Identification and Characterization of Aspartate Residues That Play Key Roles in the Allosteric Regulation of a Transcription Factor: Aspartate 274 Is Essential for Inducer Binding in *lac* Repressor†

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ABSTRACT: To explore the roles of three aspartate residues, Asp^{88}, Asp^{130}, and Asp^{274}, found in the proposed inducer binding site of *lac* repressor [Sams, C. F., Vyas, N. K., Quiocho, F. A., & Matthews, K. S. (1984) *Nature 310,* 429–430], each site was substituted with alanine, glutamate, lysine, or asparagine by site-specific mutagenesis. The mutations at the Asp^{88} site resulted in a 5–13-fold decrease in inducer binding affinity, largely due to an increase in the inducer dissociation rate constants for these mutants. In addition, the mutant proteins Asp^{88}→Ala and Asp^{88}→Lys exhibited altered allosteric behavior for inducer binding. These data conflict with the original hypothesis placing Asp^{88} in the inducer binding site, but are in agreement with a recent model that places this amino acid close to the subunit interface involved in cooperativity associated with inducer binding [Nichols, J. C., Vyas, N. K., Quiocho, F. A., & Matthews, K. S. (1993) *J. Biol. Chem. 268,* 17602–17612; Chen, J., & Matthews, K. S. (1992) *J. Biol. Chem. 267,* 13843–13850]. Substitution at Asp^{130} did not alter the inducer binding affinity nor other binding activities. Thus, this amino acid is not crucial in the binding to β-substituted monosaccharides or in protein function. In stark contrast, all mutant proteins with substitutions at the Asp^{274} site exhibited no detectable inducer binding. With the exception of Asp^{274}→Lys, the structures of these mutant proteins appear to be similar to wild-type. The data demonstrate that Asp^{274} plays a crucial role in inducer binding of this transcriptional regulator.

The lactose repressor inhibits transcription of the three genes in the *lac* operon by binding specifically to the *lac* operator sequence and inhibiting initiation by RNA polymerase (Schmitz & Galas, 1979; Miller & Reznikoff, 1980; Horowitz & Platt, 1982; Deuschle *et al.*, 1986; Straney & Crothers, 1987). The operator binding specificity of the *lac* repressor can be modulated by association with sugar ligands, and different sugars have distinct effects on operator binding capacity (Miller & Reznikoff, 1980). Inducers are sugars that diminish operator binding specificity upon binding to the repressor protein due to a conformational change that accompanies ligand binding (Ohshima *et al.*, 1972; Laiken *et al.*, 1972; Wu *et al.*, 1976; Matthews, 1987). The set of inducer sugars primarily comprises β-substituted galactosides (Barkley *et al.*, 1975) and includes allolactose (galactosyl-1,6-β-D-glucose), which is the natural inducer (Jobe & Bourgeois, 1972). In contrast, anti-inducers, such as phenyl β-D-thiogalactoside, glucose, or lactose, stabilize the operator–repressor complex (Riggs *et al.*, 1970; Jobe & Bourgeois, 1972, 1973; Barkley *et al.*, 1975). Although the binding site of anti-inducers overlaps with that of inducers, these sugars do not induce a significant conformational change in *lac* repressor (Laiken *et al.*, 1972; O'Gorman & Matthews, 1977; Alexander *et al.*, 1977; Boschelli *et al.*, 1981). Identification of the sugar moieties and amino acids that generate inducer binding can provide insight into this key process for induction.

On the basis of the binding affinities of various β-galactoside inducers, a bulky apolar group at the C1 position of galactosides is required for high binding affinity (Barkley *et al.*, 1975). Data from studies using a series of fluoro-substituted β-D-galactosides also revealed that the C3 and C6 hydroxyls significantly contribute to inducer binding, while the C4 hydroxyl provides only a nominal contribution (Chakerian *et al.*, 1987). Phenotypic characterization of *lac* repressor mutants *in vivo* (Miller, 1979; Miller & Schmeissner, 1979; Kleina & Miller, 1990) has shown that amino acids involved in inducer binding are spread throughout the core domain (amino acids 60–360), a region that displays homology with periplasmic sugar binding proteins (Müller-Hill, 1983; Nichols *et al.*, 1993). On the basis of this similarity with the periplasmic binding proteins, the major forces involved in the interaction between inducer and *lac* repressor are presumed to be hydrogen bonds, with the inducer providing hydroxyl hydrogens as hydrogen-bond donors and *lac* repressor providing various amino acid side chains as receptors.

Two models for the inducer binding site of *lac* repressor have been presented (Sams *et al.*, 1984; Nichols *et al.*, 1993). In the first case, the partial homology found between *lac* repressor and arabinose binding protein (Müller-Hill, 1983), for which the X-ray crystal structure had been solved (Quiocho & Vyas, 1984), and data from previous genetic studies of repressor (Miller, 1979; Miller & Schmeissner, 1979) were used to propose specific residues in the inducer binding site (Sams *et al.*, 1984). Subsequent studies using site-specific mutagenesis confirmed the role of Arg^{197}, which was predicted to be involved in multiple interactions with inducer, in inducer binding with a contribution of up to 50% of the free energy of interaction (Spotts *et al.*, 1991). The second model was produced by alignment of the *lac* repressor core protein (amino acids 60–360) with several periplasmic sugar binding proteins with known X-ray crystallographic structures to generate a

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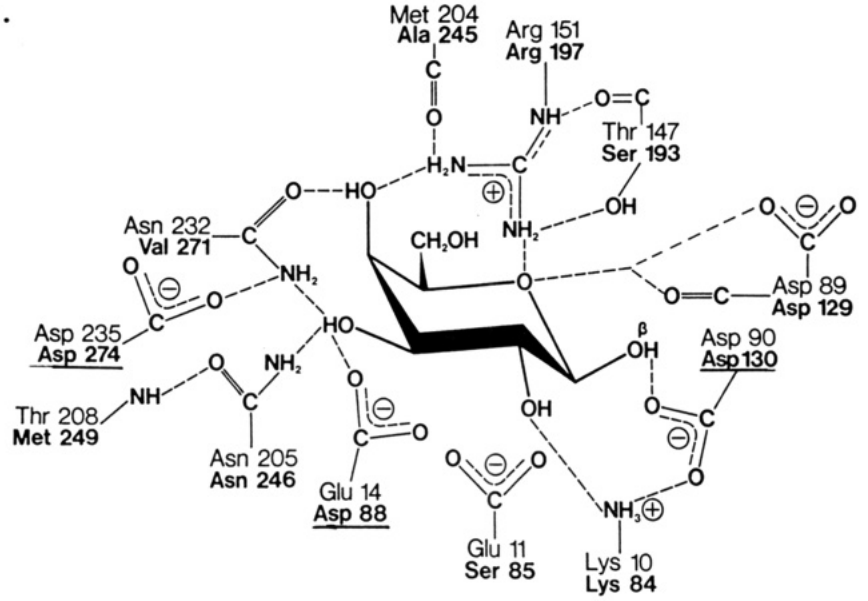

