Role of Asp^{274} in *lac* Repressor: Diminished Sugar Binding and Altered Conformational Effects in Mutants†

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Received November 3, 1994; Revised Manuscript Received May 10, 1995⊗

ABSTRACT: The role of Asp^{274} in inducer binding of *lac* repressor has been explored by spectroscopic measurements, fluorescence quenching, *in vitro* induction assays, and chemical modification of mutants with conservative substitutions at this site. Although no fluorescence emission shift or characteristic UV difference spectrum was observed at high inducer concentration, fluorescence quenching, effects on operator binding, and chemical modification results indicate indirectly that the mutants Asp^{274}→Asn and Asp^{274}→Glu bind sugar, albeit with very low affinity (>0.1 M). Consistent with very weak inducer binding indicated by protection from fluorescence quenching by iodide, operator binding activity of these two mutant proteins is altered at very high IPTG concentration, although in opposite directions. The distinct effects of inducer on operator binding in these two mutant proteins as well as substantial differences in the effect of sugar ligand on chemical modification of Cys^{107} and Cys^{140} by 2-(bromoacetamido)-4-nitrophenol suggest that the conformation of the protein before and after association with sugar may differ in these mutant proteins. Fluorescence quenching assays of *lac* mutant proteins at Asp^{274} indicate the proximity of Trp^{220} to the side chain at position 274, consistent with the location of this residue in the structural model of *lac* repressor and in the crystallographic structure of the homologous purine repressor. From these results, we conclude that Asp^{274} is in the inducer binding site, that the character of this residue is crucial to inducer binding, and that interaction of sugar with the side chain at this position may be associated with the conformational change necessary for generating high affinity ligand binding.

Inducer binding is essential to the regulatory role of the *lac* repressor protein, the key negative regulator of *lac* operon expression (Miller & Reznikoff, 1980). The inducer binding site of *lac* repressor is presumed to consist of a large number of hydrophilic amino acids, scattered throughout the core domain, capable of forming hydrogen bond interactions with inducer sugars (Miller, 1979; Sams *et al.*, 1984; Kleina & Miller, 1990; Nichols *et al.*, 1993). Despite the complexity of elucidating the components of the inducer binding site in the absence of a high resolution X-ray crystallographic structure (Pace *et al.*, 1990), progress has been made by alternate approaches. The binding affinity of various sugars to *lac* repressor revealed that most sugars that bound were β-galactosides, and a bulky group at the C1 position of the sugar was required for tight binding (Barkley *et al.*, 1975). Using a series of fluoro-substituted methyl β-D-galactosides, Chakerian *et al.* (1987) were able to show that the C3 and C6 hydroxyls of the sugar contribute significantly to inducer binding, while the C4 hydroxyl provides only a nominal contribution. In addition, using site-specific mutagenesis in the context of a hypothetical inducer binding site of *lac* repressor (Sams *et al.*, 1984), the importance of Arg^{197} and Trp^{220} for inducer binding activity has been demonstrated (Gardner & Matthews, 1990; Spotts *et al.*, 1991). Selection of side chains based on the alignment with periplasmic binding proteins and a new molecular replacement model (Nichols *et al.*, 1993) led to the demonstration that Asp^{274} is essential for inducer binding activity (Chang *et al.*, 1994), a result concordant with *in vivo* phenotypic analysis (Kleina & Miller, 1990).

Asp^{274} is found at the bottom of the inducer binding site in the molecular replacement model for *lac* repressor (Nichols *et al.*, 1993), and the homologous residue occupies a similar position with contacts to purine ligand and to other side chains in the recently solved X-ray crystallographic structure of the purine holorepressor complexed with DNA (Schumacher *et al.*, 1994). The side chain of Asp^{274} appears to be essential for sugar binding in *lac* repressor, as no inducer binding of mutant proteins, even with conservative substitutions at this position, was detected in conventional assays (Chang *et al.*, 1994). Proteins with substitutions at the Asp^{274} site did not bind [^{14}C]IPTG[1] at up to 3 × 10^{-4} M protein concentration, and no fluorescence shift was observed at 1 M IPTG (Chang *et al.*, 1994). However, the ability of these mutant proteins to bind sugar molecules at higher concentrations (*i.e.*, with a lower affinity) was not explored in detail in these studies. The absence of a shift in fluorescence emission at high IPTG concentrations could be rationalized by circumstances other than the absence of binding. First, IPTG binding in the mutant protein may not elicit the conformational change that produces the environ-

†This work was supported by grants from the National Institutes of Health (GM 22441) and the Robert A. Welch Foundation (C-576) and employed facilities of the Keck Center for Computational Biology.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1995.

[1] Abbreviations: ABP, arabinose binding protein; BNP, 2-(bromoacetamido)-4-nitrophenol; BSA, bovine serum albumin; G/GBP, D-glucose/D-galactose binding protein; HPLC, high-pressure liquid chromatography; IPTG, isopropyl 1-thio-β-D-galactoside; NATA, *N*-acetyltryptophanamide; PBS, phosphate-buffered saline; PG, phenyl β-D-galactoside; RBP, D-ribose binding protein.

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mental alteration associated with the characteristic spectral shift on sugar binding. Second, Trp^{220}, the amino acid in the inducer binding site that is responsible for the fluorescence emission shift in *lac* repressor upon inducer binding, may not contact the sugar in the mutant in the same manner as in the wild-type protein.

In order to explore further whether Asp^{274} mutant proteins bind inducers and whether such binding is accompanied by a conformational change, we designed experiments using two mutant proteins with conservative substitutions that display no detectable structural perturbations and wild-type operator affinities, namely, Asp^{274}→Asn and Asp^{274}→Glu. The data gathered in this study demonstrated that these two mutant proteins were able to bind IPTG with an extremely low affinity (>10^5-fold lower than wild-type). IPTG binding to these mutant proteins appears to elicit conformational changes either similar to (in the case of the mutation Asp^{274}→Asn) or distinct from (in the case of the mutation Asp^{274}→Glu) that of the wild-type repressor which presumably adopts a closed form to contact the sugar (Miller *et al.*, 1983; Sams *et al.*, 1984; Spurlino *et al.*, 1991; Sharff *et al.*, 1992; Mowbray & Cole, 1992; Olah *et al.*, 1993; Nichols *et al.*, 1993; Schumacher *et al.*, 1994).

