Characterization of Mutations in Oligomerization Domain of Lac Repressor Protein*

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A series of mutant lac repressor proteins at positions 281 or 282 was isolated for detailed characterization. Although Cys281 modification by sulfhydryl reagents abrogates pH effects on inducer binding and diminishes operator binding (Daly, T. J., Olson, J. S., and Matthews, K. S. (1986) Biochemistry 25, 5468-5474), substitution at this site by alanine, serine, phenylalanine, isoleucine, or methionine did not abolish completely the pH shift nor affect operator affinity. Thus, ionization of the sulfhydryl residue does not account fully for the alterations in inducer affinity and cooperativity of binding observed with elevated pH. Substitution for Cys281 did, however, alter the kinetic parameters for inducer association with the protein. The polarity of the side chain at 281 influenced the rates of sugar binding, presumably by altering the rate of opening/closing of the binding site. Furthermore, the presence of the branched side chain of isoleucine at position 281 disrupted oligomerization of the repressor. In contrast to the tolerance for substitution at 281, the only amino acid side chain exchanges for Tyr282 which yielded tetrameric protein with near normal operator binding characteristics were phenylalanine and leucine; this result is consistent with studies of suppressed nonsense mutations at position 282 which indicated repression occurred only for the corresponding substitutions (Kleina, L. G., and Miller, J. H. (1990) J. Mol. Biol. 221, 285-318). Despite the tetrameric character of the Cys281 mutant protein, the pH dependence and cooperativity of inducer binding for this mutant protein were altered. All amino acid substitutions other than phenylalanine and leucine at this position resulted in either monomeric protein or no detectable repressor in the cell. Thus, the hydrophobic character of the side chain at position 282 is essential for tetramer formation, and the phenyl ring alone alters inducer binding parameters. The monomeric mutant proteins with substitutions for Tyr282 exhibited lower stability than their tetrameric counterparts, and the absence of dimer formation suggests alterations at this site affect both dimer and tetramer interfaces. Based on previous genetic studies and our detailed mutant characterization, the region encompassing 281 and 282, indicated by secondary structure prediction to be a turn or coil, is essential for oligomer formation and additionally exerts a strong influence on the dynamic properties of the protein, presumably mediated by interactions at the subunit interface which regulate the rate of opening and closing of the inducer binding cleft.

The lactose repressor protein from Escherichia coli regulates transcription of genes for the lactose-metabolizing enzymes (Miller and Reznikoff, 1980). Repression is effected by high affinity binding of the repressor protein to operator DNA; this binding sterically hinders RNA polymerase initiation of transcription (Miller and Reznikoff, 1980; Straney and Crothers, 1987). The affinity of the protein for operator DNA is modulated dramatically by the binding of a variety of inducer molecules (Barkley et al., 1975), while these sugar derivatives have minimal effect on nonspecific DNA binding affinity. The large molar excess of nonspecific DNA sites thus competes effectively with operator for repressor binding in the presence of inducer molecules (Lin and Riggs, 1975).

The wild-type repressor protein is a tetramer of identical subunits 360 amino acids in length, and its amino acid sequence has been determined (Gilbert and Müller-Hill, 1966; Riggs and Bourgeois, 1968; Beyreuther et al., 1975; Beyreuther, 1978; Farabaugh, 1978). The multimeric nature of the protein is essential to its ability to bind operator DNA (Gilbert and Müller-Hill, 1966; Riggs et al., 1970; Schmitz et al., 1976; Brenowitz et al., 1991). The tetramer dissociates spontaneously upon long term storage at 5°C; however, operator DNA binding activity is lost (Riggs et al., 1970). Although the tetramer dissociates easily in the presence of SDS1 (Hamada et al., 1973), the application of high pressure is required to dissociate the native protein into dimer at neutral pH with a dissociation constant of ~4 nM (Royer et al., 1986, 1990). Inducer binding to repressor is cooperative at neutral pH in the presence of operator DNA or in the absence of operator at elevated pH (O’Gorman et al., 1980b; Friedman et al., 1977), observations that suggest that the conformational state at high pH and bound to operator are similar (Daly and Matthews, 1986b). Modification of Cys281 in wild-type repressor protein with sulfhydryl reagents eliminates the pH-dependent effects on inducer binding; therefore, Cys281 was implicated in cooperativity and consequently in subunit interactions (Daly and Matthews, 1986b). Furthermore, pressure dissociation experiments with protein modified by methyl methanethiosulfonate (MMTS) indicated that the tetramer was stabilized by this reaction (Royer et al., 1986).

A role for the region of the primary structure surrounding

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The abbreviations used are SDS-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MMTS, methyl methanethiosulfonate; sDNA, single strand DNA; PBS, phosphate-buffered saline; dansyl, N,N,N-trimethylamino-naphthalene-1-sulfonyle.
Cys^{82} has been indicated by multiple genetic studies (Miller-Hill, 1975; Schmitz et al., 1976; Miller, 1979; Miller et al., 1979; LeClerc et al., 1988; Gordon et al., 1988; Kleina and Miller, 1990). Mutations in the region between 289 and 291 have been isolated as r-r repressors, and several of these have been examined and found to be primarily monomeric in nature (Miller et al., 1979; Schmitz et al., 1976). One mutant, T41, was identified as a monomer (Schmitz et al., 1976) and characterized in detail (Daly and Matthews, 1986a); the DNA used to isolate this mutant has been sequenced recently and contains a single mutation of Y282D. This T41 mutant exhibits equilibrium constants for inducer binding similar to wild-type at neutral pH, but displays no detectable DNA binding nor pH shift for inducer binding (Daly and Matthews, 1986a). Cys^{82} is fully reactive to cysteine-specific reagents in this protein, in contrast to wild-type repressor, indicating that this region of the protein is buried in the tetramer form of the protein. Recognition of the T41 mutant by B2 monoclonal antibody indicates that the epitope in the C-terminal 80 amino acids of the protein is fully exposed in the T41 protein, while recognition of this epitope in wild-type protein requires the presence of SDS for tetramer dissociation (Daly and Matthews, 1986a; Sams et al., 1985).

