyophilized samples (~0.5 mg) were dissolved in 20 μL of 0.05 M sodium phosphate, pH 7.0, and injected onto a Gilson high-pressure liquid chromatographic system. Separation was achieved with an Aquapore RP-300 10-μm column, and peptides were eluted with a 0–40% acetonitrile gradient. Modified cysteines were detected by using an LKB 2238 Uvicord SII with a 405-nm interference filter.

Native 2-(Bromoacetamido)-4-nitrophenol Modification. Repressor was modified with a 200-fold molar ratio of BNP over repressor monomer concentration for 1.5 h at 25 °C in 0.1 M Tris-HCl, pH 9.0, and 1 M NaCl in the presence and absence of 10^{-3} M IPTG. Following modification, unreacted BNP was removed by dialysis against 0.1 M Tris-HCl, pH 7.5. Following this procedure, the samples were reacted with a 150-fold molar ratio of MMTS for 0.5 h. Operator and inducer binding parameters were measured for these samples.

Reaction of Repressor with Other Sulfhydryl-Specific Reagents. For kinetic measurements, operator assays, and fluorescence inducer titrations, native repressor was modified with a series of cysteine-specific reagents. The following molar ratios of probe over monomer concentration were employed: MNP, 2-fold; BNP, 200-fold; FMA, 3-fold from 1×10^{-3} M stock in DMF; DTNB, 10-fold; DDC, 100-fold; IAEDANS, 50-fold added as solid. Unreacted reagents were removed by dialysis before the protein was analyzed.

Kinetic Measurements. The association rate constants for repressor binding to inducer were measured by using a Gibson-Durrum (Dionex) rapid mixing stopped-flow spectrometer. The excitation wavelength was 285 nm, and fluorescence emission was measured by using a Corning filter which transmitted light of wavelengths greater than 350 nm. Pseudo-first-order conditions were used to measure IPTG binding to wild-type and chemically modified repressors at 25 °C. Protein concentrations unless otherwise noted were 2×10^{-6} M before mixing. The rate of inducer binding was followed by measurement of the decrease in total fluorescence. Kinetic traces were fit to either one- or, if necessary, two-exponential expressions using an iterative least-squares algorithm (Bevington, 1969).

RESULTS

Determination of Cysteine Modification. (A) Methyl Methanethiosulfonate. The extent of sulfhydryl modification at varying molar ratios of reagent was determined by titration of the reacted repressor protein with 2-(chloromercuri)-4-nitrophenol (Figure 1). An equivalence point of 2.8 ± 0.3 cysteines was obtained for unreacted repressor samples. Stoichiometric reaction of MMTS with two sulfhydryl groups in the repressor was observed up to a 2-fold molar ratio of reagent to monomer. Modification of the third equivalent of sulfhydryl in each protein monomer was nonstoichiometric, required molar ratios of MMTS per monomer greater than 3, and was nearly complete at a 150-fold excess of reagent.

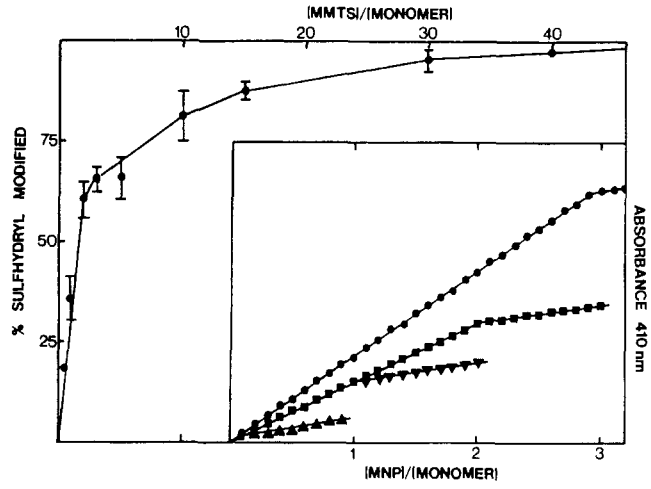

FIGURE 1: Titration of repressor protein with 2-(chloromercuri)-4-nitrophenol. Titrations were conducted as described under Materials and Methods. A profile of percent total sulfhydryl reacted with increasing molar ratios of MMTS over repressor monomer as determined by titration with 2-(chloromercuri)-4-nitrophenol is presented. Inset: Individual titration curves. (●) Titration of control unmodified repressor (1.25×10^{-5} M monomer); (■) titration of repressor (1.25×10^{-5} M monomer) modified with a 1-fold molar ratio of MMTS over protein monomer; (▼) 2-fold molar ratio of MMTS over repressor monomer; (▲) 40-fold molar ratio of MMTS over repressor monomer.