MATERIALS AND METHODS

Protein Purification and Activity Analysis. The mutant proteins were generated by site-specific mutagenesis and purified from *Escherichia 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$^+$] as described previously (O'Gorman *et al.*, 1980; Chang *et al.*, 1994). The purity of the protein was greater than 95% as determined by silver staining of SDS−PAGE gels, and the activity of the mutant proteins was ≥95% as determined by operator binding as described previously (Chang *et al.*, 1994). The stoichiometry of 40 bp operator DNA binding to the wild-type and mutant proteins is 2 operators per repressor tetramer (Chang *et al.*, 1994). Any structural disturbance in these mutant proteins was slight since there were no alterations in circular dichroism spectra, operator binding affinity, or antibody binding assays (Chang *et al.*, 1994).

Fluorescence Emission Spectroscopy. The protein was diluted in TMS buffer (0.01 M Tris-HCl, pH 7.4, 0.2 M KCl, 0.01 M $MgCl_2$, 1 mM EDTA, 0.1 mM DTT) to a concentration of 5×10^{-7} M monomer and excited at 285 nm at room temperature as described previously (O'Gorman *et al.*, 1980). The fluorescence emission data were collected from 300 to 450 nm with a scan speed of 50 nm/min. IPTG (5 mM final concentration) was then added to the protein (1/200 dilution of 1 M IPTG), and fluorescence emission data were collected again. In a parallel control experiment, the same volume of TMS was added to obtain the dilution correction factor. For examination of spectra at high IPTG concentrations, the protein was diluted directly into 1 M IPTG (dissolved in TMS buffer).

UV Difference Spectroscopy. The ultraviolet difference spectrum (Matthews *et al.*, 1973) was recorded on a Shimadzu UV-2101PC UV−vis scanning spectrophotometer from 275 to 320 nm. The repressor concentration was adjusted to (2.7−8) × 10^{-5} M monomer (1−3 mg/mL) in 1 M Tris-HCl (pH 7.4) in order to obtain sufficient signal. Dual-chamber cuvettes were used in which protein and inducer were placed in different chambers of the same cuvette for both reference and sample cuvettes, and the mixture of protein−inducer solution was obtained by mixing solutions from these two chambers in the sample cuvette.

Fluorescence Quenching. The iodide and acrylamide quenching experiments were performed as described previously (Gardner & Matthews, 1990). Potassium iodide stock solution (5 M) was freshly prepared and supplemented by 1 mM $Na_2S_2O_3$ to prevent oxidation of iodide; 30% acrylamide solution (4.2 M) was purchased from National Diagnostic, Inc.; and 5 M thallium acetate stock solution was prepared in water. The protein concentration was adjusted to 5 × 10^{-7} M monomer in a volume of 1 mL of TMS (pH 7.5) for iodide and acrylamide quenching experiments. In thallium quenching experiments, TPA buffer (0.05 M Tris−acetate, pH 7.4, 0.15 M potassium acetate, 0.1 mM DTT) was used to avoid precipitation of thallium chloride, a compound with a very low solubility in water. Using an SLM 8100 spectrofluorometer, the sample was excited at 285 nm, and fluorescence intensity was monitored at 337 nm. Subsequently, stock solutions were added to the protein solution in 5 μL aliquots, and the fluorescence intensity was measured after 35 s. The final concentrations of quencher after 10 additions were 0.24 M potassium iodide, 0.2 M acrylamide, and 0.24 M thallium chloride. An identical control titration was performed in the same way except potassium chloride (saturated, about 4.5 M) was added for iodide quenching analysis, TMS was added for acrylamide quenching analysis, and potassium acetate (5 M) was added for thallium quenching analysis in order to obtain dilution factors. The fluorescence value for each addition was multiplied by its corresponding dilution correction factor. The data were then analyzed by a Stern−Volmer plot (F_0/F *vs* the concentration of the quencher ([Q])). The Stern−Volmer equation $F_0/F = 1 + k_q\tau_0[Q]$ was thus used to yield K_D ($k_q\tau_0$), the Stern−Volmer quenching constant, which is the product of k_q, the bimolecular quenching constant, and τ_0, the lifetime of the fluorophore in the absence of quencher.

Operator Binding Assays in the Presence of Inducer. Binding of sugars can be monitored indirectly by detecting the alteration of operator binding in the presence of different concentrations of inducer. Briefly, 6 × 10^{-11} M [^{32}P]-labeled 40 bp operator DNA (Chang *et al.*, 1994) was mixed with 6 × 10^{-11} M repressor protein and incubated in filtering buffer (0.01 M Tris-HCl, pH 7.5, 0.15 M KCl, 0.1 mM EDTA, 0.1 mM DTT, 5% dimethyl sulfoxide, 50 μg/mL BSA) at room temperature for 10 min. A series of increasing sugar concentrations was then added into the mixture, which was later subjected to another 10 min of incubation. The resultant mixture was then filtered on a nitrocellulose membrane using a 96-well dot-blot apparatus (Wong & Lohman, 1993). The data were collected on a Fuji phosphorimaging plate (Type BAS-IIIs) and analyzed using the MacBas Program.

2-(Bromoacetamido)-4-nitrophenol Modification. The modification was performed according to the procedures described by Chakerian *et al.* (1985) and Gardner and Matthews (1987) with some alterations. The buffer of the protein (1 mg) was exchanged to 0.1 M Tris-HCl, pH 9.0, and 1.0 M NaCl by using a Centricon-30 (Amicon) device. The concentration of protein was adjusted to 0.5 mg/mL in the same buffer or in buffer with 0.5 M IPTG, 0.1 M PG, or 8 M urea (final concentrations). A 200-fold molar excess of 2-(bromoaceta-