The only substitutions at position 282 that produce repressors with wild-type phenotype in vivo are leucine and phenylalanine; all other substitutions examined (serine, glutamine, lysine, alanine, cysteine, glutamate, glycine, histidine, proline, arginine) resulted in an r-r phenotype (Miller et al., 1979; Kleina and Miller, 1990). Extracts from strains carrying the amber mutation at position 282 when suppressed with tRNAs carrying glutamine or serine yielded IPTG binding material (Miller et al., 1979). This material sedimented on a sucrose gradient principally as monomer for the serine and glutamine substitutions (Miller et al., 1979).

In order to explore further the roles of Cys^{82} and Tyr^{82} in assembling the tetrameric structure of the protein, we have generated site-specific substitutions at these sites and have isolated and characterized the resultant altered proteins in detail. All substitutions examined at 281 were well tolerated, with the exception of isoleucine, although isoleucine and valine substitutions at position 282 altered the inducer binding properties of the protein. In contrast to Cys^{82}, substitution of amino acids other than phenylalanine or leucine for Tyr^{82} resulted in monomeric repressors, some of which appear to be unstable and are difficult to isolate; these results are consistent with the in vivo amber suppression studies (Kleina and Miller, 1990). Based on both the in vivo measurements and the behavior of the purified mutant proteins, it appears that this region of the primary sequence is crucial to both dimer and tetramer formation, in contrast to alterations in the C-terminal region of the protein which disrupt tetramer assembly but allow stable dimers to form (Lehming et al., 1987, 1988; Brennowitz et al., 1991; Mandler et al., 1990; Oehler et al., 1990; Miller, 1979; Miller-Hill, 1975; Kania and Brown, 1976; Chakerian et al., 1991; Alberti et al., 1991).

**Materials and Methods**

**Enzymes and Reagents**—Horseradish peroxidase-linked goat anti-mouse IgG antibody, ATP, and deoxyribonucleotide triphosphates were purchased from Boehringer Mannheim. IPTG was obtained from Research Organics, Inc. Crystalline uridine was obtained from Sigma. Ampicillin was from International Biotechnologies, Inc. The Sequenase™ DNA Sequencing Kit was purchased from United States Biochemical Corp. T4-dATP was obtained from Du Pont. The enzymes T4 DNA ligase and T4 polynucleotide kinase were obtained from Promega Biotech. The Low Molecular Weight Gel Filtration Calibrant Kit was obtained from Pharmacia LKB. SDS-PAGE low molecular weight standards were purchased from Bio-Rad. **Bacterial Strains**—DH5α cells (HB101, i) were used to isolate the repressor proteins and were a gift from Dr. J. L. Betz (University of Colorado Medical School, Hare and Sadler, 1978). The bacterial strain XL1-Blue (ara, strA, thi, nala, recA60, Δlac pro, phage S13lacZ ΔM15, F' [λpir]) was a gift from Dr. P. H. Driggers (Baylor College of Medicine) and was used for repressor purification. *E. coli* strains BW313 and RZ1032 were gifts from Dr. T. A. Kunkel (Kunkel, 1985). XAC bacterial strain (ara, Δlacpro)g39A argE-am pso3 thi) was a gift from Dr. J. Miller (University of California, Los Angeles) and Kleina and Miller, 1990).

**Construction of PAC1**—The 1725-base pair fragment containing the entire lacI gene and 430 base pairs of the lacZ gene was isolated from an EcoRI digest of pJ9 plasmid (a gift from Dr. J. L. Betz, 1986) and inserted into the EcoRI site in the polycloning region of pEMBL9 plasmid DNA (Dente et al., 1983). The plasmid was selected by ampicillin resistance, characteristic of the pEMBL family of plasmids, and the single-stranded form was isolated by superinfection with IR1 phage to generate packaged particles containing the ss pAC1 DNA. The structure of the plasmid was confirmed by sequencing of ss pAC1 DNA. The coding region contains Thr^{82}, consistent with a single source of plq (Betz, 1986). Each of the 1725-base pair pAC1 plasmid used to construct the clone was isolated by the method of Clewell and Helinski (1970); derivative plasmid DNAs were isolated for sequencing by the method of Holmes and Quigley (1981).

**Oligonucleotide-directed Site-specific Mutagenesis**—Mutagenic and sequencing oligonucleotides were synthesized on a Biosearch 8600 apparatus with the help of Dr. J. Miller. The oligonucleotides were 10-20 bases in length with the targeted mismatched base(s) in the center. Uracil incorporation into pAC1 wild-type single-stranded template DNA was according to Kunkel (1985). Mutagenesis followed the procedures described by Zoller and Smith (1984) using only the mutagenic primer. The mutant sequence was confirmed by diideoxy sequencing (Sanger et al., 1980). The entire gene of each mutant was sequenced to confirm its integrity.

**Isolation of Repressor Proteins**—Wild-type and mutant repressor proteins encoded by pAC1 derivatives were prepared from transformed DH8, XAC, or DH9 cells grown at 37°C for 15 h in a B. Braun Biostat E 14-liter fermentor. The medium contained yeast extract and tryptone (2YT) supplemented with ampicillin. The cells were harvested by centrifugation for 20 min in a Sorvall RC-3B centrifuge at 5°C and frozen as a thick slurry in lysing buffer (0.2 M Tris-HCl, pH 7.6, 0.2 M KCl, 0.01 M Mg acetate, 0.3 mM DTT, 5% (v/v) glycerol, 50 μg/liter of phenylmethylsulfonyl fluoride) containing 1.5 mM EDTA.