To determine the sites of MMTS modification, 2-(bromoacetamido)-4-nitrophenol (BNP) was used to irreversibly modify unreacted sulfhydryl groups. Following MMTS modification, the protein was denatured and reacted with a 200-fold molar ratio of BNP. Peptides from modified and proteolytically digested repressor were separated by HPLC with the following order of elution: Cys-107, Cys-281, Cys-140 (Figure 2). At low molar equivalents of MMTS, Cys-107 and Cys-140 reacted stoichiometrically, while Cys-281 was minimally affected. Cys-281 gradually reacted with MMTS at increasing molar ratios. At a 50-fold molar ratio, this sulfhydryl was approximately 85% modified (Figure 3). MMTS modification of repressor in the presence of inducer yielded a slightly different reactivity pattern: although MMTS reaction at Cys-281 and Cys-107 was unaltered, Cys-140 reactivity was significantly decreased at low molar ratios of MMTS to repressor monomer (Figure 3).

(B) Didansylcystine. Cysteine-140 and cysteine-107 exhibited approximately equivalent reactivity with didansylcystine at all molar ratios studied. At a molar ratio of 200 per monomer, both Cys-107 and Cys-140 were approximately 70–80% modified, whereas only ~30% of Cys-281 had reacted (Figure 4A). These mapping results indicated a maximum incorporation of approximately two dansyl groups per monomer which corresponded well with that obtained from the absorbance at 340 nm (2.2 dansyl groups per monomer).

(C) 5,5'-Dithiobis(2-nitrobenzoic acid). When the BNP mapping procedure was used, Cys-140 and Cys-107 were significantly modified at low molar ratios of DTNB per monomer, while cysteine-281 was less reactive (Figure 4B). The

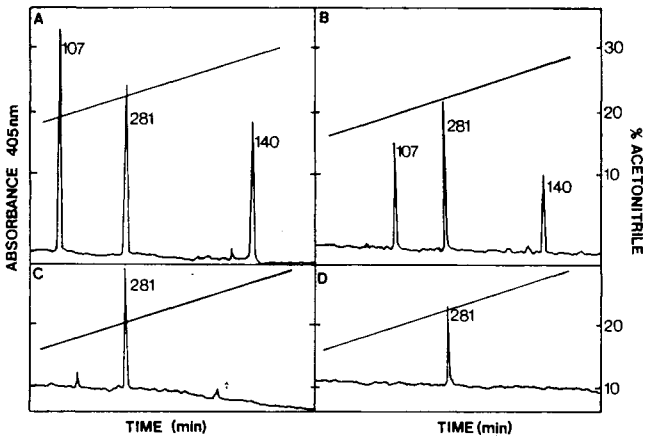

FIGURE 2: High-pressure liquid chromatography of nitrophenol-labeled peptides from repressor. Repressor was modified with MMTS and subsequently denatured and reacted with 2-(bromoacetamido)-4-nitrophenol to modify the unreacted cysteine residues. Control repressor was reacted with only 2-(bromoacetamido)-4-nitrophenol. The protein was digested with trypsin and chymotrypsin, and the peptides were separated on a C_{18} reverse-phase column and eluted with a 0–40% acetonitrile gradient. (A) Control repressor; (B) repressor modified with a molar ratio of MMTS over monomer of 1; (C) repressor modified with a 2-fold molar ratio of MMTS over repressor monomer; (D) repressor modified with a 10-fold molar ratio of MMTS over monomer.