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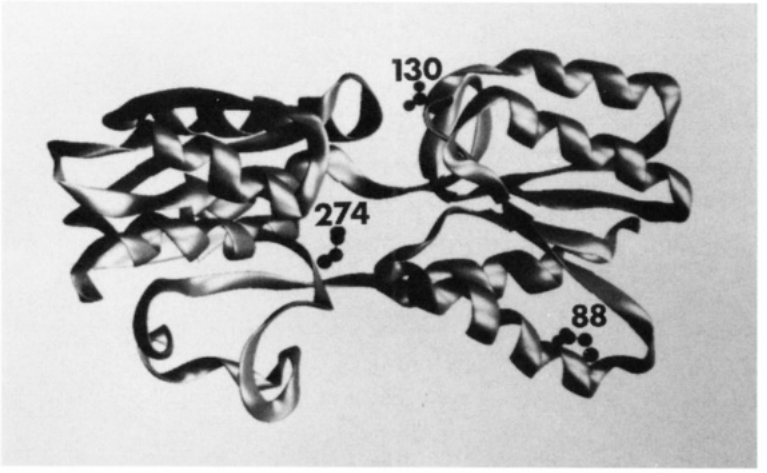

FIGURE 1: Models for the inducer binding site. (A) Proposed inducer binding site (Sams *et al.*, 1984) of *lac* repressor. The binding site is superimposed on that of ABP, which has a β-galactose bound (Quiocho & Vyas, 1984). The amino acids in regular type are from ABP, while those in boldface type are from *lac* repressor derived from homology and genetic data (Sams *et al.*, 1984). (B) Ribbon drawing of *lac* core protein based on sequence alignment and constructed on sugar binding protein structures (Nichols *et al.*, 1993). Note that the structure represents the protein with the inducer binding site closed. The three aspartates examined in this study (Asp^{88}, Asp^{130}, and Asp^{274}) are underlined in panel A and highlighted in panel B.

molecular replacement structure for this domain (Nichols *et al.*, 1993). Several differences from the earlier hypothetical inducer site were observed in the placement of residues in the binding cleft of this model for the core domain.

To experimentally test these two models, we chose to examine three aspartates, potential hydrogen-bond acceptors, found in the inducer binding site initially proposed by Sams *et al.* (1984) (Figure 1A). Asp^{274} was proposed to serve a role in inducer binding in both models; Asp^{88} was predicted to contribute a hydrogen bond to inducer binding in the site proposed by Sams *et al.* (1984), but was positioned away from the binding cleft in the more recent molecular replacement model (Nichols *et al.*, 1993); and Asp^{130} interacted with the C1 hydroxyl group of galactose in the earlier model, but was placed at the edge of the sugar binding site in the molecular replacement model (Figure 1B). In order to elucidate the involvement of these amino acids in the inducer binding site, four mutations (Asp→Ala, Lys, Asn, or Glu) were generated at each position by site-specific mutagenesis, and the purified mutant repressors were characterized *in vitro*. The data derived from these mutant repressors do not indicate a role for the side chain of Asp^{130} in binding to β-substituted galactosides, but do support the direct involvement of Asp^{274} and the indirect involvement of Asp^{88} in inducer binding, thus favoring the more recent molecular replacement model.

MATERIALS AND METHODS

Plasmids and Strains. A plasmid (pAC1) containing the *lacI* gene driven by the i^q promoter was constructed as described by Chakerian and Matthews (1991). *Escherichia coli* strain BW313 [*dut*, *ung*, *thi*-1, *rel*A, *spo*T1/F′(*lys*A)] was used for uracil-incorporated template preparation because of its unique dut^- and ung^- mutations (Kunkel, 1985). *E. coli* strain 71/18 [*supE*, *thi*, Δ(*lac–pro*AB), F′(*pro*AB^+, *lac*I^q, *lac*Z ΔM15)] was used for the selection of non-uracil-containing DNA and its preparation, while *E. coli* strain PD8 [*ara*, *str*A, *thi*, *nal*A, *rec*A56, Δ(*lac–pro*), φ80d*lac*Z ΔM15, F′(*lys*A–*fuc*)] or TB-1 [*ara*, Δ(*lac–pro*), *str*A, *thi*, φ80d*lac*Z ΔM15r^-, m^+] served as the host strain for the expression of *lac* repressors.

Site-Specific Mutagenesis. Site-directed mutagenesis was performed as described by Kunkel (1985). Uracil-incorporated template was obtained by IR1 superinfection of BW313 transformed by pAC1 in 2YT medium (16 g/L Tryptone, 10 g/L yeast extract, and 5 g/L NaCl, pH 7.4) containing uracil (0.25 mg/mL). Synthetic oligonucleotide produced by a Biosearch 8600 DNA synthesizer was purified from a desalting column and then phosphorylated at the 5′-end by T4 polynucleotide kinase. After hybridization to the uracil template at 75 °C for 2 min, the phosphorylated oligonucleotide acted as primer for synthesis of the complementary DNA strand, which was accomplished by the Klenow fragment of *E. coli* DNA polymerase and T4 polynucleotide ligase at 15 °C for about 16 h. The final reaction mixture was then transformed into *E. coli* 71/18 cells for selection of the newly synthesized DNA. Colonies were picked from ampicillin plates for single-stranded or double-stranded DNA preparation, and mutations were identified by dideoxy sequencing (Sanger *et al.*, 1980). DNA containing a mutation at the desired site was sequenced throughout the entire *lac*I gene to eliminate any possibility of mutation(s) at other sites. The confirmed mutants were then transformed into PD8 or TB-1 cells for *lac* mutant repressor expression and purification.

Isolation of lac Repressor Mutant Proteins. Wild-type and mutant repressor proteins were prepared from pAC1-transformed PD8 or TB-1 cells. After growing in ampicillin-containing 2YT at 37 °C for 20 h in a B.Braun Biostat E fermentor or several 2-L flasks, the cells were harvested and frozen as a thick slurry in lysing buffer (0.2 M Tris-HCl (pH 7.6), 0.2 M KCl, 0.01 M MgAc, 0.3 mM DTT, 5% glucose, and 50 μg/mL of phenylmethanesulfonyl fluoride) (1 g of cells/1 mL of lysing buffer) supplemented with 150 mg/mL lysozyme. The repressor proteins were purified from the frozen cell paste as described previously (Rosenberg *et al.*, 1977; O'Gorman *et al.*, 1980a), and activity was monitored by IPTG binding during the process, applying the ammonium sulfate precipitation method of Bourgeois (1971).

Circular Dichroism. Measurement of the circular dichroic spectra of the mutants (Chou *et al.*, 1975) was performed at room temperature on a Jasco J-500A instrument using a cuvette of 0.1-cm path length. The scan speed was 50 nm/min from 250 to 200 nm. The protein had been dialyzed in 0.12 M potassium phosphate buffer (pH 7.5) with 5% glucose overnight, and the concentration was adjusted to 0.3 mg/mL in the same buffer.

Gel Filtration. A Sephadex G-150 column (3 × 50 cm) was equilibrated in TMS buffer (0.01 M Tris-HCl (pH 7.4), 0.2 M KCl, 0.01 M $MgCl_2$, 1 mM EDTA, and 0.1 mM DTT). The column was calibrated at room temperature, and the eluates were monitored by an LKB 2238 Uvicord S II monitor at 280 nm.

Measurement of Inducer Binding. The fluorescence emission spectrum for each protein was recorded from 300 to 450 nm with the excitation wavelength at 285 nm on an SLM 400 spectrofluorometer at room temperature (Friedman *et al.*, 1977). The protein concentration was adjusted in TMS buffer (pH 7.4) to 1.5×10^{-7} M for IPTG measurements and to 5×10^{-7} M for other sugar measurements (methyl β-D-thiogalactoside, galactose, and fucose).