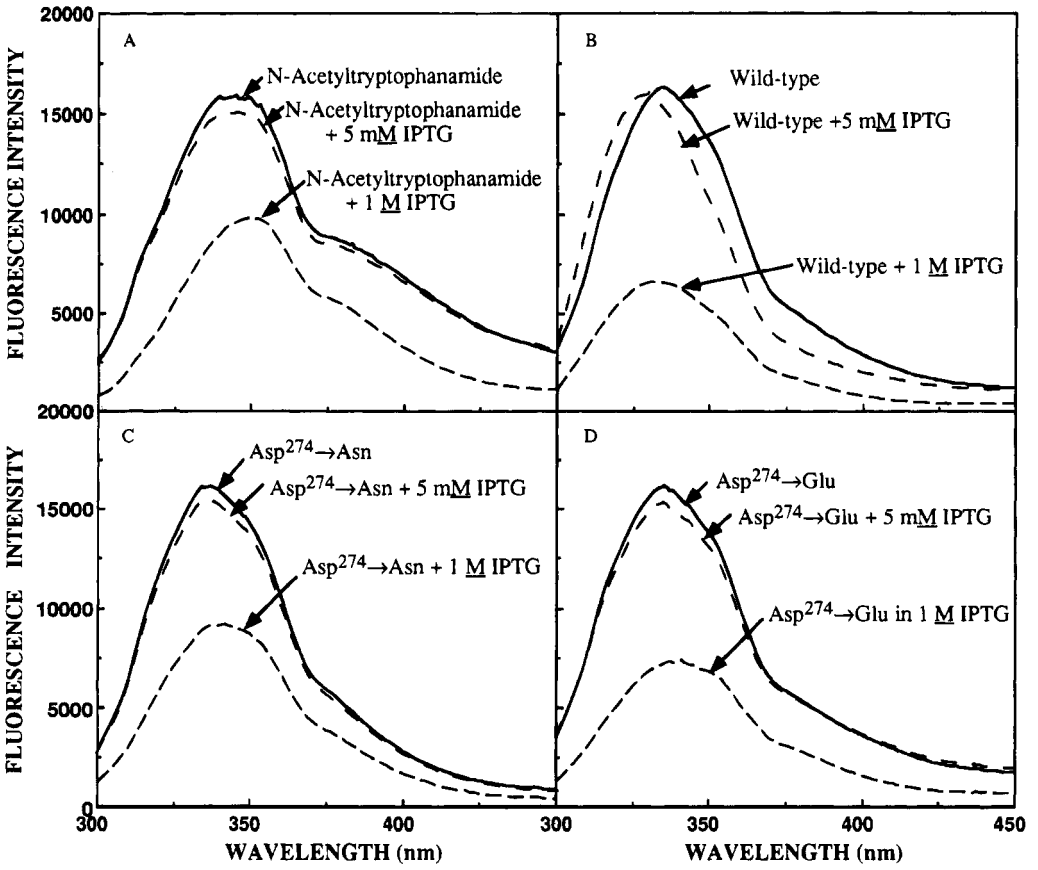

FIGURE 1: Changes in fluorescence emission of repressor proteins upon inducer addition. A solution of NATA or protein at 5×10^{-7} M monomer was excited at 285 nm at room temperature, and the emission spectrum was collected from 300 to 450 nm. When 1 M IPTG was used to monitor the fluorescence change, the protein was diluted directly into concentrated IPTG solution (in TMS buffer) before excitation at 285 nm due to the solubility of the sugar. (A) *N*-Acetyltryptophanamide; (B) Wild-type; (C) Asp^{274}→Asn; (D) Asp^{274}→Glu.

mido)-4-nitrophenol (BNP) (0.5 M stock solution in *N,N*-dimethylformamide) was added to the protein, and the resultant yellow mixture was incubated at room temperature for 1.5 h. Excess reagent was removed by a Bio-Rad 10DG desalting column equilibrated with 0.05 M ammonium bicarbonate (pH 9.0) and 4.0 M urea. The protein solution was dialyzed extensively against 0.05 M ammonium bicarbonate. The protein was then lyophilized in a Savant vacuum concentrator and resuspended in 1 mL of 0.05 M ammonium bicarbonate, pH 9.0, followed by digestion by trypsin and chymotrypsin (1% w/w final concentration). The digestion was maximized by a second addition of enzyme and by shaking the mixture at 37 °C for 3 h following each addition. The digested protein was then lyophilized and stored at 4 °C. The product peptides were dissolved in 0.05 M ammonium bicarbonate (pH 7.0) and separated on a 4.6 × 250-mm Customsil 5 μm, 300 Å C-18 reverse-phase column connected to a LDC/Milton Roy HPLC system. The column was equilibrated with 0.05 M ammonium bicarbonate (pH 7.0), and the peptides were eluted with a linear gradient of 0−30% acetonitrile. The modified peptides were detected at 405 nm, and the relative extent of modification of Cys^{107} and Cys^{140} was compared following integration of peak areas.

RESULTS AND DISCUSSION

Probing Inducer Binding by Spectroscopic Methods. Upon inducer binding, *lac* repressor undergoes a conformational change (Ohshima *et al.*, 1972; Laiken *et al.*, 1972; Matthews *et al.*, 1973; Wu *et al.*, 1976), and the inducer binding site presumably adopts a closed form in which the amino acids of the inducer binding site have close contact with ligand as observed for the homologous periplasmic sugar binding proteins and purine repressor (Spurlino *et al.*, 1991; Sharff *et al.*, 1992; Mowbray & Cole, 1992; Olah *et al.*, 1993; Schumacher *et al.*, 1994). Either direct contact with the sugar or the environmental alterations in the vicinity of tryptophan residues consequent to the conformational change are thought to be the basis of the blue shift observed in the fluorescence emission curve of *lac* repressor, as shown in Figure 1B (Laiken *et al.*, 1972; O'Gorman *et al.*, 1980; Spodheim-Maurizot *et al.*, 1985; Gardner & Matthews, 1990). However, *lac* mutant proteins Asp^{274}→Asn and Asp^{274}→Glu did not show any fluorescence shift to shorter wavelength even in the presence of 1 M IPTG (Figure 1C,D). It is interesting to note that, at high concentrations of IPTG, the fluorescence intensity of the protein decreases, and the emission peak shifts to a slightly longer wavelength. This phenomenon appears to be due to absorbing and/or quenching contaminants present in IPTG rather than to be the result of interaction between IPTG and the protein, as the model compound *N*-acetyltryptophanamide (NATA) displays a similar decrease and shift in the presence of the same concentration of IPTG (Figure 1A).