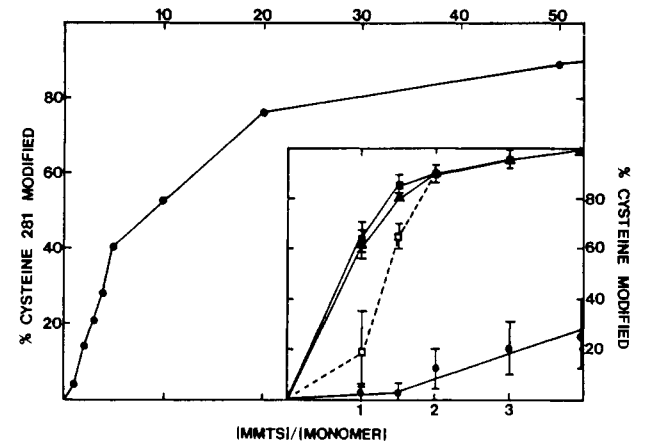

FIGURE 3: Extent of methyl methanethiosulfonate modification at individual cysteines at increasing molar excesses of reagent. Modification of repressor with MMTS and peptide production and separation were performed as described under Materials and Methods. Results are expressed as percent MMTS-modified sulfhydryl at increasing molar ratios of MMTS over monomer concentration. (●) Cysteine-281; (■) cysteine-140; (▲) cysteine-107; (□) cysteine-140 reacted with MMTS in the presence of inducer.

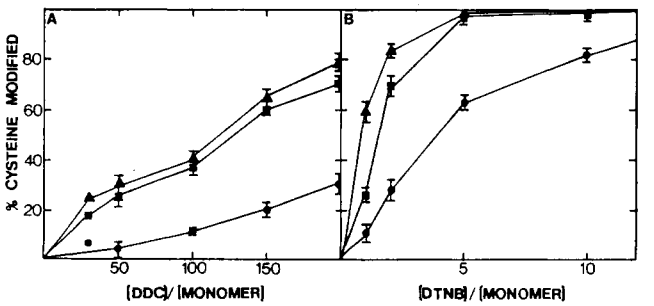

FIGURE 4: Extent of reaction of individual cysteines at increasing molar ratios of reagent over repressor monomer. Repressor was reacted with reagent as described under Materials and Methods followed by denaturation of the protein and modification with 2-(bromoacetamido)-4-nitrophenol. Proteolysis and peptide separation were performed as described under Materials and Methods. Results are expressed as percent sulfhydryl reacted. (A) Didansylcystine; (B) DTNB; (●) cysteine-281; (■) cysteine-107; (▲) cysteine-140.

Table I: Binding Constants for Ligands

protein	molar ratio of reagent	ligand	K_d (M)
repressor[a]		40 bp O	1×10^{-11}
MMTS–repressor[a]	150	40 bp O	3×10^{-10}
DDC–repressor[a]	150	40 bp O	4×10^{-11}
DTNB–repressor[a]	10	40 bp O	5×10^{-10}
repressor[b]		IPTG	2×10^{-6}
MMTS–repressor[b]	150	IPTG	2×10^{-6}
DDC–repressor[b]	150	IPTG	2×10^{-6}
DTNB–repressor[b]	10	IPTG	2×10^{-6}
repressor[c]		NS DNA	2×10^{-8}
MMTS–repressor[c]	150	NS DNA	2×10^{-8}
DDC–repressor[c]	150	NS DNA	7×10^{-9}
DTNB–repressor[c]	10	NS DNA	6×10^{-8}

[a] Protein samples at varying concentrations were mixed with ^{32}P-labeled 40 bp operator (40 bp O) DNA fragments (8×10^{-12} M) to generate a binding curve at pH 7.4 as described under Materials and Methods. [b] Inducer titrations were performed spectrofluorometrically as described under Materials and Methods. [c] Nonspecific DNA (NS DNA) binding assays were performed by using λ*plac* with saturating IPTG present (O'Gorman et al., 1980a) in the absence of DTT in standard FB buffer (Riggs et al., 1968). The value reported corresponds to the repressor concentration at half-saturation.

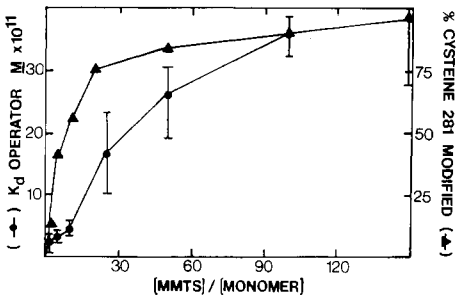

FIGURE 5: Correlation of operator binding affinity and cysteine-281 modification with increasing molar ratios of MMTS over monomer concentration. Reaction of Cys-281 was determined by BNP mapping data as described under Materials and Methods. (●) K_d for 40 base pair operator; (▲) percent modification of Cys-281.

extent of Cys-281 modification increased to ~80% at a 10-fold molar ratio and was complete at ratios >20 per protein monomer. In agreement with the results of Janatova et al. (1968), the total absorbance change at 405 nm did not correspond to the values obtained by BNP mapping.