Since a fluorescence emission shift upon IPTG binding similar to that of the wild-type repressor was observed in all mutants substituted at Asp[88] and Asp[130] sites, the inducer binding measurements (kinetic binding parameters and equilibrium binding parameters) for these mutants were monitored as the fluorescence intensity change upon IPTG addition, as described previously (O'Gorman *et al.*, 1980b). Equilibrium constants for the inducer binding activity of all mutants were measured on an SLM 400 spectrofluorometer at 20 °C as described previously (O'Gorman *et al.*, 1980b). The sample was excited at 285 nm, and the fluorescence emission was monitored with a 340-nm cutoff filter (Corning 0-52). The *lac* repressor was diluted to 1.5×10^{-7} M in TMS buffer, and all inducer stock solutions were also prepared in TMS buffer. Titration was performed to determine the fluorescence intensity decrease upon the addition of inducers. Data were analyzed by Igor, a fitting program containing the binding equation, $1/R = (1 + K_d^n)/[\text{IPTG}]^n$, to obtain equilibrium dissociation constant K_d and Hill value n. R is the fractional change of fluorescence intensity, determined by $(F_o - F_i)/(F_o - F_\infty)$, where F_∞ is the fluorescence at which additional inducer elicits no further fluorescence change. F_o is the initial fluorescence value, and F_i values at each inducer concentration are corrected for dilution effects by using data from parallel buffer titrations. The saturation value for fits was not fixed at 1.0 to ensure that the data reached saturation.

Association and dissociation rate constants for IPTG binding to the repressor were measured at 20 °C in a Gibson-Durrum rapid-mixing stopped-flow spectrometer with fluorescence optics (Friedman *et al.*, 1977; Dunaway *et al.*, 1980; Daly *et al.*, 1986). Proteins were dialyzed against TMS buffer overnight, and the association processes were continuously monitored as the fluorescence intensity decrease due to IPTG binding. Several traces were collected and averaged to minimize background noise. The measurement was performed under pseudo-first-order conditions, with the concentration of *lac* repressor much lower than that of the inducer.

Since no fluorescence emission shift was observed for mutations at the Asp[274] site, a filter binding assay with [^{14}C]-IPTG was performed (Sams & Matthews, 1988). A series of dilutions of each Asp[274] mutant protein in TMS buffer (pH 7.4) was mixed with 10^{-7} M [^{14}C]IPTG containing TMS solution and equilibrated for 10 min at room temperature before filtration. The background binding was defined as the nonspecific binding of [^{14}C]IPTG to the nitrocellulose filter in the absence of protein.

Measurement of Operator Binding. Operator DNA binding assays were performed at room temperature by nitrocellulose filtration according to the methods described previously (Riggs *et al.*, 1968; O'Gorman *et al.*, 1980b) with some modifications (Chang *et al.*, 1993). The 40-bp oligonucleotide containing the *lac* operator was labeled at the 5′-ends by T4 polynucleotide kinase and was diluted to about 10^{-12} M final concentration in binding buffer (0.01 M Tris-HCl (pH 7.5), 0.15 M KCl, 0.1 mM EDTA, 0.1 mM DTT, 5% dimethyl sulfoxide, and 50 μg/mL of bovine serum albumin (BSA)). Since some of the mutants had no detectable IPTG binding ability, the background binding was obtained as the nonspecific binding of labeled *lac* operator to the nitrocellulose filter. A series of protein dilutions was made into the DNA solution described above. The mixture was equilibrated for 10 min at room temperature before filtration. The equilibrium dissociation constant was obtained by analysis using the program Igor, fitting the data to the binding equation $1/R = (1 + K_d)/[\text{repressor}]$, where R is the fractional saturation of operator binding and K_d is the equilibrium dissociation constant. R is determined from $[\text{CPM}_i - \text{CPM}_{\text{background}}]/[\text{CPM}_\infty - \text{CPM}_{\text{background}}]$. $\text{CPM}_{\text{background}}$ was determined by DNA retention in the absence of protein, CPM_i corresponds to the radiolabel retained at a specific protein concentration, and CPM_∞ is the retention at saturation. In fits of the data, R

was not constrained to be 1.0 at the maximum to ensure that the data reached saturation.

The assay to determine the percent activity of the protein was performed in the same way, except that unlabeled *lac* operator (40-mer) was added to a final concentration of 10^{-8} M. The activity was calculated from the ratio of operator concentration to double the protein tetramer concentration at saturation, assuming one tetramer binds to two operator 40-mers (Adler *et al.*, 1972; O'Gorman *et al.*, 1980a; Culard & Maurizot, 1981; Whitson & Matthews, 1986). The fits to these data employed an "x-break" fitting function using Igor.

Antibody Binding. The antibody binding reaction was performed according to Sams *et al.* (1985). A specified amount of each protein was incubated in phosphate-buffered saline (PBS) (2.7 mM KCl, 140 mM NaCl, 1.4 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4) in the absence or presence of 0.01% sodium dodecyl sulfate (SDS) and dot-blotted onto a nitrocellulose filter. The filter was incubated at 4 °C in the presence of 10 μg/mL B2 monoclonal antibody and 0.5% BSA in PBS overnight, followed by extensive washes in PBS. The filter was then exposed to 0.02% goat anti-mouse antibody linked with peroxidase and 0.1% BSA in PBS and incubated at 4 °C for at least 6 h. After a brief rinse with PBS, the final result was visualized by the addition of 0.01% H_2O_2 and 0.5 mg/mL 4-chloro-1-naphthol in 0.01 M sodium citrate.

Trypsin Digestion. Reactions were carried out in 0.12 M potassium phosphate (KP) buffer with 0.3 mM DTT and 5% glucose at room temperature. In a total volume of 20 μL, 5 μg of repressor protein was subjected to 2% (w/w) trypsin, which was dissolved in 1 mM HCl for 30 min. The reaction was stopped by the addition of 2% (w/v) phenylmethanesulfonyl fluoride in 100% ethanol (Matthews, 1979). One-tenth of the mixture was then denatured and analyzed by 12.5% SDS–polyacrylamide (acrylamide:bisacrylamide = 38.5:1) gel electrophoresis.

RESULTS

Generation of Mutants and Protein Purification. Mutations have been generated at Asp^{88}, Asp^{130}, and Asp^{274} sites by site-directed mutagenesis as described in Materials and Methods. For each mutant, the entire *lacI* gene was sequenced to confirm that the mutation at the desired site was the only alteration. All mutant repressors were expressed in *E. coli* cells lacking a *lacI* gene and subsequently purified using phosphocellulose chromatography. The purity of each protein was ≥95%, as determined on 12.5% SDS–polyacrylamide gel by silver staining. The activity of each protein was ≥78% as determined by the operator binding assay (Table 3).