The wild-type and mutant proteins that bound to phosphocellulose were purified by ammonium (Rosenberg et al., 1977; O'Gorman et al., 1980a). Briefly, the cells were lysed, the slurry digested with DNase, centrifuged, and the supernatant precipitated with 35% ammonium sulfate. The resuspended precipitate was dialyzed, and then resuspended to a phosphocellulose column and eluted with potassium phosphate concentration. Purification was monitored by IPTG binding using the ammonium sulfate precipitation method of Bourgeois (1971) and by SDS-PAGE. The mutant proteins which did not bind to phosphocellulose were purified by initial passage through phosphocellulose and DEAE-cellulose columns followed by separation on a Sephadex G-75 column (2.5 x 50 cm) equilibrated with 0.048 M potassium phosphate, pH 7.5, 5% glucose, 0.3 mM DTT. Pooled fractions from the purification were concentrated in a Pro-D-Con apparatus (Bio-Molecular Dynamics) against 0.12 M potassium phosphate buffer, pH 7.5, 0.3 mM DTT, 5% Glucose, and were frozen in small aliquots. The proteins purified were ≥99% pure by SDS-PAGE analysis.

**Activity Assays**—Inducer equilibrium constants were measured by fluorescence titration with IPTG at ambient temperature as described previously (Daly et al., 1986). The buffers utilized were 0.01 M Tris-HCl, 1.0 mM EDTA, 0.1 mM MgCl₂, 0.1 mM DTT, 0.2 M KCl, pH adjusted to 7.5 or 9.2 (TMS buffers). Association and dissociation rate constants for inducer binding to repressors were measured at 20°C using a Gibson-Durrum rapid-mixing stopped-flow spectrometer equipped with fluorescence optics as described previously (Daly et al., 1986). Operator DNA binding assays were performed according to O’Gorman et al. (1980b) in buffer containing 0.01 M Tris-HCl, pH 7.5, 0.3 mM EDTA, 0.1 mM MgCl₂, 0.1 mM DTT.
7.4, 0.15 M KCl, 0.1 mM DTT, 0.1 mM EDTA, 5% dimethyl sulfoxide, 50 μg/ml of bovine serum albumin. 

Evaluation of Oligomeric State—Sephadex G-150–120 chromatography (column dimensions, 1.2 X 55 cm) was used to examine the molecular weight of the repressor proteins. Samples were applied in a volume of 0.1 ml and eluted in 0.1 M potassium phosphate buffer, pH 7.5, with no glucose or DTT. Fractions containing 60 drops (~1.7 ml) were collected, the volume measured, and the presence of protein determined by monitoring fluorescence emission with a 350-nm cut off filter (Corning 0–52) with excitation at 285 nm. The column was standardized using bovine serum albumin and ovalbumin supplied in the Low Molecular Weight Gel Filtration Calibration Kit from Pharmacia LKB as well as wild-type lac repressor and arabinose-binding protein from E. coli. Sodium chloride (100 mg/ml, 0.1 ml) in column buffer was used to determine the total column volume; the elution volume of sodium chloride was determined by conductivity measurements. Blue Dextran 2,000,000 (2 mg/ml in column buffer) was used to determine the void volume.

Sedimentation velocity experiments were conducted in collaboration with Dr. James C. Lee and Thomas Callaci (St. Louis University Medical Center) using a Beckman Model E analytical ultracentrifuge. Samples were dialyzed prior to centrifugation against 0.12 or 0.048 M potassium phosphate, pH 7.5, 0.5 mM DTT, 5% glucose at 5°C, and experiments were performed on 0.5 mg/ml of protein samples at 20°C. Viscosity and density corrections were made for the presence of glucose in the buffer. Analysis of 2-fold dilution was performed for several mutants. The results were graphed, and s_{20,w} values were determined.

Measurement of Circular Dichroism—Circular dichroic spectra were measured on a Jasco J-500A instrument using a cuvette with 0.1-cm pathlength. Scan speed was 50 nm/min from 260 to 190 nm. Protein concentration was 1.0 mg/ml in 0.12 M potassium phosphate buffer, pH 7.6, 5% glucose containing no DTT. The data were normalized for protein concentration.

Antibody Reaction—B2 monoclonal antibody to the lactose repressor (Sams et al., 1985) was used to monitor the exposure of epitopes in the C-terminal domain of the mutant proteins; B2 does not react with the repressor fully with monomeric lactose repressor (Daly and Matthews, 1986a). Repressor proteins were incubated with varying concentrations of SDS up to 0.1% at room temperature for 30 min and applied to nitrocellulose filters prewashed in phosphate-buffered saline (PBS) in a dot-blot apparatus (Sams et al., 1985). BLOTTO (Johnson et al., 1984) was used to block available sites on the nitrocellulose, and following extensive washes with PBS the nitrocellulose was incubated at 5°C overnight in the presence of 10 μg/ml of B2 monoclonal antibody in PBS containing 0.5% bovine serum albumin. After extensive washing in PBS, the nitrocellulose was incubated with goat anti-mouse antibody linked with 125 I. The resulting cleavage fragments were analyzed by 15% SDS-PAGE.

Proteolytic Digestion—Limited digestion of the mutants was performed in the presence of 0.5% trypsin (w/w, 1 mg/ml in 1 mM HCl) at ambient temperature for 20 min; addition of 2% phenylmethyl sulfonyl fluoride (w/w, 4 mg/ml in 100% ethanol) was used to stop the reaction (Matthews, 1979). The resulting cleavage fragments were analyzed by 15% SDS-PAGE.

Fluorescence Measurements—Fluorescence measurements were performed in the Laboratory for Fluorescence Dynamics at the University of Illinois, Champaign-Urbana, in collaboration with Dr. Catherine Royer. Lifetime measurements of selected dansyl-modified and unmodified proteins were performed as described previously (Royer et al., 1989).

Chemical Modification of Repressors—Modification of proteins with MMTS followed the method described by Daly et al. (1986).

