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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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to isolate a native monomeric repressor species (R. B. O'-Gorman and K. S. Matthews, unpublished results; Royer et al., submitted for publication).

Analysis of mutations within the lactose repressor gene has identified specific domain regions within the amino acid sequence. Mutations which alter the quaternary structure of the protein have been mapped primarily to the carboxy-terminal region of the amino acid sequence (Schmitz et al., 1976). These mutants maintain normal inducer binding, are unable to repress β-galactosidase synthesis in vitro, and sediment more slowly than wild-type repressor in a sucrose gradient (Schmitz et al., 1976). One of these mutants was determined to be the result of a point mutation at amino acid 282 of the primary sequence (Ganem et al., 1973). This mutant T-41 repressor exhibits different physical properties compared to wild-type repressor and consequently could not be isolated by the standard purification techniques (Rosenberg et al., 1977). We report in this paper the isolation, characterization, and chemical modification of the T-41 mutant monomer repressor.

Materials and Methods

Growth of Bacterial Strain Containing T-41. The *E. coli* strain containing the mutant repressor T-41 was the kind gift of Dr. Ponzy Lu (University of Pennsylvania). Cells were grown at 32 °C, and standard induction procedures were used for the heat-sensitive prophage (Miller, 1972). After induction at A_{550} = 1.6, the cells were allowed to grow at 32 °C for 4 additional h. Cells were harvested by centrifugation and frozen in 250-g packets until use.

Purification of Monomeric Mutant T-41. The presence of the monomer was monitored during purification by inducer binding using the ammonium sulfate precipitation procedure of Bourgeois (1971). Frozen cells were ruptured by thawing in buffer containing 0.2 M Tris-HCl, pH 7.6, 0.2 M KCl, 0.01 M magnesium acetate, 3×10^{-4} M DTT, 5% glucose, and 50 μg/mL PMSF. Cells were blended to a homogeneous suspension, DNase was added, and the mixture was allowed to incubate for approximately 0.5 h. The suspension was centrifuged for 50 min at 7500 rpm, and the supernatant was brought to 25% in ammonium sulfate at 4 °C. Following incubation for 2 h and centrifugation, the supernatant from this step was brought to 37% ammonium sulfate and incubated for 2 h, and the mixture was centrifuged at 7500 rpm for 40 min. The supernatant was removed, a small volume of column buffer containing 0.048 M potassium phosphate, pH 7.5, 5% glucose, and 3×10^{-4} M DTT was added to the precipitate, and the suspension was dialyzed against this buffer with several changes of dialysate. Following dialysis, any precipitated material was removed by centrifugation, and the supernatant was introduced onto a column containing DEAE-cellulose matrix layered on top of phosphocellulose and previously equilibrated with column buffer. Flow-through fractions containing inducer binding activity were pooled and brought to 30% in ammonium sulfate at 4 °C. Following centrifugation, the supernatant was then brought to 36% in ammonium sulfate and recentrifuged at 7500 rpm for 40 min. The precipitate from this step was resuspended, dialyzed against column buffer, and loaded onto a Sephadex G-100-40 column equilibrated with column buffer. Fractions containing inducer binding activity were pooled, divided into small volumes, and frozen.

Lactose Repressor Specific B1 Monoclonal Antibody. Antibodies (B1) specific for native tetrameric *lac* repressor were isolated as previously described and screened by ELISA assay (Sams et al., 1985). Nitrocellulose sheets onto which proteins were dot blotted were analyzed by the methods described by Sams et al. (1985) for electrophoresis transfers.

Measurement of Binding Parameters. Fluorescence measurements were carried out on an SLM Instruments Series 400 spectrofluorometer. The excitation wavelength for emission spectra, quenching experiments, and inducer titrations was 285 nm. Emission was measured by using either a monochromator (8-nm band-pass) for scanning spectra or a Corning 0-52 filter only. Inducer binding to wild-type repressor and mutant monomer was measured fluorometrically as described previously (O'Gorman et al., 1980; Daly et al., 1986). The association and dissociation rate constants for wild-type and T-41 mutant repressor were measured by using a Gibson-Durrum rapid-mixing stopped-flow spectrometer as described in Daly et al. (1986).