Secondary Structure and Oligomeric Structure. Circular dichroism (CD) spectroscopy provides information on the gross secondary structure of the protein. Significant alterations in the secondary structure can be detected by comparing the CD spectrum of wild-type repressor with that of a mutant protein. In Figure 2, the CD spectra of all of the mutant proteins are shown together with that of the wild-type repressor. All of the mutants except Asp^{88}→Glu and Asp^{274}→Lys display a CD spectrum similar to that of the wild-type, indicating no dramatic change in the secondary structure of the mutant proteins. The mutant proteins Asp^{88}→Glu and Asp^{274}→Lys exhibit a small increase in CD intensity that may suggest structural alterations that increase helicity. In order to determine the oligomeric form of the mutant proteins, gel filtration was used, and the results are shown in Figure 3. All

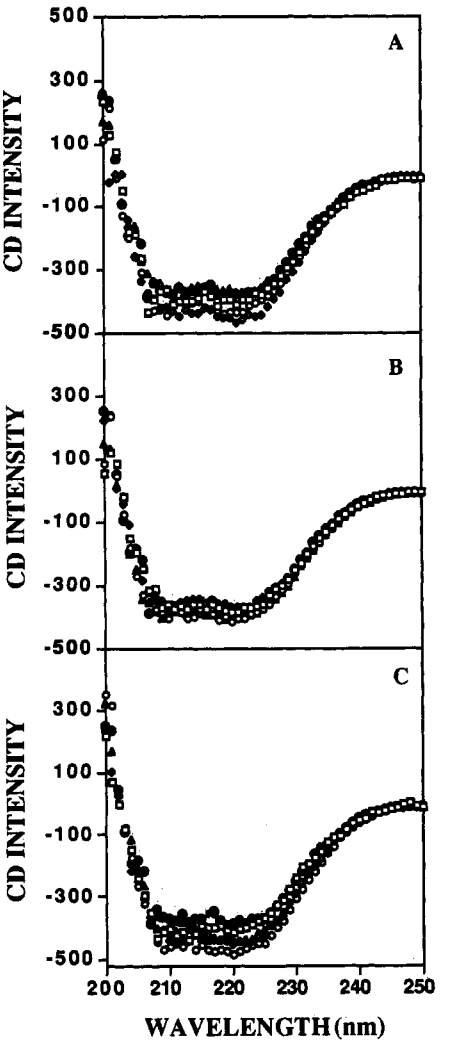

FIGURE 2: Circular dichroism spectra of mutant proteins. Circular dichroism spectra were collected using a Jasco J-500 instrument as described in Materials and Methods. The CD spectra of mutant and wild-type proteins are compared. The protein concentration is 0.3 mg/mL. (A) Mutants at Asp^{88} site; (B) mutants at Asp^{130} site; (C) mutants at Asp^{274} site. ●, Wild-type; □, Asp→Ala mutant; ◇, Asp→Glu mutant; ○, Asp→Lys mutant; △, Asp→Asn mutant.

mutant proteins exhibited the same molecular mass as the wild-type tetrameric repressor, indicating the same oligomeric state as the wild-type protein.

Inducer Binding. Inducer binding properties of mutant repressors at Asp^{88} and Asp^{130} sites have been examined by equilibrium and kinetic measurements of the fluorescence decrease upon inducer binding, as shown in Figure 4A. Interestingly, none of the proteins substituted at position Asp^{274} showed any fluorescence shift upon the addition of IPTG (Figure 4B). The IPTG binding affinity of mutants at Asp^{88} decreases from 5- to 13-fold, with mutant proteins Asp^{88}→Asn and Asp^{88}→Lys showing the largest alterations, as shown in Table 1 and Figure 5. The decrease in IPTG binding affinity for the Asp^{88} mutants largely results from an increase in dissociation rate constants (Table 1). It is interesting to note that the IPTG binding affinity of Asp^{88}→Glu displays the smallest decrease (5-fold). In this mutant, a 12-fold increase in the dissociation rate constant is compensated in part by a 3-fold increase in the association rate constant. The pH effect on IPTG binding of the mutant proteins at the Asp^{88} site is shown in Table 2. In contrast to wild-type repressor, the mutants Asp^{88}→Ala and Asp^{88}→Lys show cooperative binding at neutral pH. At pH 9.2, the apparent binding affinity for

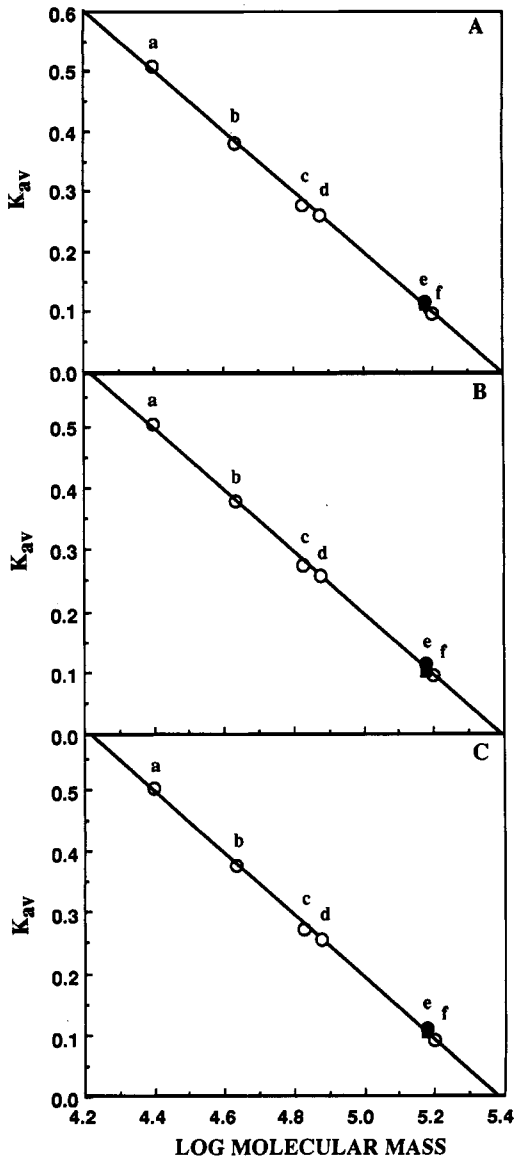

FIGURE 3: Gel filtration chromatography of *lac* mutants. A Sephadex G-150 column was eluted with TMS (pH 7.5) at room temperature and calibrated by molecular mass standards, as well as the tetrameric *lac* wild-type repressor and a dimeric *lac* repressor [deletion of C-terminal 11 amino acids; gift from J. Chen (Chen & Matthews, 1992)]. $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the sample, V_t is the elution volume of L-tryptophan, and V_o is the elution volume of Blue Dextran 2000. ○ corresponds K_{av} for standard proteins with the following molecular masses (in daltons): a, chymotrypsinogen A, 25 000; b, ovalbumin, 43 000; c, bovine serum albumin, 67 000; d, dimeric *lac* repressor, 75 000; e, alcohol dehydrogenase, 150 000; f, aldolase, 158 000. △ corresponds to K_{av} for wild-type repressor; □ corresponds to K_{av} for Asp→Ala mutants; + corresponds to K_{av} for Asp→Glu mutants; ■ corresponds to K_{av} for Asp→Lys mutants; and ▲ corresponds to K_{av} for Asp→Asn mutants. (A) Mutants at Asp[88] site; (B) mutants at Asp[130] site; (C) Mutants at Asp[274] site.

IPTG is decreased only 4–5-fold for the mutants at the Asp[88] site, compared to the 11-fold change found for wild-type repressor. For mutations at Asp[130], the equilibrium dissociation constant and kinetic parameters for IPTG binding are identical to those for the wild-type repressor (Table 1). Similar results were obtained for methyl β-D-thiogalactoside and galactose (data not shown).