Effect of Cysteine Reaction on Binding Activities. (*A*) *Methyl Methanethiosulfonate.* The affinity of MMTS-modified repressor for 40 base pair operator fragment was 30 times smaller than that observed for unmodified repressor (Table I). As shown in Figure 5, the increase in the K_d for operator DNA correlates with modification of cysteine-281. However, this decrease in operator affinity appeared to occur only after 50% of the total Cys-281 residues are modified. This result suggests a quaternary effect in which at least two of the Cys-281 residues in the tetramer must be modified before operator DNA binding is affected. The nonspecific DNA binding activity of the repressor protein was unaffected by modification up to a 150-fold molar ratio of MMTS over monomer (Table I). At all molar ratios of MMTS to monomer studied, the half-saturation concentration of IPTG in fluorometric titration corresponded to that observed for wild-type repressor (Table I).

(*B*) *Didansylcystine.* A 4-fold decrease in operator binding affinity of the repressor was observed at molar ratios of didansylcystine >30 per monomer. At these high ratios, nonspecific DNA binding affinity was increased ~3-fold; at lower levels of reagent, no effect was observed (Table I). Precipitation of the protein occurred at molar ratios >200 per monomer. Inducer affinity was unaffected regardless of the extent

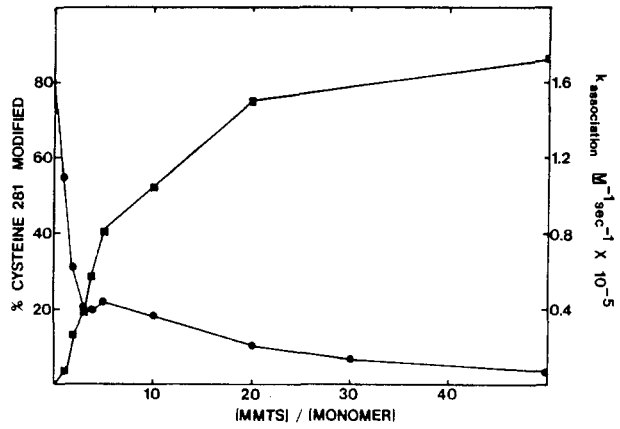

FIGURE 6: Correlation between the inducer association rate constant and modification of cysteine-281 at increasing molar ratios of MMTS over monomer concentration. Reaction at cysteine-281 was determined from 2-(bromoacetamido)-4-nitrophenol mapping data. Inducer association rate constants for inducer binding to modified repressor were determined as described under Materials and Methods. (■) Percent cysteine-281 modified with MMTS; (●) inducer association rate constant.

Table II: Effects of Sulfhydryl Modification on Ligand Affinity and Binding Rates

protein	IPTG k_{assoc}[a] (M^{-1} s^{-1})	IPTG k_{dissoc}[a] (s^{-1})	IPTG K_d[b] (M)	40 bp O K_d[c] (M)
repressor	1.5×10^5	0.3	2.0×10^{-6}	1×10^{-11}
MMTS–repressor	3.5×10^3	0.007	2.0×10^{-6}	3×10^{-10}
DDC–repressor	1.1×10^5	0.18	1.6×10^{-6}	4×10^{-11}
DTNB–repressor	4.9×10^3	0.008	1.6×10^{-6}	5×10^{-10}
MNP–repressor	2.9×10^3	0.005	1.6×10^{-6}	2×10^{-10}
BNP–repressor	1.1×10^5	0.22	2.0×10^{-6}	2×10^{-11}
BNP–IPTG–repressor	1.4×10^5	0.28	2.0×10^{-6}	2×10^{-11}
BNP–repressor + MMTS	8.8×10^3	0.016	1.8×10^{-6}	3×10^{-10}
BNP–IPTG–repressor + MMTS	7.8×10^3	0.016	2.0×10^{-6}	3×10^{-10}

[a] Inducer association and dissociation rates were measured by using stopped-flow rapid mixing techniques. The individual rates were determined by examining the dependence of the observed rate on sugar concentration ($k_{obsd} = k_{assoc}[IPTG] + k_{dissoc}$). The values for k_{dissoc} are obtained as the y intercept of a k_{obsd} vs. [IPTG] plot and are therefore subject to the greatest error. [b] IPTG affinity was determined by fluorometric titrations from the concentration of inducer at half-saturation of protein as described under Materials and Methods. [c] Operator affinity was determined from binding curves generated from varying protein concentrations mixed with ^{32}P-labeled 40 bp operator DNA using nitrocellulose filter binding.

of didansylcystine modification.