Upon inducer binding, the interaction between sugar and *lac* repressor and the associated conformational change result in environmental changes of tryptophans and tyrosines that are reflected in the UV absorbance difference spectrum of wild-type *lac* repressor (Matthews *et al.*, 1973). Specifically,

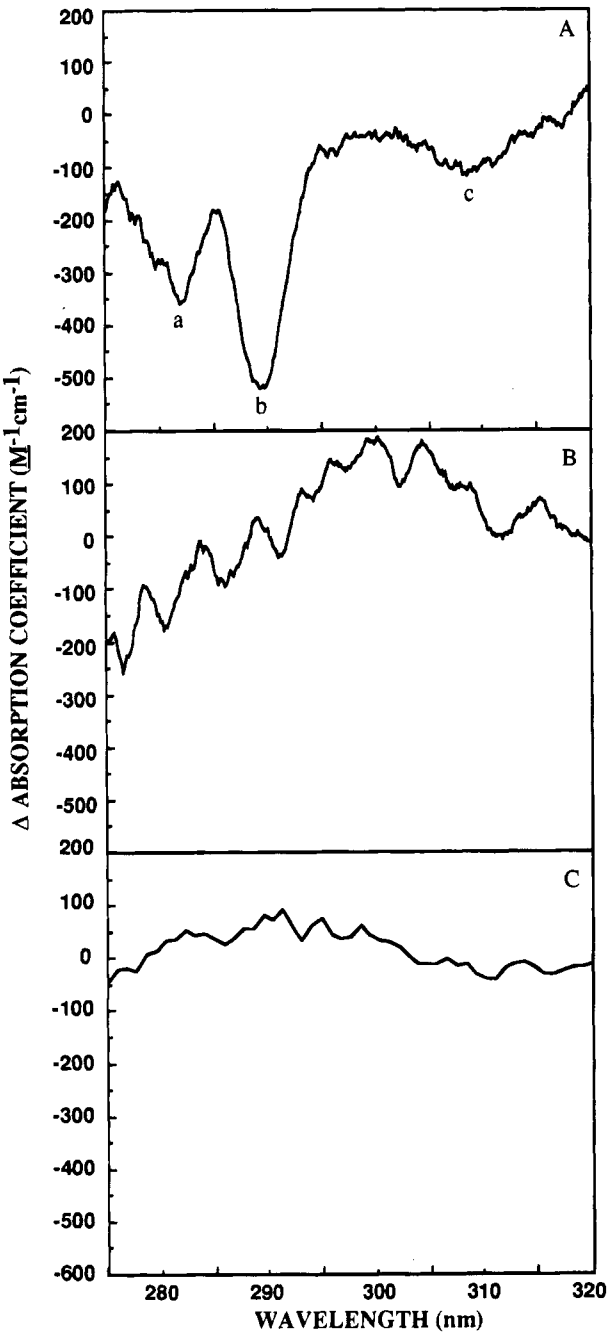

FIGURE 2: Ultraviolet difference spectrum of wild-type repressor and mutant proteins at Asp^{274}. The UV difference spectrum between unbound and bound repressor was scanned from 320 to 275 nm at room temperature as described in Materials and Methods. Protein concentration was 2.7×10^{-5} to 8×10^{-5} M monomer, and the final IPTG concentration was 0.5 M. (A) Wild-type repressor; (B) Asp^{274}→Asn mutant protein; (C) Asp^{274}→Glu mutant protein. The characteristic decreases in absorbance at 282, 290, and 308 nm for wild-type protein were labeled as a, b, and c, respectively.

IPTG binding to wild-type repressor results in UV absorbance decreases at 282, 290, and 308 nm (O'Gorman & Matthews, 1977; Gardner & Matthews, 1990), corresponding to environmental changes for tryptophans, tryptophans and tyrosines, and Trp^{220}, respectively (labeled a, b, and c in Figure 2A). Since these absorbance decreases are characteristic of inducer binding to *lac* repressor (Gardner & Matthews, 1990), the UV difference spectrum of *lac* repressor mutants in the absence of inducer *vs* in the presence of inducer could be another indication of inducer binding to these proteins and/or the associated conformational change. As shown in Figure 2, there is no characteristic absorbance decrease at the wavelengths found for wild-type in mutant proteins Asp^{274}→Asn and Asp^{274}→Glu in the presence of 0.5 M IPTG, a result that suggests either that inducer does not bind or that there is no characteristic conformational change in the mutant proteins in the presence of inducer.

Probing Inducer Binding by Protection of Fluorescence Quenching. Two tryptophans (Trp^{201} and Trp^{220}) are present in *lac* repressor. Trp^{220} is quenched by iodide to a large extent, while Trp^{201} is minimally quenched (Sommer *et al.*, 1976; Gardner & Matthews, 1990), suggesting Trp^{220} is more accessible to the solvent. The binding of inducers can reduce the quenching of Trp^{220}, consistent with the presence of this residue at the inducer binding site (Sommer *et al.*, 1976; Gardner & Matthews, 1990; Kleina & Miller, 1990; Nichols *et al.*, 1993). The homologous residue in purine repressor (Phe^{221}) stacks with the purine ligand (Schumacher *et al.*, 1994), and in the sugar binding proteins corresponding aromatic residues are involved in stacking interactions with the sugar ligands (*e.g.*, Vyas *et al.*, 1988, 1991; Mowbray & Cole, 1992). Both tryptophans in *lac* repressor are quenched by acrylamide, and quenching of Trp^{220} can be partially protected by binding to the inducer (Gardner & Matthews, 1990). Thallium is a positively-charged quencher not previously used with *lac* repressor, and we have employed this agent to examine the effects of charge on quenching properties.