None of the mutations at Asp[274] shows any detectable fluorescence shift upon the addition of a high concentration of IPTG (Figure 4B). In addition, these mutants did not show any fluorescence shift upon the addition of any other

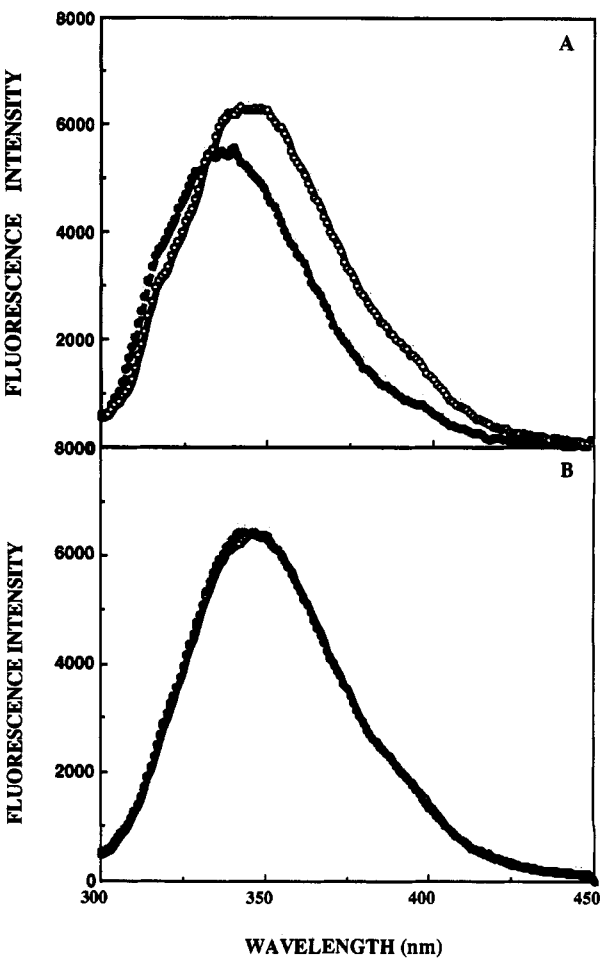

FIGURE 4: Fluorescence shift observed upon inducer binding. A solution of mutant protein at 1.5×10^{-7} M was excited at 285 nm at room temperature, and the emission spectrum was collected from 300 to 450 nm. (A) Fluorescence emission spectrum of the Asp[130]→Asn mutant protein. Similar spectra were observed for all mutant proteins at Asp[88] and Asp[130] sites. (B) Fluorescence emission spectrum of the Asp[274]→Asn mutant protein. ○, Spectrum upon the addition of 15 μL of buffer; ●, spectrum upon the addition of the same volume of 1 M IPTG (final IPTG concentration was 0.01 M).

inducers, including galactose, methyl β-D-thiogalactoside, and fucose (data not shown). In addition, within the protein concentration range from 2×10^{-7} to 3.3×10^{-4} M, there was no detectable binding observed in a [^{14}C]IPTG binding assay using nitrocellulose filtration (Figure 6). Therefore, the equilibrium dissociation constants for these mutants are greater than 10^{-3} M and are undetectable by tryptophan fluorescence change up to 0.1 M IPTG.

Operator Binding. Since operator binding activity is very sensitive to the alteration of tertiary and/or quaternary structures of the *lac* repressor, this property was examined. Theoretically, operator binding activity requires proper tertiary folding, as well as a favorable orientation of helix–turn–helix motifs in the assembled quaternary structure (Lehming *et al.*, 1990; Kisters-Woike *et al.*, 1991). The wild-type operator binding affinity under the conditions employed is 8 pM, as shown in Table 3. All substitutions at the Asp[88] and Asp[130] sites exhibit operator binding affinities comparable to that of the wild-type repressor (Table 3). At the Asp[274] site, mutant proteins with Asp[274]→Ala, Asp[274]→Glu, or Asp[274]→Asn show binding affinity similar to the wild-type, while mutant protein Asp[274]→Lys displays a 30-fold decrease in operator binding. This decrease is not due to a lower level of active protein, as all mutants were ≥78% active in operator binding (Table 3).

Table 1: IPTG Binding Activity of *lac* Repressor Mutants at pH 7.4

	kinetic parameters[a]			equilibrium
	k_{assoc} (10^{-5} M^{-1} s^{-1})	k_{dissoc} (s^{-1})	k_{dissoc}/k_{assoc} (μM)	constant[b] K_d (μM)
wild-type	1.4	0.1	0.7	1.0 (±0.3)
Asp^{88}→Ala	1.0	0.6	6.0	6.1 (±0.3)
Asp^{88}→Glu	4.4	1.2	2.7	4.8 (±1.4)
Asp^{88}→Lys	1.3	1.1	8.5	13 (±1)
Asp^{88}→Asn	1.1	1.2	11	13 (±2)
Asp^{130}→Ala	1.5	0.1	0.7	0.7 (±0.1)
Asp^{130}→Glu	1.3	0.1	0.8	0.7 (±0.1)
Asp^{130}→Lys	1.8	0.1	0.6	0.6 (±0.1)
Asp^{130}→Asn	1.5	0.1	0.7	1.5 (±0.4)
Asp^{274}→Ala	UD[c]	UD	UD	>1000[d]
Asp^{274}→Glu	UD	UD	UD	>1000[d]
Asp^{274}→Lys	UD	UD	UD	>1000[d]
Asp^{274}→Asn	UD	UD	UD	>1000[d]

[a] The kinetic parameters were calculated from $k_{obs} = k_{assoc}[IPTG] + k_{dissoc}$, where k_{assoc} is the association rate constant, k_{dissoc} is the dissociation rate constant, and k_{obs} is the rate constant measured at different inducer concentrations. [b] The equilibrium dissociation constant was obtained from fluorescence titration, as described in Materials and Methods. The titration points were collected and fit to the equation, $1/R = (1 + K_d^n)/[IPTG]^n$, to obtain the equilibrium dissociation constant. n, the Hill value, is shown in Table 2 for Asp^{88} substitutions. [c] Undetectable. No IPTG binding activity was detected in any of the Asp^{274} mutants, either using the fluorescence shift assay ([IPTG] ≤ 0.1 M), or the [^{14}C]IPTG binding assay ([protein] ≤ 3.3×10^{-4} M). Since no fluorescence shift was detected at the highest concentration of IPTG obtained, the kinetic parameters for these mutants could not be measured. [d] Estimated from the [^{14}C]IPTG binding assay.

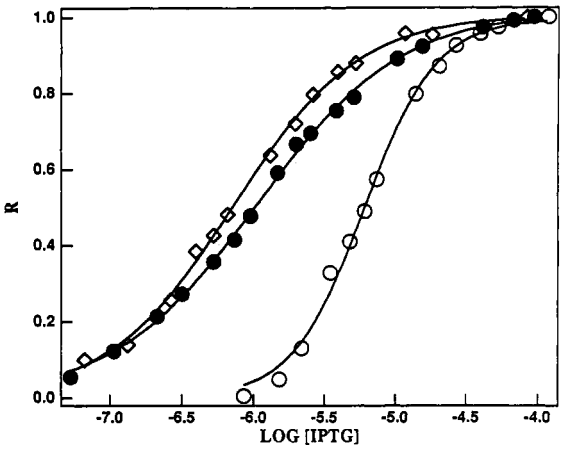

FIGURE 5: IPTG titration curves of mutant proteins. IPTG titration of Asp^{88} and Asp^{130} mutants was performed on the basis of the fluorescence emission shift of *lac* repressor upon IPTG binding. The initial concentration of protein was 1.5×10^{-7} M, which is well below the equilibrium dissociation constant of wild-type repressor binding to IPTG. The experimental data points for each mutant have been fit with the binding equation, $1/R = (1 + K_d^n)/[IPTG]^n$: ●, wild-type; ○, Asp^{88}→Ala; ◇, Asp^{130}→Ala. Note the increased cooperativity in sugar binding observed for the Asp^{88}→Ala mutant protein.