Binding of 1-anilinonaphthalene-8-sulfonic acid [ANS, bis(ANS) free; Molecular Probes, Inc.] to wild-type repressor and T-41 monomer was measured spectrofluorometrically by using an excitation wavelength of 355 nm and an emission wavelength of 488 nm. Protein solutions were diluted to 1 mg/mL in 0.12 M potassium phosphate, pH 7.6, 3×10^{-4} M DTT, and 5% glucose. The fluorescence intensity of the solution was measured after each addition of 2 μL of a 0.005 M ANS stock to a 2-mL volume of protein. Fluorescence intensities of buffer titrated with ANS were subtracted from comparable ANS–protein values. The data were not corrected for inner filter effects.

Chemical Modification of T-41 Mutant Repressor. Monomeric mutant repressor was modified with MMTS, and the products were analyzed as described previously for wild-type repressor (Daly et al., 1986).

Results

Isolation of Purified T-41 Monomeric Repressor. Due to differences in the physical properties of the mutant, the purification scheme used for wild-type repressor was not applicable. At all buffer concentrations studied, the T-41 monomeric repressor did not bind to phosphocellulose, although small amounts of other proteins were removed from the mixture. When DEAE-cellulose chromatography was employed in the purification, monomer activity was detected in the flow-through fractions, and significant amounts of contaminating proteins were bound to the column (Figure 1). Although a portion of the monomer (~25%) was irreversibly lost on the column, the extent of purification justified utilizing combined DEAE-phosphocellulose chromatography in the isolation procedure. A series of ammonium sulfate precipitations was used to further purify the monomer. The elution profile of the partially purified protein from a molecular sieve column consisted of one major peak and several smaller peaks; the fractions containing inducer binding activity were pooled. The results of SDS–polyacrylamide gel electrophoresis on this purified protein are shown in the inset to Figure 1. A single major species whose migration corresponds to an approximate molecular weight of 38 000 was observed. The purity of the monomer was estimated to be ~95%. Inducer binding activity measured at pH 7.5 by ammonium sulfate precipitation and nitrocellulose filter binding was identical with wild-type activity. No DNA binding was detectable, a result consistent with the inability of this protein to bind phosphocellulose.

Determination of Molecular Weight of Monomer by Gel Filtration. The elution volumes of mutant and wild-type repressor from a Sephadex G-100-40 column were compared to those of proteins of known molecular weight (Figure 2A). Fractions from columns on which T-41 mutant or wild-type repressor was run were assayed for inducer binding activity. The T-41 elution volume (Figure 2B) corresponds to a protein

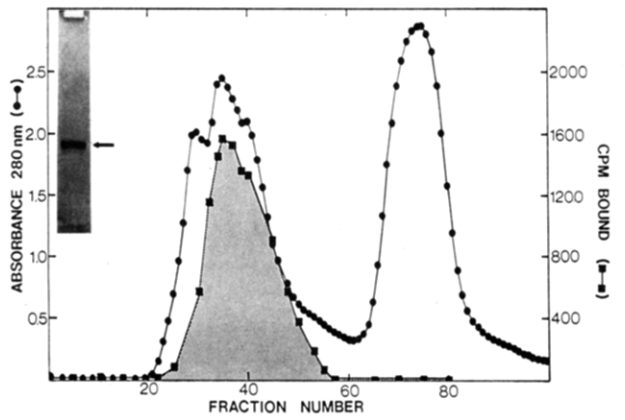

FIGURE 1: Elution of T-41 mutant repressor from a layered DEAE-phosphocellulose column. The ion-exchange resins were equilibrated in 0.048 M potassium phosphate, pH 7.5, 3×10^{-4} M DTT, and 5% glucose. Protein elution was monitored by absorbance at 280 nm. Inducer binding activity of T-41 repressor monitored by the ammonium sulfate precipitation method is indicated by shading. Inset: Electrophoresis pattern for purified T-41 monomeric mutant. Protein was denatured by boiling in the presence of 0.1% SDS and 1% β-mercaptoethanol. The denatured protein was electrophoresed on a 12.5% SDS–polyacrylamide gel at 5 mA/well for 4 h. The protein in the gel was fixed in 10% trichloroacetic acid and stained with silver. Arrow indicates mobility of wild-type repressor.