(C) 5,5′-Dithiobis(2-nitrobenzoic acid). Repressor modified with a 10-fold molar ratio of DTNB over monomer exhibited a 50-fold decrease in affinity for 40 bp operator. This DTNB-modified repressor bound nonspecifically to DNA with an affinity 3-fold lower than unmodified repressor (Table I). IPTG binding was unaffected by DTNB modification.

Effects of MMTS Modification on Inducer Binding Rates. The pseudo-first-order rates of IPTG binding were decreased ~50-fold when the protein was completely modified with MMTS. The individual association and dissociation rate constants for inducer binding decreased as a function of increased molar ratios of reagent (Figure 6, Table II); however, the equilibrium constant remained the same as unmodified protein and was invariant at all molar ratios of MMTS studied. The decrease in the inducer association rate correlated directly with the extent of modification of Cys-281 as determined by BNP mapping (Figure 6). The kinetic rate constants were

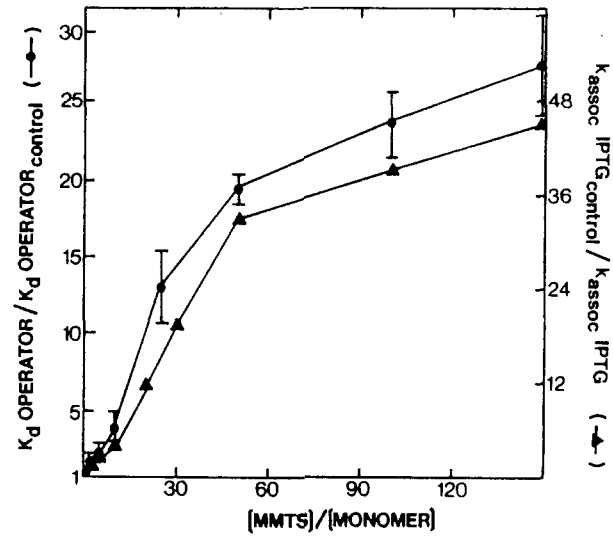

FIGURE 7: Correlation between the increase in the K_d for operator binding and the decrease in the inducer association rate constant at increasing molar ratios of MMTS over repressor monomer. Results are plotted as the ratio of either the operator DNA K_d or the inducer k_{assoc} for the modified protein compared to these values for unmodified repressor. (●) Operator DNA affinity; (▲) inducer association rate.

Table III: Effects of Cysteine Reagents on Repressor–IPTG Association

reagent	cysteine modified	k_{assoc} (M^{-1} s^{-1})	K_d (M)
none		1.9×10^5	2.0×10^{-6}
2-(bromoacetamido)-4-nitrophenol	107, 140[a]	1.5×10^5	2.0×10^{-6}
didansylcystine	107, 140	1.1×10^5	1.6×10^{-6}
(iodoacetamido)-DNS	140[b]	1.2×10^5	1.6×10^{-6}
methyl methanethiosulfonate	107, 140, 281	4.6×10^3	2.0×10^{-6}
2-(chloromercuri)-4-nitrophenol	107, 281[a]	2.9×10^3	1.6×10^{-6}
5,5′-dithiobis(2-nitrobenzoic acid)	107, 140, 281	4.9×10^3	1.6×10^{-6}
fluorescein mercuric acetate	107, 140, 281[c]	1.4×10^3	1.6×10^{-6}

[a] Yang et al. (1977). [b] Schneider et al. (1984). [c] Burgum & Matthews (1978).

obtained by fitting the observed time courses to a single-exponential expression regardless of the extent of modification. This analysis was approximate when the protein was treated with intermediate levels of MMTS. It is noteworthy that the decreased operator affinity and decreased association rate constant for inducer binding as the molar ratio of MMTS is increased to 150 per monomer correlate directly with one another and with modification of cysteine-281 (Figures 5–7).