Antibody Binding Assay. The epitope recognized by B2 monoclonal antibody to *lac* repressor is found between amino acids 280 and 328, and this region is buried in tetrameric wild-type repressor due to subunit association (Sams *et al.*, 1985; Chen & Matthews, 1992; Chang *et al.*, 1993). Only dissociated repressors such as the wild-type in the presence of a small amount of detergent SDS (Hamada *et al.*, 1973; Sams *et al.*, 1985), monomeric (Daly & Matthews, 1986a), or dimeric protein (Chen & Matthews, 1992) react with B2 monoclonal antibody. As shown in Figure 7, all of the mutant proteins except Asp^{274}→Lys show an antibody binding pattern similar to that of the wild-type, suggesting no dramatic change in quaternary structure in these mutant proteins. However, the mutant protein Asp^{274}→Lys reacts with the antibody even in the absence of SDS, a phenomenon similar to the monomeric protein (Figure 7), despite the tetrameric structure shown by gel filtration (Figure 3). The examination of this protein by gel filtration was repeated at the same concentration employed for the antibody reaction, and the protein remained tetrameric. Thus, the anomalous antibody reaction pattern does not derive from dissociation of the subunits in this mutant protein.

Table 2: IPTG Binding Activity of *lac* Repressor Asp^{88} Mutants at pH 7.4 and 9.2

	pH 7.4		pH 9.2		
	K_d (μM)	Hill value[a]	K_d (μM)	Hill value	ratio[b]
wild-type	1.0 (±0.3)	0.9 (±0.1)	11 (±2)	1.7 (±0.2)	11
Asp^{88}→Ala	6.1 (±0.3)	1.7 (±0.1)	26 (±6)	1.2 (±0.1)	4
Asp^{88}→Glu	4.8 (±1.4)	0.7 (±0.1)	24 (±3)	1.5 (±0.2)	5
Asp^{88}→Lys	13 (±1)	1.3 (±0.1)	46 (±2)	1.5 (±0.1)	4
Asp^{88}→Asn	13 (±2)	1.0 (±0.2)	47 (±1)	2.1 (±0.1)	4

[a] The Hill value was obtained by fitting the experimental data according to the equation, $1/R = (1 + K_d^n)/[IPTG]^n$, where n is the Hill value. [b] Ratio of K_d at pH 9.2 over that at pH 7.4.

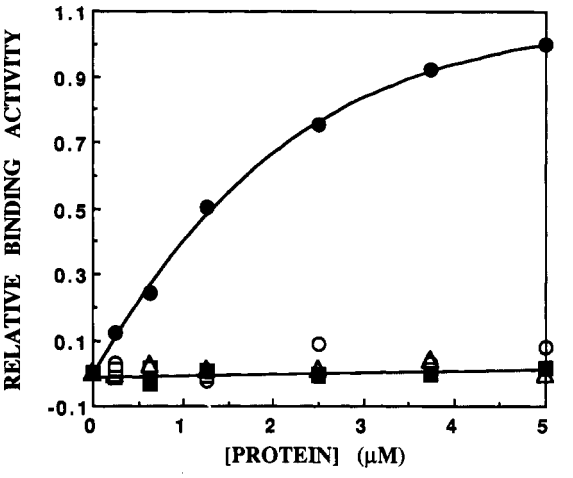

FIGURE 6: [^{14}C]IPTG binding ability of Asp^{274} mutants. The binding assay was performed in TMS buffer using nitrocellulose filtration, as described previously (Sams & Matthews, 1988; Chang *et al.*, 1993). The protein concentration ranged from 0.2 to 5 μM, with the labeled IPTG concentration kept constant at 2×10^{-6} M: ●, wild-type; □, Asp^{274}→Ala; ■, Asp^{274}→Glu; ○, Asp^{274}→Lys; △, Asp^{274}→Asn.

Table 3: Operator Binding Affinity of Asp Mutants at pH 7.4[a]

	K_d ($\times 10^{11}$ M)	activity (%)[b]
wild-type	0.8 (±0.3)	98
Asp^{88}→Ala	0.6 (±0.3)	99
Asp^{88}→Glu	1.1 (±0.6)	93
Asp^{88}→Lys	0.4 (±0.1)	96
Asp^{88}→Asn	0.7 (±0.1)	97
Asp^{130}→Ala	1.2 (±0.4)	100
Asp^{130}→Glu	0.5 (±0.1)	98
Asp^{130}→Lys	1.5 (±0.3)	100
Asp^{130}→Asn	3.3 (±0.3)	88
Asp^{274}→Ala	2.5 (±0.9)	80
Asp^{274}→Glu	0.6 (±0.2)	100
Asp^{274}→Lys	24 (±12)	78
Asp^{274}→Asn	0.9 (±0.1)	100

[a] Both the equilibrium dissociation constant and % operator activity were obtained by the filter binding assay, as described in Materials and Methods. [b] Activity corresponds to the operator concentration divided by double the tetrameric repressor concentration at saturation, assuming that one repressor tetramer binds to two *lac* operators. This fractional value was converted to percent.

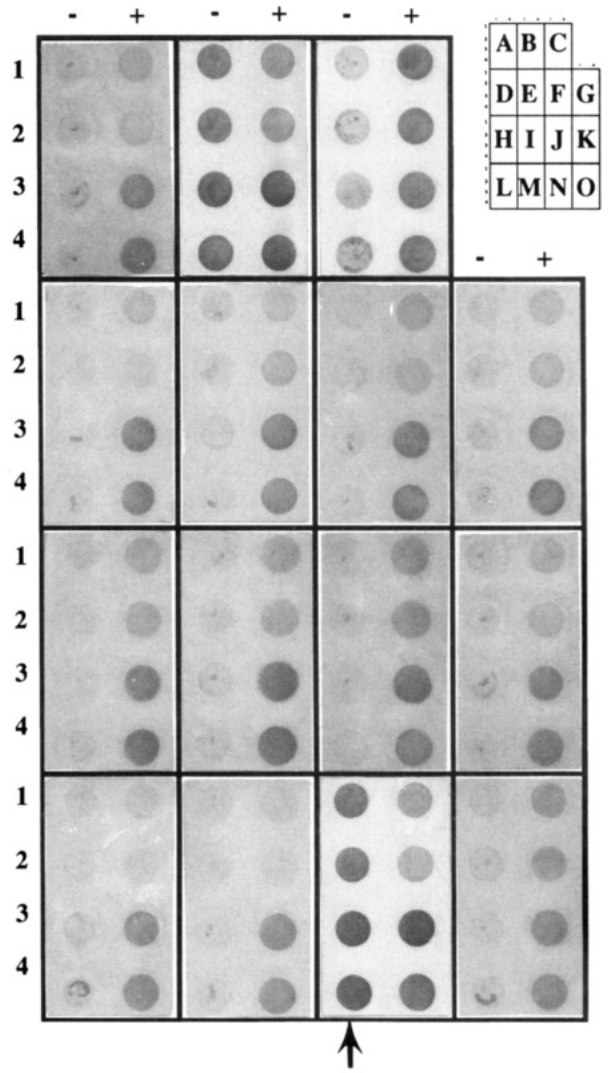

FIGURE 7: Immunoblotting assay. Monoclonal antibody B2 was used to examine binding to *lac* mutant proteins dot-blotted on a nitrocellulose filter in the absence (–) or in the presence (+) of 0.01% SDS, as described in Materials and Methods: (A) wild-type repressor (tetramer); (B) Trp^{282}→Asp (monomer); (C) –18 aa (dimer); (D) Asp^{88}→Ala; (E) Asp^{88}→Glu; (F) Asp^{88}→Lys; (G) Asp^{88}→Asn; (H) Asp^{130}→Ala; (I) Asp^{130}→Glu; (J) Asp^{130}→Lys; (K) Asp^{130}→Asn; (L) Asp^{274}→Ala; (M) Asp^{274}→Glu; (N) Asp^{274}→Lys; (O) Asp^{274}→Asn. Two different amounts of protein were used: 1 and 2, 2 μg; 3 and 4, 10 μg. The arrow indicates the pattern for Asp^{274}→Lys, which differs from that of the remainder of the mutant proteins examined in this study.