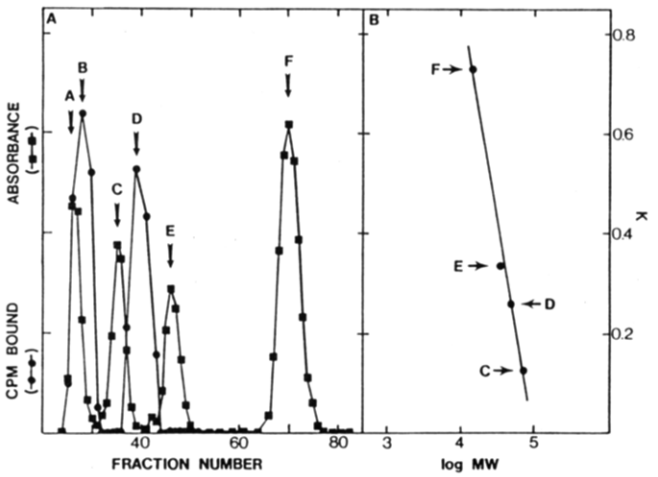

FIGURE 2: Gel filtration chromatography of wild-type and T-41 monomeric repressors. Approximately 2 mg (in 1 mL) of each protein was loaded onto a G-100-40 Sephadex column (45 cm × 2 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5. Absorbance at 280 or 660 nm and IPTG binding activity were determined for the eluted fractions (1.4 mL). Blue dextran was used to determine the void volume of the column. (Panel A) Elution profiles: (A) Blue dextran (2×10^6 daltons); (B) wild-type repressor (150000 daltons); (C) bovine serum albumin (66700 daltons); (D) T-41 mutant repressor; (E) arabinose binding protein (33170 daltons); (F) lysozyme (14400 daltons). (Panel B) Determination of the molecular weight of the T-41 mutant repressor. The elution volumes of the molecular weight standards in panel A were used to determine κ $[(V_e - V_o)/(V_t - V_o)]$; these κ values were plotted vs. the log of the molecular weight.

of molecular weight ~40000, and the protein elutes as a single peak. No dimeric mutant was detectable at the concentrations studied.

Reaction of Monomer with Lac Repressor Monoclonal Antibody. Purified wild-type and monomeric repressors were filtered onto nitrocellulose paper in both native and partially denatured forms. SDS was added to a final concentration ranging from 0.01% to 0.1%. The protein on the nitrocellulose paper was allowed to react with monoclonal antibody generated to wild-type repressor (Figure 3). The two protein species display significantly different antibody reactivities. In the native form, only the monomeric mutant repressor reacted with

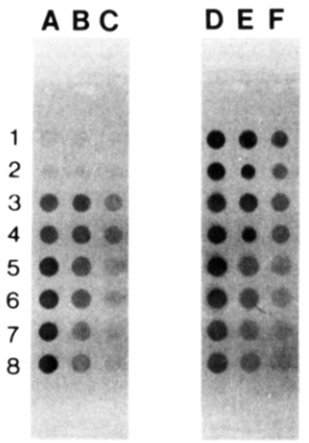

FIGURE 3: Antibody reaction with protein species on nitrocellulose filter paper. Wild-type (A–C) and T-41 (D–F) repressors were filtered onto nitrocellulose paper following addition of 0.0% (1, 2), 0.01% (3, 4), 0.03% (5, 6), or 0.1% SDS (7, 8). (A, D) 50 μg of protein; (B, E) 10 μg of protein; (C, F) 2 μg of protein. Following incubation with monoclonal antibody, the nitrocellulose paper was developed as described under Materials and Methods.