Effects of Prior Modification of Cys-140 and Cys-107. Repressor was modified with BNP in the absence of IPTG (Cys-107 ~35% modified; Cys-140 ~90% modified; Yang et al., 1977) and in the presence of IPTG (Cys-107 ~85% modified; Cys-140 ~90% modified). The inducer association rates and operator affinities of the product proteins are shown in Table II. Neither of the BNP-modified samples demonstrated any significant decrease in the rate constants for inducer binding or in operator affinity. Decreases in the sugar binding rate and operator binding affinity similar to those observed without prior reaction with BNP were found when these modified proteins were reacted further with MMTS (Table II). These data confirm that modification of Cys-281 is responsible for the decreases observed in operator affinity and in the rate constants for inducer binding.

Effects of Sulfhydryl-Specific Reagents on Binding Parameters. As shown in Table III, only those reagents which

modify Cys-281 were observed to significantly decrease the rate of inducer binding to the protein. Those probes which react only at Cys-107 and/or Cys-140 did not alter the inducer association rate compared to unmodified repressor. There was no appreciable change observed in the affinity of any of the proteins for inducer. Of the five reagents studied, only the three that modify Cys-281, MMTS, DTNB, and MNP elicited significant decreases in operator affinity (Table II).

Discussion

Lactose repressor protein has been modified by a series of reagents whose mixed disulfide adducts are structurally diverse. MMTS reaction with free sulfhydryl groups introduces a small, nonpolar, non-hydrogen-bonding methanethiol group. Didansylcystine modification results in the incorporation of a strongly hydrophobic, sterically bulky group, whereas DTNB modification results in the addition of a moderately bulky, negatively charged moiety. MMTS and DTNB reacted with all three cysteine residues on the repressor monomer, while didansylcystine reacted with only two sulfhydryl equivalents. The inability of didansylcystine to react with all three cysteine residues can be rationalized in terms of steric constraints within the repressor. Previous work (Sams et al., 1977; Manly & Matthews, 1979) has demonstrated the lack of reactivity of Cys-281 toward many sulfhydryl reagents. Cys-281 is apparently either buried within a monomer or located on the surface of the monomer in a region involved in subunit aggregation. In this context, it is noteworthy that a mutant repressor (T-41) containing a single amino acid change at position 282 is unable to aggregate into tetramers (Schmitz et al., 1976). The location of this mutation indicates that Cys-281 may be positioned at or near a subunit interface and thus would be inaccessible to both solvent and more bulky modifying reagents.

This hypothesis is substantiated by the results of MMTS modification of the isolated mutant monomer (T-41); reaction at Cys-281 in T-41 monomer is observed at stoichiometric ratios of reagent and parallels reaction at Cys-107 and -140 (Daly & Matthews, 1986). This increased exposure of Cys-281 in the monomer relative to the tetramer structure and the genetic data are consistent with location of this residue in a region which is involved in subunit assembly. There is spectroscopic evidence to suggest that Cys-281 is in close proximity to acidic residues (Brown & Matthews, 1979b). The similarity of the wavelength of maximum absorbance for nitrophenol bound to Cys-281 to that for free organomercurial indicates that the region surrounding Cys-281 is probably very polar. Glycerol perturbation studies of nitrophenol-labeled sulfhydryls have indicated that the cysteine residues are not fully accessible to solvent molecules (Sams et al., 1977). A buried but polar region would be expected to diminish Cys-281 reactivity with nonpolar bulky sulfhydryl reagents such as DDC, IAEDANS, and *N*-pyrenylmaleimide [see Table III and Brown & Matthews (1979a,b)].

The equilibrium dissociation constant for inducer binding to the repressor was unaffected by sulfhydryl modification at any of the cysteine residues. Previous work involving sulfhydryl reagents which reacted at Cys-281 (e.g., FMA; Burgum & Matthews, 1979) suggested that inducer binding affinity was increased for modified repressor compared to control by using the nitrocellulose filter binding assay. In the present study, fluorescence titrations of repressor reacted with these reagents indicated no alteration in the K_d for inducer binding. The decreased dissociation rate constant observed with reaction of Cys-281 results in an apparent increase in binding using the filter binding assay, since a portion of the complex which normally dissociates from unreacted repressor during the filtration period would remain bound to the modified protein.