Since the ability to protect Trp^{220} fluorescence quenching by inducer (Gardner & Matthews, 1990) approximately coincides with binding affinity, it was conceivable that this phenomenon could be used to monitor sugar binding for the mutant proteins (Sommer *et al.*, 1976; Gardner & Matthews, 1990). At a concentration of ~40 mM, IPTG is able to protect slightly the Asp^{274}→Asn mutant from iodide quenching (Figure 3), indicating possible weak binding of IPTG to the inducer binding site of this mutant protein. A high concentration of IPTG *per se* does not affect iodide quenching, as iodide quenching of the model molecule *N*-acetyltryptophanamide is not altered by the presence of sugar (Table 1). Thus, the increase in fluorescence intensity in the presence of high IPTG concentration appears to be the result of protection of *lac* repressor protein from iodide quenching provided by the sugar. This protection from iodide quenching increased with increasing inducer concentration for Asp^{274}→Asn mutant protein, and the half-protection point exceeded 0.1 M IPTG (Figure 4, Table 2). It must be emphasized that this value can serve only as a *crude* estimate of IPTG binding affinity, since at high concentration the sugar alone diminishes fluorescence intensity and shifts the emission maximum (Figure 1). That is, with a given concentration of IPTG, the actual increase in protein fluorescence might be greater if it were possible to dissect the effects of binding from absorption/quenching by IPTG. Due to the complexities of working with high concentrations of sugar, no attempt was made to refine this value further. There is no indication of protection from iodide quenching for Asp^{274}→Glu mutant protein in the presence of ~40 mM IPTG (Figure 3), indicative of even lower inducer binding affinity of this protein as compared to Asp^{274}→Asn. However, IPTG binding to the Asp^{274}→Glu mutant is suggested by the ability of >10-fold higher concentrations of sugar to protect Trp^{220} from iodide quenching (Table 1).

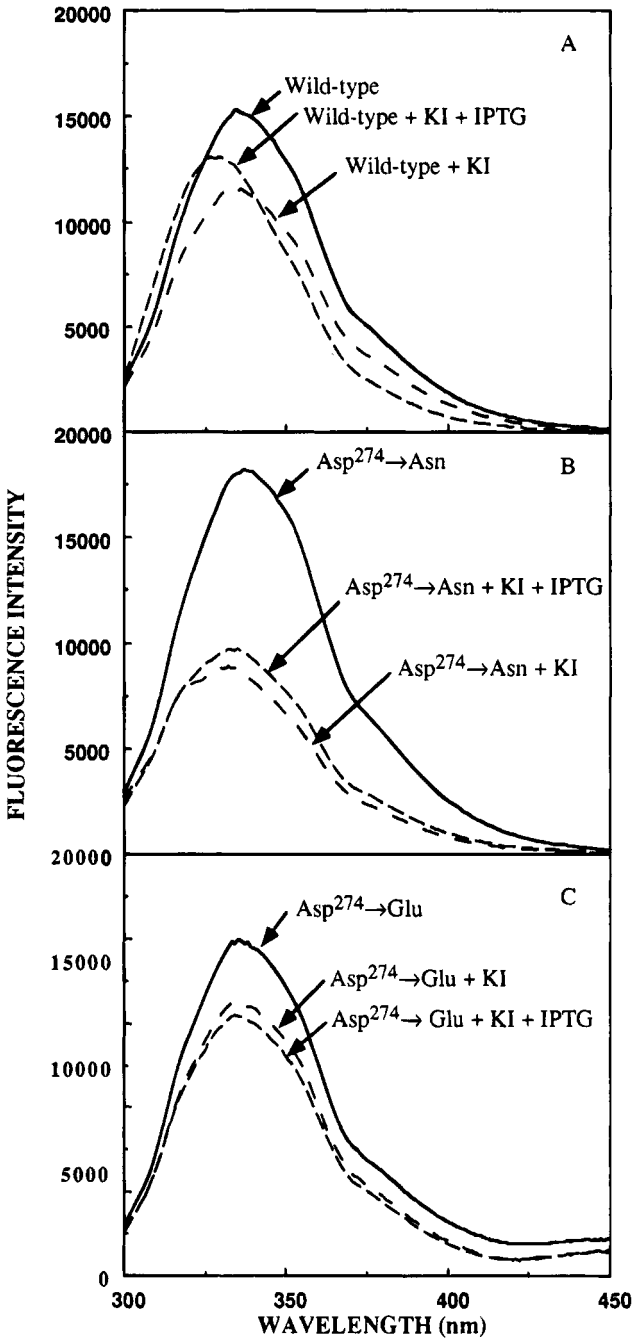

FIGURE 3: Protection from iodide quenching by IPTG binding to *lac* repressor. The fluorescence emission curve of *lac* repressor was recorded from 300 to 450 nm. (A) Wild-type repressor; (B) Asp^{274}→Asn; (C) Asp^{274}→Glu. Protein concentration was 5×10^{-7} M monomer. The designation "protein + KI" indicates the fluorescence emission curve collected for the protein in the presence of 0.2 M KI. The designation "protein + KI + IPTG" indicates the fluorescence emission curve of the protein after subsequent addition of 40 μL of 1 M IPTG to a final concentration of 38.5 mM.

It is interesting to note that the Stern−Volmer quenching constant for iodide, a bulky and negatively charged quencher, in the Asp^{274}→Asn mutant protein was much greater than that of the wild-type repressor, while that in Asp^{274}→Glu was similar to wild-type repressor; the quenching constant for all three proteins is decreased by 0.5 M IPTG (Table 1). In contrast, the fluorescence quenching of the mutant proteins by acrylamide, a neutral and smaller quencher, was affected minimally by the substitutions and only slightly by sugar (Table 1). The quenching constant with thallium for mutant protein Asp^{274}→Asn was the smallest of the three proteins. This result contrasts to the behavior of this mutant protein with the other two quenchers, especially the negatively charged quencher iodide. It is conceivable that the negative electrostatic environment around Trp^{220} contributed by Glu or Asp side chains at position 274 is the major cause for this difference (Table 1) (Lakowicz, 1983). The $C_\alpha-C_\alpha$ distance between the homologous residues, Asp^{275} and Phe^{221}, in the purine repressor holorepressor structure is 11 Å, with the nearest approach of the side chains ~5 Å, distances consistent with the effects of charge at position 274 on quenching of the Trp^{220} indole ring. Increased quenching in the presence of 0.5 M IPTG with thallium is opposite to protection observed with sugar for iodide quenching. The origin of this behavior is unclear, but this effect could derive from the presence of Arg^{197} in the binding site in close proximity to Trp^{220} [$C_\alpha-C_\alpha$ <13 Å, side chains <5 Å based on the Phe^{221}−Arg^{196} distance in purine repressor (Schumacher *et al.*, 1994)].