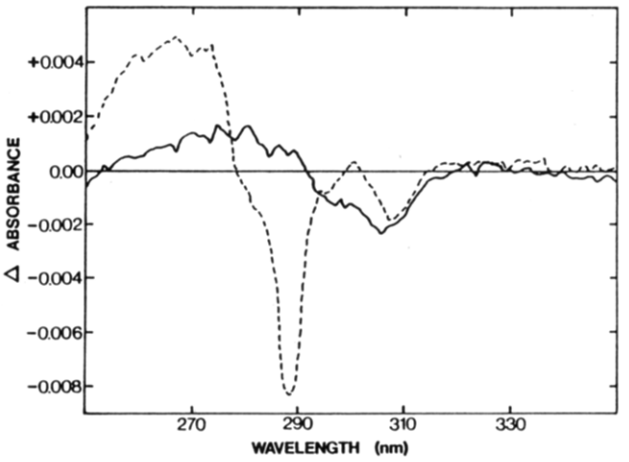

FIGURE 4: Ultraviolet difference spectra for wild-type and T-41 mutant repressors. Protein (1.2 mg/mL) in 0.1 M Tris-HCl, pH 7.5, was placed in each of two 1-mL cuvettes. The base line was determined, and 10 μL of 0.1 M IPTG was added to the sample cuvette and an identical volume of buffer to the reference cuvette. The difference spectrum obtained for T-41 mutant repressor is the solid line, and the wild-type repressor difference spectrum is the dashed line.

the antibody. Both species of repressor reacted as the SDS concentration was increased to 0.1%. As demonstrated previously (Sams et al., 1985), the antibody recognition site is accessible for reaction only on the native T-41 monomer or on SDS-dissociated wild-type repressor.

Difference Spectra of Mutant Monomer. Ultraviolet difference spectra for mutant and wild-type repressors are shown in Figure 4. The pattern for T-41 monomer repressor is significantly different from wild-type repressor (Matthews, 1974). At 288 and 280 nm, no minima are observed for the mutant protein, whereas the minimum at 308 nm is equivalent for monomer and wild-type repressor. The difference spectrum observed for the monomer suggests that the environments of tryptophan and/or tyrosine residues are affected by inducer binding but the pattern is significantly altered from wild-type repressor.

Fluorescence Emission Spectra of T-41 Monomer. The fluorescence emission spectra for wild-type and monomeric repressor were measured both in the absence and in the presence of inducer. The fluorescence intensity for the T-41 monomer is ~80% that observed for the wild-type repressor.

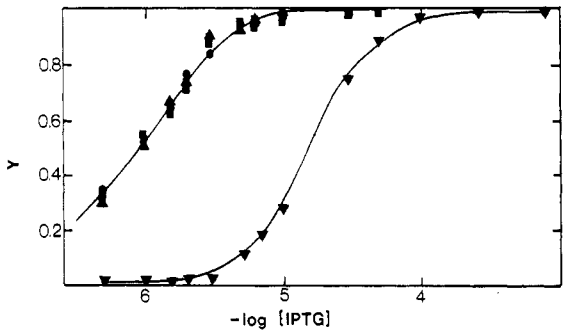

FIGURE 5: Fluorescence titrations of IPTG binding to wild-type and T-41 monomer repressors at pH 7.5 and 9.2. Concentration of protein monomers was 1×10^{-7} M. Titrations with IPTG were performed as described under Materials and Methods with excitation at 285 nm and emission greater than 350 nm (Corning filter 0–52). The fractional degree of saturation, Y, was measured as the ratio of the change in the fluorescence observed compared to the total change in fluorescence. Titrations were carried out in 0.01 M Tris-HCl, at either pH 7.5 or pH 9.2, 0.2 M KCl, 0.01 M $MgCl_2$, and 0.001 M EDTA. (●) Wild-type repressor, pH 7.5; (▼) wild-type repressor, pH 9.2; (▲) T-41 repressor, pH 7.5; (■) T-41 repressor, pH 9.2.