RESULTS

Purification of Mutant Repressors—Mutant lac repressor proteins were generated by oligonucleotide-directed site-specific mutagenesis using the pAC1 vector constructed as described under "Materials and Methods." This vector is derived from the pEMBL family and exists as double-stranded plasmid DNA in the cell; however, upon superinfection with phage, ss DNA is packaged and extruded into the media (Dente et al., 1983). Use of this system allowed for isolation of mutant repressor from cells bearing a genomic deletion for lac repressor and transformed by ss pAC1 DNA carrying the desired mutation. The entire coding region of each mutant was sequenced to confirm that the designed amino acid change was the only change in DNA sequence. The wild-type and mutant repressors were purified from bacterial cells as described under "Materials and Methods." Phosphocellulose elution profiles of all the Cys621 mutant repressors, with the exception of C281I, and the Y282F and Y282L repressors were similar to wild-type protein. However, repressor proteins C281I, Y282A, C281S/Y282L, Y282S, and Y282D did not bind well to phosphocellulose. Some heterogeneity in phosphocellulose binding was observed with Y282A, indicating that some oligomer formation may occur in this mutant, but the extent was very low. Purification of proteins that did not adhere to phosphocellulose was accomplished with DEAE 52-cellulose and Sephadex G-75 chromatography. A small amount of the C281I mutant repressor (identified as C281I-A) was purified from phosphocellulose; however, further purification of the pool which did not bind to phosphocellulose by DEAE 52 and Sephadex G-75 chromatography yielded repressor pools C281I-B and C281I-C. The purified proteins were ≥90% pure on SDS-PAGE (see Fig. 5). The mutant protein C281S/Y282L degraded with time during purification and produced a faster moving band on SDS-PAGE; it is interesting to note that the individual substitutions do not yield these effects. In addition, several mutants were produced (Y282E and Y282S) which were monomeric and also exhibited lower stability, particularly in some strains of E. coli. The strain XAC (Kleina and Miller, 1990) was utilized to demonstrate the presence of monomeric Y282E and Y282S.

Size Analysis of the Mutant Proteins—Proteins were examined by gel filtration chromatography and/or sedimentation velocity analysis. All substitutions at position 281 yielded tetrameric proteins (Table I); however, gel filtration results for C281I revealed the presence of dimer and monomer at higher levels than tetramer. The elution profiles for the mutant repressors C281F, Y282F, and Y282A are given in Fig. 1A; the standardization curve for the column and K values for the other mutant proteins are shown in Fig. 1B. The only substitutions for tyrosine at position 282 to yield tetrameric repressor were phenylalanine and leucine (Fig. 1, Table I).

Sedimentation velocity data are given in Table II for C281S/Y282L and Y282A, which yielded s_{20,w} values consistent with

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>141</td>
</tr>
<tr>
<td>C281S</td>
<td>ND</td>
</tr>
<tr>
<td>C281A</td>
<td>ND</td>
</tr>
<tr>
<td>C281F</td>
<td>141</td>
</tr>
<tr>
<td>C281M</td>
<td>141</td>
</tr>
<tr>
<td>C281I-A</td>
<td>148, 76</td>
</tr>
<tr>
<td>C281I-B</td>
<td>71</td>
</tr>
<tr>
<td>C281I-C</td>
<td>48</td>
</tr>
<tr>
<td>Y282F</td>
<td>141</td>
</tr>
<tr>
<td>Y282A</td>
<td>45, 44</td>
</tr>
<tr>
<td>Y282L</td>
<td>141</td>
</tr>
<tr>
<td>C281S/Y282L</td>
<td>35, 32</td>
</tr>
</tbody>
</table>

* Molecular weights were determined from gel filtration.
* ND, not determined.

* Molecular weights were determined by sedimentation velocity as described under "Materials and Methods."
monomeric repressor protein. These data are concordant with the observations during purification that tetrameric and dimeric repressor proteins bind to phosphocellulose (Müller-Hill et al., 1971; Lehming et al., 1988; Chakerian et al., 1991), while monomeric repressor does not adhere to this ion exchanger (Daly and Matthews, 1986a). The monomeric character of the C281S/Y282L double mutant is striking, given that the individual changes result in tetrameric and functional repressor protein. This result suggests a limited geometry in the side chains that can be accommodated at the subunit interface.

For the Y282F and Y282A mutants, fluorescence parameters were measured to confirm the oligomeric states of these proteins. Measurement of the lifetimes and anisotropy of dansyl-modified Y282F indicated that the average rotational correlation times and lifetimes are consistent with wild-type tetramer, with an anisotropy value of 0.2. In contrast, for dansyl-Y282A the steady-state anisotropy value is 0.11 and average rotational correlation time is 5.2 ns compared to 17 ns for wild-type. These analyses confirm the tetrameric character of Y282F and monomeric nature of Y282A.

Reaction with B2 Monoclonal Antibody—The B2 monoclonal antibody for the lac repressor reacts only with wild-type repressor dissociated by the presence of ≥0.01% SDS (Sams et al., 1985; Daly and Matthews, 1986a). This antibody was used to confirm target epitope exposure in the mutant proteins that were deduced on the basis of gel filtration and sedimentation analysis to be dimeric or monomeric. None of the substitutions for Cys281, with the exception of isoleucine, nor substitution of phenylalanine for Tyr282 resulted in mutant proteins which reacted with B2 antibody in the absence of SDS (Fig. 2). The native C281I-A protein reacted with antibody, indicating that only low levels of undissociated tetramer were present. In contrast, the remaining substitutions at 282 yielded proteins which reacted maximally with B2 antibody in the absence of SDS (Fig. 2). The native C281I-A protein reacted with antibody, indicating that only low levels of undissociated tetramer were present. In contrast, the remaining substitutions at 282 yielded proteins which reacted maximally with B2 antibody in the absence of SDS, consistent with their monomeric nature (Fig. 2). The only exception was the substitution of leucine at 282 which produced protein with an antibody staining pattern similar to dimeric repressor proteins, although this protein eluted on gel filtration as a tetramer.