Probing Inducer Binding and Conformational Change by Operator Binding. Upon inducer binding to *lac* repressor, the protein undergoes a conformational change (Ohshima *et al.*, 1972; Matthews *et al.*, 1973; Wu *et al.*, 1976), which is evidently transmitted to the N-terminal DNA binding region, to reorient the helix−turn−helix DNA binding arms. As a result, the specific operator binding is diminished or stabilized depending on the nature of the sugar bound (Riggs *et al.*, 1970; Barkley *et al.*, 1975). Operator binding affinity can be decreased by inducer binding to the protein and increased by anti-inducer binding to the protein (Müller-Hill *et al.*, 1964; Gilbert & Müller-Hill, 1966, 1967; Riggs *et al.*, 1968; Barkley *et al.*, 1975). Using protein and DNA concentrations just above the K_D for operator binding, it is possible to monitor changes in affinity of the protein elicited by sugar ligand by detecting increases/decreases in retention of protein−DNA complex on nitrocellulose filters.

IPTG presence elicits different effects on operator binding by mutant proteins Asp^{274}→Asn and Asp^{274}→Glu. As shown in Figure 5, wild-type protein dissociates from operator at low inducer concentrations, while the mutant protein Asp^{274}→Asn showed a decrease in operator binding with a midpoint at approximately 0.1 M IPTG, consistent with the half-protection point observed in iodide quenching. In contrast, the mutant protein Asp^{274}→Glu showed an increase in operator binding affinity at higher IPTG concentration, suggesting a different effect of IPTG on conformation in this mutant protein. The increase in operator binding is similar to the influence of anti-inducers on wild-type protein. To explore the effect of a sugar identified as an anti-inducer for the wild-type protein, we examined the influence of PG on operator binding. PG has a lower affinity than IPTG for wild-type protein (Barkley *et al.*, 1975) and would therefore be presumed to bind more weakly to the mutants as well. However, at high concentrations, this sugar elicited a slight increase in operator binding for wild-type and Asp^{274}→Glu proteins but did not affect the Asp^{274}→Asn mutant protein. To explore the effects of high concentrations of hydroxyl-containing compounds on the filter binding assay, we have used glycerol, which presumably does not function as either an inducer or anti-inducer in this system. Glycerol increases operator binding of wild-type and mutant proteins at high concentrations as does glucose (an anti-inducer with weak affinity for repressor); whether this result indicates a specific

Table 1: Effect of Inducer on the Fluorescence Quenching of *lac* Repressor Proteins by Different Quenchers[a]

	thallium acetate[b]			acrylamide[c]			potassium iodide[d]		
	−IPTG	+IPTG[e]	ratio[f]	−IPTG	+IPTG	ratio	−IPTG	IPTG	ratio
wild-type	1.9 (±0.1)	5.6 (±0.3)	0.3	5.4 (±0.2)	3.8 (±0.2)	1.4	1.2 (±0.4)	0.3 (±0.1)	4.0
Asp^{274}→Asn	1.4 (±0.0)	3.4 (±0.1)	0.4	5.7 (±0.2)	4.2 (±0.2)	1.4	4.2 (±0.3)	1.3 (±0.1)	3.2
Asp^{274}→Glu	2.1 (±0.1)	4.8 (±0.3)	0.4	5.0 (±0.1)	4.4 (±0.4)	1.1	1.0 (±0.0)	0.3 (±0.1)	3.3
NATA[g]	25.4 (±0.4)	24.1 (±0.4)	1.1	33.0 (±0.8)	30.4 (±4.2)	1.1	10.1 (±0.1)	7.4 (±0.2)	1.4

[a] The fluorescence quenching experiments were performed as described in Materials and Methods. The K_D values shown are calculated from $F_0/F = 1 + K_D[Q]$, where F_0 is the initial fluorescence intensity at the zero quencher concentration, F is the fluorescence intensity at a defined quencher concentration [Q], and K_D is the Stern−Volmer quenching constant, which is the product of k_q, the bimolecular quenching constant, and τ_0, the lifetime of the fluorophore in the absence of quencher. The results shown are averages ± standard deviation ($n = 3$). [b] The buffer used for thallium quenching was TPA instead of TMS (see Materials and Methods). Potassium acetate (5 M) was added to protein instead of thallium acetate (5 M) in the parallel control experiment. [c] The buffer TMS was added to protein instead of acrylamide in the parallel control experiment. [d] Potassium chloride (saturated solution, about 4.5 M) was added to protein instead of potassium iodide (5 M) in the parallel control experiment as described in Materials and Methods. [e] Protein was diluted in 0.5 M IPTG, followed by subsequent addition of quencher. [f] Ratio of Stern−Volmer quenching constant in the absence of IPTG to that in the presence of 0.5 M IPTG. [g] *N*-Acetyltryptophanamide.

Table 2: IPTG Binding Activity for Wild-Type and Asp^{274} Mutant Proteins[a]

	[IPTG] (M) at half-maximum effect			
	iodide quenching	operator dissociation[b]	fluorescence titration[c]	[^{14}C]IPTG binding[c]
wild-type	0.5×10^{-6}	4.0×10^{-6}	$1.0 (\pm 0.3) \times 10^{-6}$	$\sim 1 \times 10^{-6}$
Asp^{274}→Asn	>0.1	0.1	ND[d]	>0.001
Asp^{274}→Glu	>0.2	>0.5	ND[d]	>0.001

[a] IPTG binding activity of *lac* repressor proteins was measured using different approaches as described in Materials and Methods. For each method, the concentration at the half-maximum effect is shown. Due to the extremely low binding ability of the mutant protein Asp^{274}→Glu, all the numbers shown are lower limits for this mutant. [b] Inducer binding to the repressor−operator complex was monitored by alteration in operator binding activity of the protein. [c] From Chang *et al.*, 1994. [d] Not detectable.