Table I: Inducer Binding Parameters[a]

protein	pH	k_{assoc} (M^{-1} s^{-1})	k_{dissoc} (s^{-1})	K_d (M)
wild-type	7.5	1.7×10^5	0.3	1.5×10^{-6}
wild-type	9.2	2.7×10^4	0.4	1.5×10^{-5}
T-41 repressor	7.5	2.5×10^5	0.3	1.2×10^{-6}
T-41 repressor	9.2	3.6×10^5	0.6	1.7×10^{-6}

[a] Inducer binding parameters were measured as described under Materials and Methods.

The magnitude of the blue shift in the wavelength of maximum emission in response to IPTG binding is somewhat less for the monomer (~6.5 nm) vs. wild-type repressor (8 nm). Inducer binding to T-41 and wild-type repressors was monitored by the decrease in fluorescence emission intensity at wavelengths >350 nm (Figure 5). The affinities of the two proteins for IPTG were identical at pH 7.5. At pH 9.2, wild-type protein exhibits a decreased affinity (Friedman et al., 1977; Table I); however, the monomer affinity for IPTG was not altered by the increase in pH. Apparently, the pH effect on inducer binding requires an intact tetrameric structure.

ANS Binding Studies. The quantum yield of ANS, which binds noncovalently in hydrophobic pockets within proteins, is increased by an apolar environment. Worah et al. (1978) have shown that ANS binds at one site per monomer in the wild-type repressor and have postulated the location of a hydrophobic pocket between the amino-terminal and the core domains. Similar ANS binding curves were observed for wild-type and monomer repressors (data not shown). Regions on the surface of the monomer which are involved in intersubunit contacts in the tetramer apparently contain no significant hydrophobic regions which are able to bind ANS. If the site of ANS binding is assigned to a core/NH_2-terminus interface, the similarity in ANS binding for T-41 mutant and wild-type repressors suggests that conversion of monomer into tetramer does not dramatically affect these contacts.

Measurement of Inducer Association and Dissociation Rates. At neutral pH, the inducer association rate constant for the mutant monomer is less than 2-fold greater than the corresponding value for wild-type repressor. Increasing the pH from 7.5 to 9.2 causes only a small (50%) increase in the IPTG association rate constant for T-41 repressor, whereas a ~6-fold decrease is observed for the IPTG association rate constant for wild-type repressor. Thus, quaternary structure appears to be essential for the decreased IPTG binding rate seen for wild-type repressor.

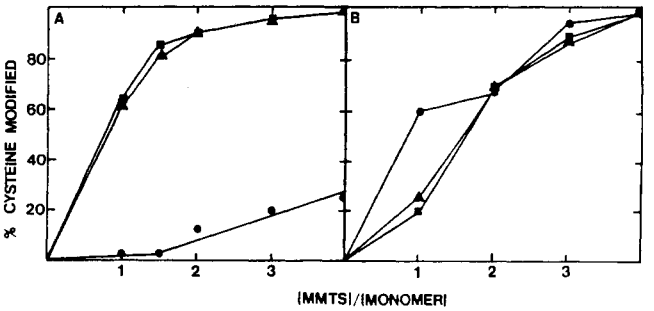

FIGURE 6: Extent of methyl methanethiosulfonate modification of individual cysteines at increasing molar ratios of reagent. Modifications of wild-type and T-41 mutant repressors with MMTS and BNP were performed as described under Materials and Methods. Following reaction with MMTS, protein samples were denatured, reacted with BNP and digested with proteases, and the product peptides were separated by reverse-phase HPLC. Peptides containing nitrophenol-labeled cysteines were detected by absorbance at 405 nm. Results are presented as percent modified sulfhydryl. (A) Wild-type repressor; (B) T-41 mutant repressor; (●) cysteine-281; (■) cysteine-107; (▲) cysteine-140.

Modification of Monomer with MMTS. At a 4-fold molar ratio of MMTS over monomer, all three cysteine residues in the mutant were completely reacted (Figure 6). Unlike wild-type repressor, equivalent reaction of each of the three T-41 mutant sulfhydryls was observed at MMTS molar ratios less than 3-fold over monomer concentration. The availability of Cys-281 toward MMTS modification suggests that this sulfhydryl is located on the surface of the monomer but is buried between subunits in the tetrameric repressor.

