Diethyl Pyrocarbonate Reaction with the Lactose Repressor Protein Affects both Inducer and DNA Binding†

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ABSTRACT: Modification of the lactose repressor protein of *Escherichia coli* with diethyl pyrocarbonate (DPC) results in decreased inducer binding as well as operator and nonspecific DNA binding. Spectrophotometric measurements indicated a maximum of three histidines per subunit was modified, and quantitation of lysine residues with trinitrobenzenesulfonate revealed the modification of one lysine residue. The loss of DNA binding, both operator and nonspecific, was correlated with histidine modification; removal of the carbethoxy groups from the histidines by hydroxylamine was accompanied by significant recovery of DNA binding function. The presence of inducing sugars during the DPC reaction had no effect on histidine modification or the loss of DNA binding activity. In contrast, inducer binding was not recovered upon reversal of the histidine modification. However, the presence of inducer during reaction protected lysine from reaction and also prevented the decrease in inducer binding; these results indicate that reaction of the lysine residue(s) may correlate to the loss of sugar binding activity. Since no difference in incorporation of radiolabeled carbethoxy was observed following reaction with diethyl pyrocarbonate in the presence or absence of inducer, the reagent appears to function as a catalyst in the modification of the lysine. The formation of an amide bond between the affected lysine and a nearby carboxylic acid moiety provides a possible mechanism for the activity loss. Reaction of the isolated NH_2-terminal domain resulted in loss of DNA binding with modification of the single histidine at position 29. Results from the modification of core domain paralleled observations with intact repressor.

The lactose repressor protein controls the expression of the lactose metabolizing enzymes in *Escherichia coli* (Miller & Reznikoff, 1980). The repressor protein binds to a control region of the operon (the operator), thereby physically blocking transcription of the coding region. The interaction of the repressor with the operator is altered by the binding of small sugar molecules (inducers) that elicit dissociation of the repressor from the operator to permit initiation of transcription by RNA polymerase. The repressor may be divided into two domains with distinct binding properties by limited digestion with proteolytic enzymes (Adler et al., 1972; Platt et al., 1973). The amino-terminal domain (amino acids 1–59) is associated with DNA binding (Platt et al., 1973; Geisler & Weber, 1977; Jovin et al., 1977; Ogata & Gilbert, 1978, 1979), while the core domain (amino acids 60–360) contains the inducer binding site and possible determinants for operator DNA recognition (Platt et al., 1973; Butler et al., 1977; Matthews, 1979). Genetic evidence supports this correlation of binding activities with the individual domains (Miller, 1979). Chemical modification and genetic data have indicated some of the essential residues involved in DNA binding (Miller, 1979, 1984; Manly & Matthews, 1979; Burgum & Matthews, 1978; Brown & Matthews, 1979; Hsieh & Matthews, 1981). Genetic evidence for residues required for native inducer binding has been presented (Miller, 1979). Although a hypothetical inducer binding site has been suggested on the basis of sequence homologies between *lac* repressor and arabinose binding protein (ABP) (Sams et al., 1984), no direct information concerning the amino acids involved in inducer binding has been reported. Diethyl pyrocarbonate has been widely used as an acetylating agent for amino acids in proteins (Miles, 1977). We have utilized this reagent to investigate the contributions of various amino acids to the binding activities of the repressor protein.

Materials and Methods

Materials. Diethyl pyrocarbonate, isopropyl β-D-thiogalactoside (IPTG), imidazole, and hydroxylamine were purchased from Sigma. [^{14}C]IPTG was obtained from Research Products International. [^{14}C]Ethanol was from ICN or New England Nuclear. All other reagents were of reagent grade or better.

Isolation of Repressor Protein. Lactose repressor was isolated from *Escherichia coli* CSH 46 as previously described (Müller-Hill et al., 1971; Rosenberg et al., 1977; O'Gorman et al., 1980). Cells were grown in 100-L batches and stored frozen before use. The yield of pure repressor was 1–2 mg/g of frozen cells. The purity of the isolated protein (>95%) was assessed by sodium dodecyl sulfate (SDS) gel electrophoresis (Weber et al., 1972). Core protein was purified as previously described (Sams et al., 1985), and NH_2 termini were isolated by the procedure of Arndt et al. (1981). Inducer binding was assayed by the nitrocellulose filter binding or precipitation assays of Bourgeois (1971). Operator binding was measured by nitrocellulose filter binding using [^{3}H]λp*lac* DNA for stoichiometric conditions or a ^{32}P-labeled 40-bp operator fragment (Riggs et al., 1968; Whitson & Matthews, 1986). Repressor binding to nonspecific DNA was determined as described previously (O'Gorman et al., 1980).

Synthesis of [^{14}*C*]*Diethyl Pyrocarbonate.* ^{14}C-Labeled diethyl pyrocarbonate (DPC) was synthesized by an adaptation of the procedure of Melchior and Fahrney (1970). The syn-

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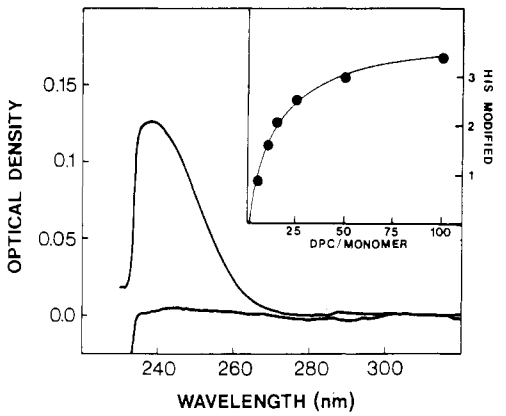

FIGURE 1: Difference spectrum of unmodified and diethyl pyrocarbonate modified *lac* repressor. Repressor (0.5 mg/mL in 0.24 M potassium phosphate, pH 7.5, 5% glycerol) was reacted with a 100-fold molar excess of diethyl pyrocarbonate over monomer as described under Materials and Methods. The base line was measured prior to the addition of reagent and the modified spectrum after 30 min of reaction. The inset depicts the number of modified histidines per monomer vs reagent excess. Repressor was reacted with varying amounts of reagent for 30 min on ice. The number of modified histidines was calculated from the optical density at 240 nm by difference spectroscopy.

thesis was carried out as described except that the amounts of reagents were one-tenth those used in the published protocol.