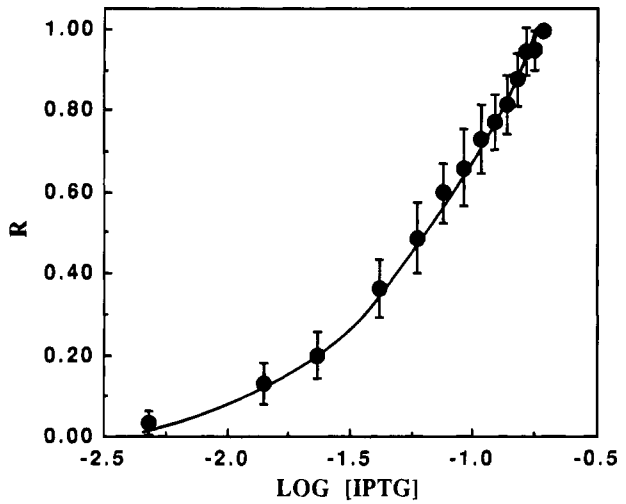

FIGURE 4: Titration curve of protection from iodide quenching by IPTG for Asp^{274}→Asn substitution. R is obtained from the apparent fluorescence intensity change ($F_i - F_0$) after a specific amount of IPTG was added divided by the correction factor ($F_i' - F_0'$) after the same amount of TMS buffer was added.

anti-inducer effect at the binding site or, more likely, an influence on retention of DNA in the filter binding assay is not clear. However, it is apparent that IPTG is the only sugar that *decreases* the affinity of wild-type repressor and Asp^{274}→Asn for operator and that this effect occurs at concentrations consistent with the apparent affinity of IPTG for these two proteins. In addition, the effect of IPTG on Asp^{274}→Glu suggests that the response of this mutant repressor to IPTG presence differs from wild-type and Asp^{274}→Asn, a result confirmed by data from chemical modification.

Probing the Conformational Change by Chemical Modification. Chemical modification can be used to detect conformational changes in proteins. For *lac* repressor, it has been shown that the conformational change upon inducer binding results in differential modification of cysteines by cysteine modifiers such as 2-(bromoacetamido)-4-nitrophenol (Yang *et al.*, 1977; Daly *et al.*, 1986; Chakerian *et al.*, 1985). Since all three cysteines (Cys^{107}, Cys^{140}, Cys^{281}) are located outside of the inducer binding site (see Conclusion; Kleina & Miller, 1990; Nichols *et al.*, 1993), using these cysteines to monitor the conformational change associated with inducer binding might provide information on regions not involved in direct interaction with the sugar ligand. In the absence of inducer, Cys^{107} is modified to a small extent by BNP (~35−40%), while Cys^{140} is more extensively modified (~85%) (Yang *et al.*, 1977; Daly *et al.*, 1986). Although Cys^{140} modification is unaltered by ligand binding, modification of Cys^{107} increases upon inducer binding (to ~85−90%), presumably as a result of the global conformational change in the protein (Yang *et al.*, 1977; Daly *et al.*, 1986). Therefore, the ratio of Cys^{107} modification to Cys^{140} modification by BNP is one of the parameters that can monitor sugar binding and the associated conformational change in *lac* repressor (Chakerian *et al.*, 1985; Daly *et al.*, 1986). We found the percent modification of Cys^{140} and the ratios for Cys^{107}/Cys^{140} modification for wild-type consistent with previous studies (Table 3). Cys^{140} for both mutant proteins reacted to an extent similar to that for the wild-type repressor. The mutant protein Asp^{274}→Asn shows a modification pattern similar to that of the wild-type repressor, but with an increase in the ratio of modification in the presence of anti-inducer. In contrast, the mutant protein Asp^{274}→Glu shows a significantly different pattern (Table 3). The modification of Cys^{107} is high in the absence of inducer and decreases significantly in Asp^{274}→Glu in the presence of 0.5 M IPTG, indicating a conformational change in this protein

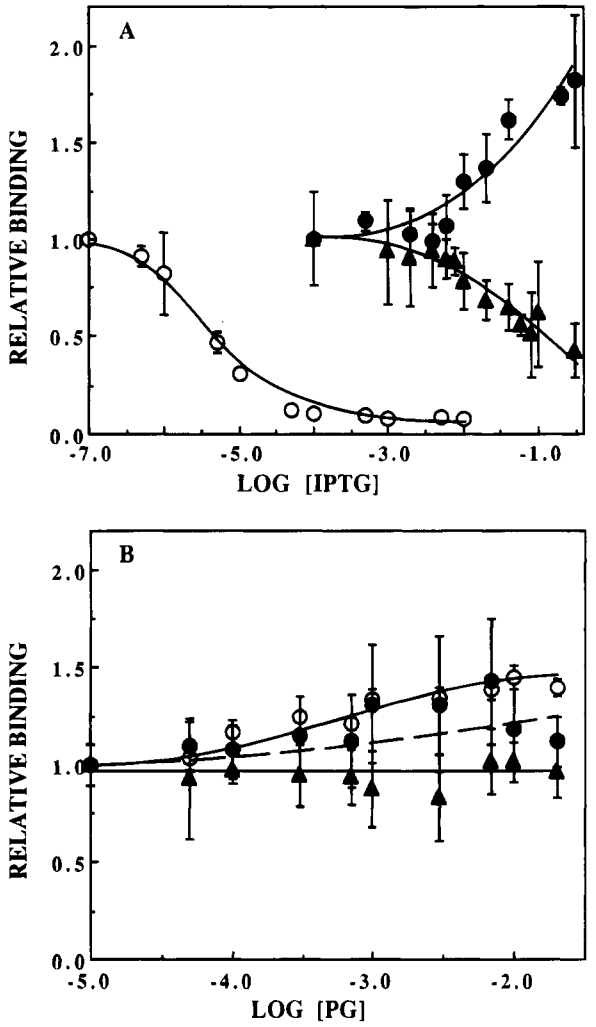

FIGURE 5: Operator binding curves for *lac* repressors in the presence of sugar ligands. The relative operator binding activity was defined as the operator binding (in cpm) observed at the indicated sugar concentration divided by operator binding in the absence of inducer. (○) Wild-type; (▲) Asp^{274}→Asn; (●) Asp^{274}→Glu. (A) IPTG; (B) PG.