Discussion

T-41 mutant monomeric repressor was purified to >95% homogeneity by using a series of ammonium sulfate precipitation and chromatographic steps. The inducer binding affinity of partially purified mutant repressor was previously determined to be comparable to that of wild-type repressor (Schmitz et al., 1976), and similar results were obtained for the isolated protein. As anticipated, the mobility of denatured mutant on an SDS–polyacrylamide gel was equivalent to the mobility of denatured wild-type repressor. Gel filtration was used to determine the state of aggregation of the mutant repressor, since previous work with partially purified monomer suggested that a dimeric species also existed along with monomeric protein (Schmitz et al., 1976). No dimeric protein was detected by gel filtration, and the elution volume of the mutant repressor corresponded to a protein species of molecular weight ~40 000. It is feasible that at higher concentrations of T-41 repressor the dimeric species may be observed.

A monoclonal antibody to denatured wild-type repressor exhibits high affinity for the native monomeric repressor (Sams et al., 1985). This result suggests that the region of the native wild-type repressor involved in antibody recognition is located at or near a subunit interface. The epitopes recognized by the antibody are in the region between residues 281 and 360 (Sams et al., 1985). The hypothesis that this carboxyl-terminal region is involved in subunit interactions is also supported by the genetic evidence that mutations affecting subunit assembly are located in this segment of the protein (Miller, 1979; Schmitz et al., 1976).

Mutant T-41 repressor yielded the characteristic wild-type shift in fluorescence emission maximum upon binding to inducer (Laiken et al., 1972), although the magnitude of the shift was slightly decreased for the T-41 monomer. Similar ANS binding to both wild-type and mutant repressors suggested that the absence of quaternary structure does not alter the ANS

binding site. Assuming the assignment of this site to a core/NH_2-terminal interface (Worah et al., 1978), these results indicate that this region of the protein is not significantly altered in the mutant protein. In contrast, ultraviolet difference spectra demonstrated significant variance in environmental changes of aromatic amino acids elicited by inducer binding to T-41 monomer compared to wild-type repressor (Matthews, 1974).

The low reactivity of Cys-281 toward MMTS in wild-type repressor suggested that this residue was buried within the monomer or located between monomers at a subunit interface (Daly et al., 1986). In contrast, the three cysteine residues in the T-41 mutant repressor displayed equivalent reactivity, and Cys-281 was completely reacted at molar ratios of MMTS less than 5-fold over the protein concentration. The T-41 mutation which influences subunit aggregation occurs at position 282, and the sulfhydryl residue at position 281, buried in the tetramer, is located on the surface of the monomer available for reaction with MMTS. From these data, it appears that this region is directly involved in subunit assembly in the wild-type protein.

The inducer equilibrium dissociation constants for wild-type and T-41 mutant repressor were determined spectrofluorometrically at neutral and high pH. Unlike the tetramer, the K_d for IPTG binding to the monomeric protein was not affected by increased pH. Similar differences were observed for inducer association rate constants. Although the inducer association rate constants for wild-type and T-41 repressors at pH 7.5 are similar, they differ markedly at high pH. The pH dependence for wild-type repressor is therefore not due to alterations in amino acids in the inducer binding site but instead is a consequence of effects on the quaternary structure. The ionization of sulfhydryl residues occurs in the pH range examined, and reaction of Cys-281 with MMTS replaces the proton with an $-S-CH_3$ and abrogates the pH dependence of inducer binding (Daly & Matthews, 1986). It is tempting to speculate that the effects of pH on tetramer inducer binding characteristics derive from ionization of the free sulfhydryl at Cys-281 and consequent effects on subunit interactions. Further studies using this mutant *lac* repressor may provide insight into the relationship between structure and function in this regulatory protein.

ACKNOWLEDGMENTS

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Registry No. IPTG, 367-93-1; L-cysteine, 52-90-4; L-tyrosine, 60-18-4.

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