Previous work with 2-(chloromercuri)-4-nitrophenol-modified repressor demonstrated no effect on percent operator activity using λp*lac* DNA (Yang et al., 1977) under stoichiometric conditions ([DNA] $\gg K_d$). Accurate measurements of K_d for λp*lac* were hampered by the high affinity of this DNA species for repressor and the difficulty in obtaining molecules with a high specific activity. In contrast, the effects of modification on repressor binding to a 40 base pair operator fragment can be measured accurately, and reduced affinity for operator is observed when cysteine-281 is reacted.

The clear correlation between decreased rates of IPTG access to and egress from the inducer binding site and modification of Cys-281 indicates a role for this region of the protein in regulating the dynamic aspects of the sugar binding process. The modification of Cys-281 appears to result in a slowed rate of opening and closing of the sugar binding site, with no change in the thermodynamics of inducer binding. The conformational alterations which cause these marked changes in the dynamics of sugar binding also influence operator binding. The correlation between Cys-281 reaction, decreased operator affinity, and decreased rates of IPTG binding suggests strongly that this amino acid is located in a region of the protein which serves to link DNA and inducer binding.

Registry No. IPTG, 367-93-1; DDC, 18468-46-7; DTNB, 69-78-3; MMTS, 2949-92-0; L-cysteine, 52-90-4.

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Characterization and Modification of a Monomeric Mutant of the Lactose Repressor Protein†

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ABSTRACT: A monomeric mutant lactose repressor protein (T-41), containing serine at position 282 in place of tyrosine [Schmitz, A., Schmeissner, U., Miller, J. H., & Lu, P. (1976) *J. Biol. Chem. 251*, 3359–3366], has been purified by a series of chromatographic and precipitation methods. The molecular weight of the mutant as determined by gel filtration was approximately 40 000. The inducer equilibrium binding constant for the mutant was comparable to that of the tetrameric wild-type repressor at pH 7.5, whereas operator DNA binding was not detectable. In contrast to wild-type repressor, equilibrium and kinetic rate constants for inducer binding to the monomer were largely independent of pH; thus, the quaternary structure of the wild-type repressor is required for the pH-associated effects on inducer binding. Although ultraviolet absorbance difference spectra indicated that inducer binding to T-41 protein elicited different changes in the environment of aromatic residues from those generated in wild-type repressor, the shift in the fluorescence emission maximum in response to inducer binding was similar for T-41 and wild-type repressors. Similarity in 1-anilinonaphthalene-8-sulfonic acid binding to monomer and tetramer suggests that this fluorophore does not bind at subunit interfaces. Modification of Cys-281 with methyl methanethiosulfonate was observed at low molar ratios of reagent per T-41 monomer (4-fold). This result is in contrast to data observed for tetrameric wild-type repressor which requires high molar ratios for this cysteine to react. We conclude that Cys-281, adjacent to the site of the T-41 mutation, is located on the surface of the monomer in this region crucial for subunit interaction.

DNA binding proteins which regulate transcription have been studied extensively, and a common feature of this class of proteins is multimeric structure (Müller-Hill, 1971; Pirrotta et al., 1970; Krakow & Paston, 1973; Joachimiak et al., 1983; Monod et al., 1963). Many of the members of this family of proteins exist as dimers; in contrast, the *lac* repressor is a tetramer of identical subunits with molecular weight 38 000 (Müller-Hill, 1971). In the absence of operator DNA, inducer binds to the *lac* repressor noncooperatively; in the presence of bound operator, significant cooperativity is observed for inducer binding (O'Gorman et al., 1980). Thus, the quaternary structure provides a mode of communication within the repressor tetramer which can be used to alter binding characteristics of the protomers.

The addition of denaturants [e.g., 0.1% sodium dodecyl sulfate (SDS)[1] and 2 M guanidinium chloride] has been ineffective in producing monomers of the *lac* repressor without causing partial denaturation of the protein (Hamada et al., 1973). The low dissociation constant for repressor–subunit interactions ($<10^{-20}$ M) may explain the failure of attempts

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[1] Abbreviations: ABP, arabinose binding protein; ANS, 1-anilinonaphthalene-8-sulfonic acid; BNP, 2-(bromoacetamido)-4-nitrophenol; BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbant assay; HPLC, high-pressure liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; MMTS, methyl methanethiosulfonate; PMSF, phenylmethanesulfonyl fluoride; RAMP, rabbit anti-mouse peroxidase; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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