Table 3: Chemical Modification by BNP

	% reaction[a] of Cys^{140}	ratio[b] of reaction at Cys^{107}/reaction at Cys^{140}		
		−IPTG	+IPTG	+PG
wild-type	82	0.5 (±0.2)	1.1 (±0.1)	0.4 (±0.3)
Asp^{274}→Asn	85	0.7 (±0.2)	1.3 (±0.3)	0.9 (±0.3)
Asp^{274}→Glu	72	1.3 (±0.1)	0.4 (±0.3)	1.3 (±0.3)

[a] Percent reaction of Cys^{140} was determined by comparing the integrated area corresponding to Cys^{140} in HPLC elution traces for each protein without ligand present to the integrated area for the same protein modified in the presence of 8 M urea using samples that contained identical amounts of protein. [b] The ratio of integrated peak areas of the peptide eluted from HPLC containing modified Cys^{107} to that containing modified Cys^{140}. IPTG is an inducer, while PG is an anti-inducer for the wild-type repressor (Barkley *et al.*, 1975). The values represent the average ratio ± standard deviation ($n \geq 3$).

as a result of inducer binding that shields an already exposed Cys^{107} from reaction. The ratio observed in response to anti-inducer is similar to modification in the absence of ligand. These results demonstrate effects on residues remote from the sugar binding site (see below). From the differences in reactivity in the presence of ligand, it appears that both mutant proteins are capable of binding to ligand, albeit with a very low binding affinity. However, the conformational change upon inducer or anti-inducer binding is distinct in the mutant protein Asp^{274}→Glu, consistent with the anomalous effect of IPTG on operator binding of this protein.

CONCLUSION

Inducer binding properties of mutant proteins at the Asp^{274} site have been explored using spectroscopic methods, equilibrium binding, and chemical modification. The data obtained provide some insights into the possible role played by Asp^{274}, an amino acid conserved in a number of periplasmic sugar binding proteins including arabinose binding protein (ABP), galactose/glucose binding protein (G/GBP), and ribose binding protein (RBP), as well as sugar-binding regulatory proteins such as *gal* repressor and purine repressor (Sams *et al.*, 1984; Weickert & Adhya, 1992; Mauzy & Hermodson, 1992; Nichols *et al.*, 1993; Schumacher *et al.*, 1994). Asp^{274} appears to be located in the inducer binding site close to Trp^{220}, the residue responsible for significant spectroscopic alterations in *lac* repressor upon inducer binding. The distance between α carbons of the homologous residues, Asp^{275} and Phe^{221}, in the purine repressor crystallographic structure is ~11 Å, with the closest approach of the side chains ~5 Å (from coordinates of Schumacher *et al.*, 1994). The proximity of Asp^{274} and Trp^{220} in *lac* repressor is supported by the variations in tryptophan quenching by differently charged quenching reagents. Iodide quenching is enhanced in the mutant protein Asp^{274}→Asn, presumably due to the removal of a negatively-charged residue from the environment near Trp^{220}, while the thallium quenching of this mutant protein is slightly decreased.

Characteristic spectroscopic alterations upon inducer binding are absent in the mutant proteins. However, evidence for inducer binding in these mutant proteins was derived from the alteration of operator binding activity in the presence of sugar and the protection from fluorescence quenching by iodide, as well as the effects of sugar on chemical modification by BNP. For mutant protein Asp^{274}→Asn, at high IPTG concentrations a decrease of operator binding was observed. In addition, high concentrations of IPTG were able to provide protection for tryptophan (most likely Trp^{220}) from iodide quenching, consistent with a low binding affinity for inducer (K_D >0.1 M) in this mutant protein. Finally, modification of Cys^{107} increases in the presence of 0.5 M IPTG, similar to the behavior of the wild-type upon IPTG binding. For the mutant protein Asp^{274}→Glu, an extremely low IPTG binding affinity ($K_D \geq 0.5$ M) is suggested from the alteration in operator binding, the protection of iodide quenching, and diminished Cys^{107} modification by BNP in the presence of inducer. These cysteine residues are well removed from the site of mutation in the tertiary fold of the protein based on the positions of their homologues in the purine repressor (Schumacher *et al.*, 1994): distance between C_α carbons for Cys^{107}−Asp^{274} homologues is 29 Å, while that for Cys^{140}−Asp^{274} homologues is 31 Å. Thus, changes in the reactivity of these residues presumably reflect more global alterations in the protein structure elicited by occupancy at the sugar binding site.

Of particular interest is the evidence that IPTG binding to mutant proteins Asp^{274}→Asn and Asp^{274}→Glu results in different effects on the protein conformation. The opposite

effect of inducer binding on operator binding and chemical modification of Cys^{107} by BNP in these two mutant proteins strongly suggests that the character of the side chain at position 274 influences the nature of the conformational change upon inducer binding. Asp at this position is highly conserved in the homologous periplasmic sugar binding proteins and in repressor proteins (Vyas *et al.*, 1991; Mauzy & Hermodson, 1992; Mowbray, 1992; Weickert & Adhya, 1992; Schumacher *et al.*, 1994). In the case of ribose binding protein (RBP) and galactose/glucose binding protein (G/GBP), Asp at this site appears to contact ligand and to interact with residue(s) at the hinge connecting the two subdomains (Vyas *et al.*, 1988; Mowbray & Cole, 1992; Mowbray, 1992). In the purine repressor structure, Asp^{275} participates in hydrogen bond and charge interactions with Arg^{196}, which anchors Asp^{275} interaction with ligand (Schumacher *et al.*, 1994). Asp^{274} in *lac* repressor might function similarly, with its side chain interacting with that of Arg^{197}, a residue at which substitution significantly alters inducer affinity (Spotts *et al.*, 1991). However, it should be underscored that differences in the arrangement of side chains in these homologous proteins are required to provide the basis for distinctions in their binding and functional properties, and the presence of similar side chains does not ensure similar arrangement in binding sites specific for different ligands (*i.e.*, purine *vs* sugar). Despite the obvious limitations to structure comparisons in deciphering the detail of binding site contacts, it is apparent that a network of hydrogen-bonded interactions in *lac* repressor forms the sugar binding pocket. Although the specific contacts may differ from the homologous periplasmic binding proteins and from purine repressor, one common element for all of these proteins is a conformational change on binding to ligand, involving the closing of the binding site. The partners with which specific residues hydrogen bond may be altered by ligand binding, eliciting the structural shifts associated with the binding process, and it will be of significant interest in the future to compare the structures for open and closed forms of these proteins. The evidence provided in this paper supports the placement of Asp^{274} in the inducer binding site in proximity to Trp^{220} and indicates that the aspartate side chain at this position is essential for the characteristic conformational change associated with high affinity inducer binding of *lac* repressor.

ACKNOWLEDGMENT

We would like to express our sincere thanks to Dr. Kamalam Muthukrishnan and Ronit O. Spotts for performing HPLC analysis of BNP-modified peptides and to Ronit O. Spotts for purifying mutant proteins for some of the experiments.

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BI942574K