Trypsin Digestion. Mild trypsin digestion of wild-type *lac* repressor results in major polypeptides of ~7 and ~20–30 kDa in molecular mass (Platt *et al.*, 1973). In a 12.5% SDS gel, as shown in Figure 8, only the larger polypeptides are visible by silver staining. For the Asp^{88} and Asp^{130} substitutions, no differences from wild-type protein digestion were observed (data not shown). For the Asp^{274} substitutions, all mutants except Asp^{274}→Lys show a digestion pattern similar to that of the wild-type repressor (Figure 8). Mutant protein Asp^{274}→Lys displays additional bands of smaller molecular mass, suggesting a higher sensitivity of this mutant protein to trypsin.

DISCUSSION

Genetic and physical methods provide powerful tools to examine the role played by specific amino acid residues, despite the limitation of replacement with the remaining amino acid side chains. Two kinds of mutational approaches can be

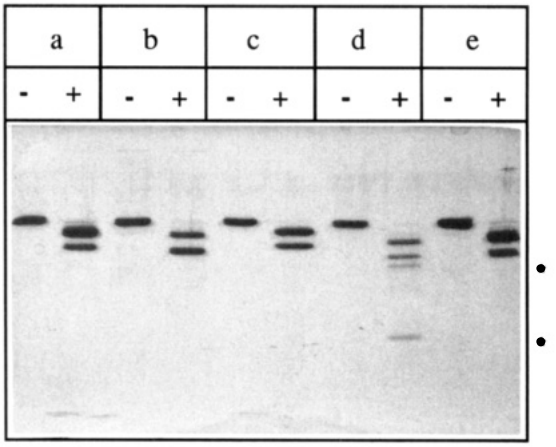

FIGURE 8: Trypsin digestion of *lac* mutant proteins. Trypsin digestion (+) was carried out for 30 min at room temperature, with a final protein concentration at 0.25 μg/μL and a final trypsin concentration of 2% (w/w). The control (–) was carried out under the same conditions, except that trypsin was excluded. (a) Wild-type repressor; (b) Asp^{274}→Ala; (c) Asp^{274}→Glu; (d) Asp^{274}→Lys; (e) Asp^{274}→Asn. The additional bands for the Asp^{274}→Lys mutant protein are marked with *.

employed: (1) *in vivo* phenotypic analysis of multiple mutants has provided useful information on the functional distribution of amino acids in *lac* repressor [e.g., Kleina and Miller (1990)]; (2) *in vitro* studies of purified mutant proteins, made possible by techniques for site-specific mutagenesis (Kunkel, 1985) and the convenient purification method of *lac* repressor (Rosenberg *et al.*, 1977; O'Gorman *et al.*, 1980a), provide information on the detailed structural and functional properties that are altered by individual substitution. Sensitivity to substitution at a site is a major criterion to determine whether the corresponding amino acid side chain plays an important role in a specific function. In addition, the context of a mutation may also play a strong role in determining the activity of a mutant (Zabin *et al.*, 1991).

In this study, we chose to examine three aspartate residues in the inducer binding site proposed by Sams *et al.* (1984) in order to assess their participation in sugar binding. According to this original model, Asp^{88} and Asp^{130} both contribute a hydrogen bond to inducer binding, while Asp^{274} is involved in multiple hydrogen bonds, and possibly a salt linkage with the surrounding amino acids, and might be presumed to be located at the base of the binding cleft (Figure 1A). In contrast, a recent molecular replacement model of core protein offers somewhat different assignments (Nichols *et al.*, 1993). In the latter model, Asp^{88} is placed near the subunit interface, distant from the inducer binding pocket; Asp^{130} is found at the edge of the sugar binding pocket, while Asp^{274} is located in a position analogous to the previous model at the base of the binding pocket (Figure 1B). The hypothetical inducer binding sites in these two models differ mainly in the amino acids preceding and including position 130 of *lac* repressor. The earlier model (Sams *et al.*, 1984) placed amino acids up to 130 in the site on the basis of data from *in vivo* mutational studies of *lac* repressor (Miller, 1979; Miller *et al.*, 1979), while the more recent model is based solely on sequence alignment between repressor and multiple periplasmic sugar binding proteins (Nichols *et al.*, 1993). The method used for the latter model has successfully predicted the structure of the ribose binding protein (RBP) when compared to its subsequently solved X-ray crystallographic structure (Vyas *et al.*, 1991; Mowbray & Cole, 1992).

To investigate more thoroughly the contributions of these three aspartates, four mutations have been introduced at each

site. The conservative substitutions include mutations to asparagine and glutamate: mutation to asparagine mainly alters the charge of the side chain, while mutation to glutamate increases the size of the side chain but preserves the charge. Two other nonconservative substitutions have also been employed: mutation to alanine introduces a smaller, hydrophobic side chain, while mutation to lysine introduces a positively charged, bulkier side chain. The assembly of all of the mutant proteins obtained by site-specific mutagenesis, except Asp^{274}→Lys, is similar to that of wild-type repressor on the basis of gel filtration, operator binding assays, immunoblotting assays, and protease digestion. Although mutant protein Asp^{88}→Glu showed slightly increased CD intensity, indicating possible alterations in secondary structure, its tertiary and quaternary structures appear to be unaltered since this mutant protein showed behavior similar to the wild-type, with the exception of alteration in properties for inducer binding. The mutant Asp^{274}→Lys appears to have altered structural characteristics compared to the wild-type protein, as shown in increased circular dichroism intensity, a 30-fold decrease in operator binding affinity, and a B2 monoclonal antibody reaction pattern different from that of wild-type. In addition, the increased susceptibility to proteolysis (Figure 8) indicates altered tertiary folding/quaternary assembly in this protein. These differences may arise from structural shifts to accommodate charge repulsion between Lys^{274} and Arg^{197} in the inducer binding pocket of *lac* repressor, as Arg^{197} is located within 5–8 Å of the side chain at position 274 on the basis of the molecular replacement model (Nichols *et al.*, 1993).

If all three aspartates, as proposed by Sams *et al.* (1984), were in the inducer binding site of the *lac* repressor, mutations of aspartate to other amino acids, especially to less conservative substitutions (e.g., alanine and lysine), would presumably result in decreased inducer binding affinity. In addition, the conservative mutation to asparagine might be assumed to maintain inducer binding affinity similar to wild-type repressor if the respective aspartate served as a hydrogen-bond acceptor. Mutations at Asp^{88} showed a 5–13-fold decrease in IPTG binding affinity (Table 1), a result that at first appears consistent with the direct involvement of this amino acid in inducer binding. However, the mutation to alanine, which abolished the hydrogen-bonding potential, resulted in the second smallest decrease in inducer binding (6-fold), while the mutation to asparagine, which retains hydrogen-bonding potential, resulted in a 13-fold decrease (Table 1). The apparent IPTG binding affinity of these mutants decreased only 4–5-fold at higher pH, in contrast to the 11-fold decrease for wild-type repressor (Table 2).