Circular Dichroism Measurements on Mutants—Circular dichroic spectra were measured for the various mutants to determine whether a secondary structure characteristic of the wild-type protein was retained. Substitutions at 281 yielded proteins with circular dichroic spectra comparable to wild-type repressor (data not shown). The spectra for Y282A and C281S/Y282L were diminished slightly in intensity compared to wild-type protein (Fig. 3A). These proteins may be partially unfolded, as their stability was lower than other mutant repressors (see below). The CD spectra for C281I-A (tetramer and dimer, purified from phosphocellulose) and for C281I-B (dimer, purified from Sephadex G-75) were similar to each other but exhibited decreased intensity compared to wild-type (Fig. 3), whereas the spectrum for C281I-C (monomer, purified from Sephadex G-75) showed that substantial structural alterations are present for this species as compared to C281I-A and C281I-B (Fig. 3B).

Operator and Inducer Binding—The observed $K_d$ values for operator binding for the mutants are found in Table III. The

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\toprule
Repressor & $K_d$ (mM) & & & \\
\midrule
Wild-type & 7.5 & & & \\
Y282A & 3.3 & & & \\
C281S/Y282L & 2.7 & & & \\
\bottomrule
\end{tabular}
\caption{Sedimentation velocity data}
\end{table}

$^a$ Sedimentation coefficients were corrected to standard conditions based on density and viscosity measurements of buffers utilized during run.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Antibody reaction for lac repressor proteins in the presence and absence of SDS. Protein was treated as described under "Materials and Methods." Protein samples are as follows: (A) wild-type, (B) C281F, (C) C281M, (D) Y282F, (E) C281I-A, (F) Y282A, (G) C281S/Y282L. Protein concentrations for each vertical column from left to right in each panel are 50, 10, and 2 ng. Horizontal rows 1 and 2 contain 0% SDS; rows 3 and 4, 0.01% SDS; rows 5 and 6, 0.03% SDS; and rows 7 and 8, 0.1% SDS. In panel G, protein was not applied to wells in rows 6 and 8, left-hand column.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Operator and Inducer Binding—The observed $K_d$ values for operator binding for the mutants are found in Table III. The}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Circular dichroism measurements on mutants.}
\end{figure
affinities of all the mutants substituted at position 281, with the exception of C281I, and the affinity of Y282F and Y282L are within 2-fold of the wild-type values. In contrast, the Y282A protein demonstrated only a low level of detectable operator binding, and binding was not observed for C281S/Y282L. This low affinity for operator binding indicates that Y282A may undergo some dimer formation, although size determination measurements for this protein did not indicate this occurrence. C281I bound to operator DNA with an affinity characteristic of dimer (Brenowitz et al., 1991).

Inducer affinity was measured by fluorescence titration of the mutant proteins at pH 7.5 and 9.2 with the results detailed in Table III (Daly and Matthews, 1986a). The wild-type protein undergoes a conformational shift with pH that results in cooperative inducer binding with weaker affinity at elevated pH (Daly and Matthews, 1986a). The ratio of the half-saturation concentration of IPTG at pH 9.2 to that at pH 7.5 is ~7 for wild-type protein. Substitution at 281 with serine, alanine, isoleucine, phenylalanine, and methionine resulted in a small progressive diminution of the pH shift (from a ratio 7 for serine to 2.5 for methionine). It is apparent from these results that ionization of the sulfhydryl residue is not responsible entirely for the pH shift characteristic of the wild-type protein. MMTS modification of C281F or C281M had no effect on inducer binding at high pH, consistent with the loss of a modifiable cysteine residue at position 281 in these mutants and in support of the conclusion that modification at this site is responsible for the abrogation of the pH effects. The association rate constant for inducer binding is decreased by the phenylalanine and methionine substitutions at pH 7.5, while minimal alterations are observed at pH 9.2.

For the substitutions at position 282, phenylalanine and leucine result in a diminished association rate constant at neutral pH (2-3-fold). The Y282A mutation yielded an increased association rate constant and no pH shift. The Y282F mutation resulted in the greatest alteration in equilibrium constants; the ratio of half-saturation concentration of IPTG at pH 9.2 to 7.5 is ~20, 2.5-fold greater than wild-type (Fig. 4). This differential appears to be primarily due to changes in the inducer dissociation rate constant. MMTS modification of this repressor results in abolition of the pH differential, as observed for the wild-type protein. Thus, introduction of a mixed disulfide of \(-\text{S-CH}_2\) at Cys\(^{281}\) significantly affects the properties of the subunit interface and abolishes cooperativity for the mutant substituted with phenylalanine and lacking the phenolic hydroxyl.

**Proteolytic Susceptibility**—The substitutions at Tyr\(^{280}\) that yielded proteins that could be readily isolated were phenylalanine and leucine, consistent with the in vivo results of Kleina and Miller (1990). The alanine, serine (Schmitz al., 1976),

![Circular dichroic spectra for mutant repressors](image)

**Fig. 3. Circular dichroic spectra for mutant repressors.** CD spectra were recorded from wavelengths 250 to 190 nm as described under “Materials and Methods.” Protein concentrations were 1 mg/ml. Panel A: wild-type (●), Y282A (○), and C281S/Y282L (●). Panel B: C281I-A (○), C281I-B (●), and C281I-C (▲).

<table>
<thead>
<tr>
<th>Protein</th>
<th>(K_a^*) (\times 10^{-5}) M(^{-1}) s(^{-1})</th>
<th>(K_d) (\times 10^4) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.2 (±0.3)</td>
<td>5.2 (±0.5)</td>
</tr>
<tr>
<td>C281S</td>
<td>1.3 (±0.7)</td>
<td>5.1 (±0.8)</td>
</tr>
<tr>
<td>C281A</td>
<td>1.2 (±0.4)</td>
<td>5.6 (±0.9)</td>
</tr>
<tr>
<td>C281F</td>
<td>1.1 (±0.2)</td>
<td>5.8 (±0.4)</td>
</tr>
<tr>
<td>MMTS-wild-type</td>
<td>1.0 (±0.9)</td>
<td>6.0 (±0.5)</td>
</tr>
<tr>
<td>MMTS-C281F</td>
<td>1.1 (±0.6)</td>
<td>6.1 (±0.8)</td>
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<td>MMTS-C281M</td>
<td>1.2 (±0.3)</td>
<td>6.2 (±0.7)</td>
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<td>Y282A</td>
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<tr>
<td>Y282F</td>
<td>1.2 (±0.5)</td>
<td>6.4 (±0.6)</td>
</tr>
<tr>
<td>Y282L</td>
<td>1.1 (±0.2)</td>
<td>6.5 (±0.3)</td>
</tr>
<tr>
<td>C281S/Y282L</td>
<td>1.0 (±0.6)</td>
<td>6.6 (±0.4)</td>
</tr>
<tr>
<td>MMTS-Y282F</td>
<td>1.1 (±0.7)</td>
<td>6.7 (±0.5)</td>
</tr>
</tbody>
</table>

\(K_a^*\) is defined as the inducer concentration at half-saturation as described in the legend to Fig. 4.

Daly and Matthews, 1986a.
Mutants in Oligomerization Domain of Lac Repressor

The selection of 281 for substitution derived from the observation that MMTS modification of this sulphydryl residue, with the introduction of an -S-CH₂ group in a mixed disulfide, abrogates the pH-dependent shift in affinity and cooperativity observed with the wild-type protein. In addition, this modification diminishes the rate constants for inducer binding by more than 2 orders of magnitude without effect on the equilibrium binding parameters (Daly and Matthews, 1986b). A previously characterized monomeric mutant T41 (recently sequenced and found to be Y282D) did not undergo the pH shift, and the sulphydryl at Cys²⁸ⁱ reacted readily with modifying agents, in contrast to behavior of this site in the wild-type tetrameric protein. The midpoint of the pH shift was 8.3, consistent with ionization of a sulphydryl residue. We therefore initially selected serine and alanine for substitution at 281 to eliminate the sulphydryl. The resultant mutant proteins displayed minimal differences from the wild-type protein, indicating that the sulphydryl group at 281 was not responsible for the pH dependence of inducer affinity and cooperativity. Subsequent substitutions with the residues phenylalanine and methionine demonstrated that the apolar nature of the side chain exerted an influence on the pH shift and dynamics of inducer binding. The ratio of the midpoint of the saturation curve for the fluorescence change on inducer binding for pH 9.2 and 7.5 decreased from ~7 for wild-type and C281S to ~2.5 for methionine substitution. MMTS modification of these mutants, which do not contain a sulphydryl residue at position 281, did not abrogate the pH shift, as expected from the assignment of this effect to Cys²⁸¹. It is noteworthy that methionine has the amino acid side chain which most closely approximates the MMTS-modified cysteine and also yields the greatest diminution of the pH shift.

The rate constants for inducer binding provide an assessment of the accessibility of the inducer binding site over time, while the equilibrium constant reflects the nature of the contacts in the bound state. Substitution for Cys²⁸¹ does not significantly affect the equilibrium parameters for inducer binding, while the association rate constant for inducer binding at pH 7.5 is diminished ~2–4-fold by the apolar environment introduced by methionine or phenylalanine substitution. Methionine presumably mimics in some manner the -S-CH₂ modification by MMTS which elicits a decrease of ~2 orders of magnitude in the rate constants with no effect on the equilibrium constant. Isoleucine in this position appears to have a deleterious effect on the protein which disrupts oligomerization. Thus, alterations in the polarity and/or steric nature of the side chain at 281, either by ionization or mutation, influence the kinetic parameters for inducer binding, presumably by altering the rate at which the binding cleft opens to allow sugar entry or exit. By analogy to the periplasmic sugar-binding proteins (Quiocho et al., 1977), access to the sugar-binding site involves an opening and closing of the sugar-binding cleft; thus, mutations which alter the rate of opening/closing affect the kinetic but not the equilibrium parameters for sugar binding (Matthews, 1987). Using homology between lactose repressor protein and arabinose-binding protein (Müller-Hill, 1983; Sams et al., 1984), we propose that Cys²⁸¹ is at the base of this cleft in the crossover stretches between subdomains of the core region in a position to participate in a subunit interface and also to influence dynamics of the opening and closing of the sugar-binding site (Quiocho et al., 1977; Matthews, 1987).

Another candidate to account for the pH-dependent behavior of inducer binding in the wild-type repressor is Tyr²⁸², which also could display an ionization in the range observed.

**Fig. 4. Fluorometric titration of Y282F mutant repressor binding to inducer.** Saturation curves were generated by addition of aliquots of IPTG at increasing concentration to repressor at 1.5 × 10⁻⁷ M monomer. The excitation wavelength was 285 nm, and emission was monitored at wavelengths greater than 350 nm (Corning filter 0–52). The fractional degree of saturation, Y, at pH 7.5 (●) and 9.2 (□) was calculated as the ratio of the change in fluorescence observed as compared to the total change in fluorescence. The IPTG concentration at half-saturation is taken as the apparent $K_a$.