Unusual allosteric behavior was observed for the mutations to alanine and lysine at position 88. The wild-type repressor exhibits lower affinity for inducer and cooperative binding at elevated pH (Daly *et al.*, 1986; Daly & Matthews, 1986b), a property that mimics the presence of operator DNA (O'Gorman *et al.*, 1980a; Daly & Matthews, 1986b). The allosteric behavior of the wild-type protein is pH-dependent and is associated with alterations in the monomer-monomer interface along the long axis of the protein, a region encompassing Lys^{84} and Tyr^{282} (Daly *et al.*, 1986; Chen & Matthews, 1992; Chang *et al.*, 1993; Chen *et al.*, 1994). Both alanine and lysine substitutions at Asp^{88} resulted in decreased affinities and cooperative binding at neutral pH, and the cooperativity persisted at elevated pH despite a smaller shift in binding affinity (Table 2). This alteration in allostery and in the pH dependence of binding is consistent with the placement of Asp^{88} at the long axis–subunit interface in the molecular replacement model (Figure 1B), given the contribution of this region to the pH shift and inducer binding cooperativity (Daly *et al.*, 1986; Chen & Matthews, 1992; Chang *et al.*, 1993; Chen *et al.*, 1994).

On the basis of homology with the periplasmic sugar binding proteins, the rate of inducer binding in *lac* repressor has been proposed to depend on the rate at which the inducer binding cleft opens and closes (Miller *et al.*, 1983; Sams *et al.*, 1984; Chakerian *et al.*, 1987; Matthews, 1987). Therefore, kinetic parameters for *lac* repressor provide information on the relative accessibility of the inducer binding site to sugar. In addition, opening and closing of this cleft may be influenced by changes in subunit interactions (Daly *et al.*, 1986; Chakerian & Matthews, 1991; Chang *et al.*, 1993). In accordance with this hypothesis, the increased dissociation rate constant (6–12-fold) found for the proteins with substitutions at Asp^{88} could result from changes in the subunit interface, in analogy to the effects of mutations at Lys^{84} that altered kinetic properties dramatically (Chang *et al.*, 1993). It is interesting to note that mutant protein Asp^{88}→Glu has an increased association rate constant, which might reflect more rapid opening of the sugar binding pocket associated with elongation of the charged side chain at position 88.

In contrast to the alterations resulting from substitution of Asp^{88}, none of the substitutions at Asp^{130} affected IPTG binding affinity or kinetic parameters (Table 1). This result seems inconsistent with the original inducer binding site proposed by Sams *et al.* (1984), since this amino acid side chain was hypothesized to serve as a hydrogen-bond acceptor from the C1 hydroxyl group of galactose (Figure 1A). On the basis of the sugars that bind to the repressor, it appears that van der Waals interactions instead of hydrogen bonds are the major forces that stabilize binding at the C1 position (Barkley *et al.*, 1975). Due to the hydrophilic nature of its side chain, Asp^{130} would not be expected to contribute energy to binding at the 1-position of isopropyl β-D-thiogalactoside, even if this amino acid residue were in the inducer binding site as proposed by Sams *et al.* (1984). Our results for Asp^{130} are consistent with such an interpretation. Because Asp^{130} is positioned at the outer edge of the sugar binding site in the recent molecular replacement model generated by Nichols *et al.* (1993) (Figure 1B), the absence of alteration in IPTG binding or other detectable properties found for the Asp^{130} mutant proteins prevents us from distinguishing these two models unequivocally. Since all sugars examined were monosaccharides, it is possible that mutant proteins at the Asp^{130} site could exhibit altered binding affinity for the disaccharide allolactose, which is the natural inducer.

Data on proteins with substitutions at Asp^{274} indicate strong involvement of this amino acid in inducer binding activity. Interestingly, none of the mutations at this site yielded protein that bound to any of the inducers examined, despite the maintenance of wild-type operator binding affinity and oligomeric structure of the protein. Even at high concentrations of inducer, none of the mutants exhibited the fluorescence shift typical for *lac* repressor upon inducer binding (Figure 4). In addition, none of the mutants at Asp^{274} could bind [^{14}C]IPTG at millimolar concentrations (Figure 6). Since operator binding, size by gel filtration, circular dichroism spectra, and immunoblotting assays showed no significant structural disturbance (except for the Asp^{274}→Lys mutant protein), the absence of inducer binding capacity in even conservative mutations such as Asp^{274}→Asn and Asp^{274}→Glu suggested that Asp^{274} plays an indispensable role in inducer

binding. None of the mutant repressors at this site showed responses to any other sugars tested, including methyl-β-D-thiogalactoside, galactose, or fucose (data not shown). These results strongly support the predicted role of Asp^{274}, which is similar in both models, with possible involvement in both hydrogen bonding and salt linkages with surrounding side chains. Given such a role, even subtle alteration of side chain hydrogen-bonding potential, charge, or size may disrupt local structure. The extreme case for this effect is illustrated by the Asp^{274}→Lys mutant, where a bulky and positively charged side chain has been introduced at this site. The structure of this mutant is disturbed, resulting in altered antibody binding, increased sensitivity to trypsin digestion, and decreased operator binding activity. These effects may arise from charge repulsion with Arg^{197}, which is nearby in the inducer binding site in the molecular replacement model (Nichols *et al.*, 1993).

Since mutations at Asp^{274} totally abolished characteristic spectroscopic changes in the presence of inducer, decreased inducer binding capacity by at least 1000-fold, and, with the exception of Asp^{274}→Lys, did not yield any significant structural alterations compared to wild-type repressor, it is conceivable that these mutant proteins are unable to undergo the conformational change associated with inducer binding and therefore remain in the form with high affinity for operator DNA (Daly & Matthews, 1986b). This conformational shift to surround the ligand is necessary for contacts that generate tight binding of inducers. On the basis of these data, we propose that Asp^{274} is located at the base of the inducer binding cleft in a region essential for either direct inducer binding and/or closure of the binding pocket. It is interesting to note that, in the alignments of ligand binding bacterial regulators (Weickert & Adhya, 1992; Mauzy & Hermodson, 1992; Schumacher *et al.*, 1993), aspartate in the position corresponding to 274 in *lac* repressor is highly conserved. It is possible that this site is critical to structural shifts in these proteins and that any interruption of interactions in this region may affect sugar binding and/or the conformational change associated with ligand binding. Further investigation to explore these possibilities for *lac* repressor mutants at Asp^{274} is underway.

In summary, from the results of amino acid substitutions at Asp^{88}, Asp^{130}, and Asp^{274} sites, we conclude that Asp^{274} plays an essential role in inducer binding, either in direct contact(s) with sugar and/or in conformational alterations consequent to inducer binding, and that Asp^{88} plays an indirect role in inducer binding, apparently via influence on subunit interactions. In contrast, the absence of an effect of Asp^{130} substitution on IPTG binding suggests that this residue is not crucial to β-substituted monosaccharide binding. Thus, the roles deduced for these aspartate residues in structure and function are consistent with their positions in the molecular replacement model of *lac* core repressor (Nichols *et al.*, 1993) and contrast at two positions with the earlier attempt to place amino acids in the binding site on the basis of *in vivo* phenotypic data (Sams *et al.*, 1984). The data presented in this article provide support for the utility of model building in attempting to elucidate the roles of specific amino acid side chains in protein structure and function (Vyas *et al.*, 1991; Nichols *et al.*, 1993).

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