**Fig. 5. Trypsin digestion of mutant repressors.** SDS-polyacrylamide gel electrophoretic analysis of fragments from partial trypsin digestion of wild-type and mutant repressors produced as described under “Materials and Methods.” Odd numbered lanes 1, 3, 5, 7, 9, and 11 contain protein in absence of trypsin; even numbered lanes 2, 4, 6, 8, 10, and 12 contain protein digested with trypsin. Lanes 1 and 2, C281S wild-type repressor; lanes 5 and 6, C281M; lanes 2, 4, and 7, Y282F; lanes 3 and 10, C281S/Y282L; lanes 11 and 12, Y282A; lane 13, wild-type repressor; lane 14, trypsin. Lane 15 contains SDS-PAGE low molecular mass markers: rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, 97 kDa; bovine serum albumin, 66 kDa; hen egg white lysozyme, 14 kDa; soybean trypsin inhibitor, 22 kDa; hen egg white lysozyme, 14 kDa. The 15% cross-linked polyacrylamide gel was run at 190 volts; the dye bands contain protein digested with trypsin. Odd numbered lanes were not contain protein in absence of trypsin, while the equilibrium constant reflects the nature of the contacts in the bound state. Substitution for Cys²⁸¹ does not significantly affect the equilibrium parameters for inducer binding, while the association rate constant for inducer binding at pH 7.5 is diminished ~2–4-fold by the apolar environment introduced by methionine or phenylalanine substitution. Methionine presumably mimics in some manner the -S-CH₂ modification by MMTS which elicits a decrease of ~2 orders of magnitude in the rate constants with no effect on the equilibrium constant. Isoleucine in this position appears to have a deleterious effect on the protein which disrupts oligomerization. Thus, alterations in the polarity and/or steric nature of the side chain at 281, either by ionization or mutation, influence the kinetic parameters for inducer binding, presumably by altering the rate at which the binding cleft opens to allow sugar entry or exit. By analogy to the periplasmic sugar-binding proteins (Quiocho et al., 1977), access to the sugar-binding site involves an opening and closing of the sugar-binding cleft; thus, mutations which alter the rate of opening/closing affect the kinetic but not the equilibrium parameters for sugar binding (Matthews, 1987). Using homology between lactose repressor protein and arabinose-binding protein (Müller-Hill, 1983; Sams et al., 1984), we propose that Cys²⁸¹ is at the base of this cleft in the crossover stretches between subdomains of the core region in a position to participate in a subunit interface and also to influence dynamics of the opening and closing of the sugar-binding site (Quiocho et al., 1977; Matthews, 1987).
and could potentially be affected by modification of the adjacent Cys. Previous genetic studies have indicated that this site can be substituted with phenylalanine and leucine to produce active repressor, but other substitutions do not repress in vivo (Kleina and Miller, 1990). Consistent with these results, both phenylalanine and leucine substitution in the site-specific mutants produced tetrameric proteins with near-normal operator affinity. Substitution of Tyr by phenylalanine or leucine yielded a tetrameric mutant repressor with intact operator binding, inducer affinity comparable to wild-type, but with a lower association rate constant for inducer binding at pH 7.5. It is apparent from the data in Table III that the pH differential for equilibrium binding of the Y282F protein not only is maintained despite the elimination of the phenolic hydroxyl, but in fact is 2.5-fold greater than the ratio for wild-type, indicating the phenol moiety may dampen this pH differential. From these results, it may be concluded that the pH shift in wild-type repressor cannot be ascribed to either Cys or Tyr and may derive from a more concerted change in the subunit interface or from other unidentified ionizing groups.

Substitutions other than phenylalanine or leucine at position 282 resulted in monomeric (and somewhat unstable) repressor, in accord with results from genetic studies indicating that substitution of this site by amino acids other than leucine and phenylalanine yields mutant proteins which do not have the capacity to repress in vivo (Schmitz et al., 1976; Miller, 1979; Miller et al., 1979; Gordon et al., 1988; Kleina and Miller, 1990). The alanine and aspartate substitutions and leucine substitution in the C281S background yielded monomeric proteins that were isolated and characterized. The combination of two mutations which singly have no significant effect on activity (C281S and Y282L) results in monomeric protein unable to assemble into tetramer and therefore unable to bind to the operator site. This result suggests that the spatial arrangement of the subunit interface is quite sensitive to changes, and although some alterations can be tolerated, accumulated reorientation disrupts the side chain packing that stabilizes the association of subunits.

Wang and Fried (1987) have proposed that protein degradation of some multimeric forms in E. coli may occur in response to exposure of signal sequences upon shifts in the monomer-multimer equilibrium. Our results indicate that the folded conformation of monomeric mutants that were isolated is less stable and therefore suggests that these proteins may be partially unfolded. The susceptibility of the Y282A and C281S/Y282L proteins to trypsin digestion was shown to be greater than for tetrameric proteins. In addition, circular dichroism spectra demonstrate that there are small differences in secondary structure between these monomeric mutant proteins and the wild-type protein that are not found in the tetramer mutants. Using the R repressor N-terminal domain, Parsell and Sauer (1989) have demonstrated that thermal stability is an important, although not exclusive, determinant of the proteolytic susceptibility of this protein in the cell. It is possible that degradation of the monomeric R mutant repressor proteins may proceed through a similar mechanism, with the unfolded form serving as an effective protease substrate. The proteolytic fragments obtained from trypsin digestion of C281M and Y282F present a pattern different from both wild-type and the Y282A and C281S/Y282L mutants; these structural differences parallel the alterations observed in the rate constants. The pattern for C281I-A is similar to wild-type, in spite of its obvious dysfunction with respect to oligomer formation.

The region of the protein encompassing residues 281 and 282 has been predicted by several methods (Bourgeois et al., 1979) to be in a coil or turn following a B-sheet domain. Turns and coils are frequently found on the surface of proteins, and the reactivity of Cys with a variety of cysteine reagents in monomeric mutant repressor (Daly and Matthews, 1986a), in contrast with its behavior in tetramer wild-type repressor, indicates that this region is indeed on the surface of the monomer, but is buried in the tetramer. The evidence from pressure dissociation of MMTS-modified wild-type protein (Royer et al., 1986), reactivity of the wild-type and T41 mutant with B2 antibody (Daly and Matthews, 1986a), and from the studies reported here indicates that this 281–282 region implicated by genetic studies is involved in a subunit interface (Schmitz et al., 1976; Miller, 1979; Miller et al., 1979; LeClerc et al., 1988; Gordon et al., 1988; Kleina and Miller, 1990). In this regard, it is noteworthy that Cys can be replaced by a number of amino acid residues with minimal effects on the overall ability of the protein to carry out its function of repression in the cell. There are subtle effects on the inducer association rates which suggest that this region of the molecule exerts influence on the opening and closing of the inducer-binding site (Matthews, 1987). In contrast, Tyr can be replaced only by phenylalanine and leucine and still maintain approximately normal function in vivo and in vitro. The role played by the hydrophobic side chain appears to be crucial to the entire process of oligomerization.

Other mutations which influence oligomerization are located in the turns residues from the C terminus, affect a leucine heptad repeat, and result in dimeric repressor proteins (Brenowitz et al., 1991; Mandal et al., 1990; Lehming et al., 1987, 1988; Oehler et al., 1990; Chakerian et al., 1991; Alberti et al., 1991). In addition, fusion of the bulk of the coding sequence for the repressor protein with other coding sequences to yield a chimeric protein has resulted in oligomeric species which exhibit DNA binding capacity (Kania and Brown, 1976; Kania and Müller-Hill, 1977). Lundeberg et al. (1990) have fused the intact lac repressor coding sequence to the sequence of the staphylococcal protein A gene (spa) encoding four IgG-binding domains and observed inducer-sensitive DNA binding activity, reflective of dimer formation, in the fusion product. The mutations at position 282, however, disrupt both monomer association to dimer and dimer association to tetramer. Brenowitz et al. (1991) suggested that the monomer-monomer and dimer-dimer interfaces may be “contiguous” based on results obtained with the dimeric repressor lac. The data amassed in our study coupled with the in vivo genetic studies (Miller et al., 1979; Kleina and Miller, 1990) provide additional evidence for an overlap in dimer and tetramer formation; the substitution of nonhydrophobic amino acids at position 282 eliminates both dimer and tetramer formation.

Folding of proteins into tertiary structures and the association of subunits to form quaternary structures are complex processes, and the requirements for folding interactions and for subunit interfaces have not been fully elucidated. From the results obtained in this study, several features of the lactose repressor structure emerge. The elimination of the phenolic hydroxyl at 282 yields significant differential in the high and low pH affinities of the mutant protein, indicating that this group plays an important role in the subunit interactions which mediate this pH-dependent phenomenon. Influence on cooperativity and kinetics of aspartate transcarboxylase have also been observed for a tyrosine to phenylalanine alteration in the catalytic subunit; the change was interpreted as a destabilization of the T-state and shift in equilibrium toward the R-state (Hsuanyu et al., 1989; Wedler
et al., 1989). Similarly, the Y282F mutant appears to be shifted toward the operator-binding form of the protein favored by elevated pH, with kinetic parameters for inducer binding similar to those observed for operator-bound wild-type protein. The inability to predict precisely the effects of substitution on the interior of a protein or at a subunit interface has been demonstrated by Sandberg and Terwilliger (1989) from studies on wild-type and mutant gene V proteins from bacteriophage f1; it is apparent from their studies that alteration of packing without alteration in hydrophobicity of the side chain can destabilize protein structure. Thus, alterations which influence both polarity and size of the side chain, such as those explored in this study, are complex to interpret in detailed molecular terms. Such effects are demonstrated by the elimination of tetramer by adjacent substitutions which singly yield protein with essentially wild-type functional characteristics. Loss of hydrogen bonds or hydrophobic interactions can elicit alterations in hemoglobin structure, and the concerted changes on different aspects of binding complicate interpretation (Perutz, 1990). Despite the complexities, it is clear that tyrosine to phenylalanine changes can have significant consequences on protein cooperativity in hemoglobin (Ishimori et al., 1989) and as found in the Y282F lactose repressor mutant.

The conclusions derived from the data accumulated on the lac repressor mutations at positions 281 and 282 can be summarized as follows. First, this region, which is predicted to form a coil/turn secondary structure (Bourgeois et al., 1979), is located on the surface of the protein in monomeric repressors and is shielded in tetramer repressors. Second, alterations in the side chain at position 281 result primarily in effects on the cooperativity and dynamics of sugar binding with minimal alterations in equilibrium parameters for inducer or operator binding. Placement of this residue at the base of the sugar-binding site by homology to arabinose-binding protein (Sams et al., 1984; Matthews, 1987) and the simultaneous requirement that this residue reside at a subunit interface suggests a mechanism whereby the observed effects could be mediated. The character of the side chain in this position could exert a significant influence on the surrounding structure with side chains that stabilize the subunit interface structure hindering the opening/closing process, and those that destabilize the surrounding region facilitating this process. Based on the observed data, the polarity and size of the residue at position 281 are both factors in the resulting kinetic effects. Finally, the hydrophobic character of the side chain at position 282 is essential for tetramer formation (Miller et al., 1979; Kleina and Miller, 1990), and the phenolic hydroxyl is crucial to aspects of cooperativity and dynamics of inducer binding. It is noteworthy that only monomeric species are observed for nonhydrophobic substitutions, with no dimer or tetramer evident in any of the methods used to assess the oligomeric state of the protein. Since dimeric repressor mutants have been observed, it is apparent that mutation at 282 disrupts not only the dimer-tetramer interface, but also precludes formation of the monomer-monomer interface. Although a detailed molecular understanding of these mutations must await the solved x-ray crystallographic structure of this protein (Pace et al., 1990), we can conclude based on the available data that the amino acid side chain arrangement in the region encompassing 281 and 282 is crucial to any oligomer formation and additionally exerts a strong influence on the dynamic properties of the protein. We suggest that the effects on dynamics and cooperativity of inducer binding to this protein are mediated by interactions at the subunit interface which regulate the rate of opening and closing of the inducer-binding cleft.

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