Reaction of Lac Repressor with Diethyl Pyrocarbonate. DPC was diluted to working concentration with ethanol. The concentration of the dilution was verified by reaction with imidazole and quantitation of the resulting carbethoxyimidazole by absorbance at 230 nm (ϵ = 3200 M^{-1} cm^{-1}). The *lac* repressor (0.5 mg/mL) was reacted on ice with a 10–100-fold excess of DPC (up to ~3 mM reagent) in 0.24 M potassium phosphate, pH 7.5, and 5% glycerol for the times indicated. The buffer was deaerated by bubbling with nitrogen. The reaction was monitored by absorbance at 240 nm (Ovádi et al., 1967), and effects of the reaction were monitored by assays for inducer, nonspecific DNA, and operator DNA binding. The time course of activity loss was determined by removing aliquots of reaction mixtures at specific time intervals, stopping the reaction with an equal volume of 10^{-3} M imidazole, and assaying the resulting mix for binding activity. Imidazole had no effect on the binding activity of the repressor protein. A similar reaction protocol was followed for isolated core and NH_2-terminal domains.

Quantitation of Other Residues. The modification of tyrosine was monitored by the change in absorbance at 278 nm (Burstein et al., 1974; Wells, 1973). Lysine residues were determined by reaction with trinitrobenzenesulfonate (TNBS) as described previously (Whitson et al., 1984). Cysteine residues were quantitated by titration with 2-(chloromercuri)-4-nitrophenol (MNP) by the procedure of Manly and Matthews (1979).

RESULTS

Reaction of Lactose Repressor with Diethyl Pyrocarbonate. Diethyl pyrocarbonate potentially modifies histidine, tyrosine, and lysine residues in proteins (Miles, 1977). Reaction with cysteine residues is theoretically possible, although reports of cysteine modification in proteins have not been widely presented. Difference spectra of the modified vs native *lac* repressor exhibited an absorbance maximum near 240 nm (Figure 1) consistent with the carbethoxylation of histidine residues. By use of an extinction coefficient of 3200 M^{-1} cm^{-1} (Ovádi et al., 1967), approximately three histidine residues per monomer were found to react at a 100-fold molar ratio

Table I: Effects of Diethyl Pyrocarbonate Reaction with *Lac* Repressor

treatment conditions[a]	residues reacted[b]		binding activities[c] (%)	
	His	Lys	IPTG	operator DNA
control	0	0	100	100
DPC	3.4 (±0.2)	1.4 (±0.5)	<10	<5
DPC + IPTG	3.5 (±0.3)	0.3 (±0.3)	80 (±15)	<5
DPC + NH_2OH	0	1.2 (±0.5)	<10	85 (±14)
DPC + IPTG + NH_2OH	0	0.3 (±0.3)	79 (±13)	78 (±12)

[a] *Lac* repressor was modified with a 100-fold molar ratio of DPC over monomer as described under Materials and Methods. Hydroxylamine was used to reverse reaction at histidine residues. [b] Histidine reaction was determined by absorbance at 240 nm and lysine reaction by trinitrobenzenesulfonate modification as described under Materials and Methods. [c] IPTG and DNA binding activities relative to unmodified protein were measured by filter binding as described under Materials and Methods.

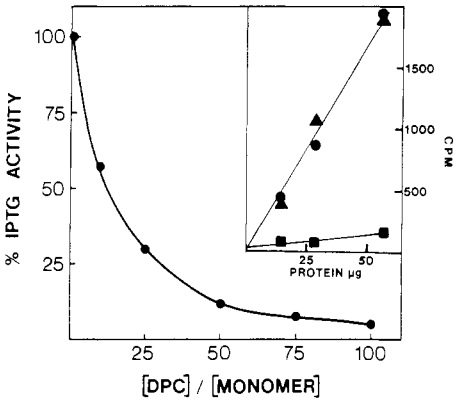

FIGURE 2: DPC-induced loss of inducer binding activity. Repressor (0.5 mg/mL in 0.24 M potassium phosphate, pH 7.5, 5% glycerol) was reacted with various amounts of DPC for 30 min on ice. After reaction, IPTG binding was determined by the filter binding assay of Bourgeois (1971). Results are expressed as percent of activity observed for unmodified repressor. Inset shows IPTG protection of DPC-induced loss of sugar binding: (●) native repressor; (■) repressor modified with a 100-fold excess of DPC; (▲) repressor modified by 100-fold DPC in the presence of 10^{-3} M IPTG.

of DPC over repressor monomer (Figure 1, inset). The absence of decreased absorbance at 280 nm demonstrated that no reaction with the tyrosine residues had occurred. Quantitation of the lysines with trinitrobenzenesulfonate indicated a loss of one lysine residue (Table I). Titration of the native and modified repressors with 2-(chloromercuri)-4-nitrophenol demonstrated the presence of 2.9 cysteine residues, in agreement with the known repressor cysteine content. Therefore, diethyl pyrocarbonate modified only histidine and lysine residues in the *lac* repressor protein.

Effects of DPC Modification on Inducer Binding Function. Treatment of lactose repressor with diethyl pyrocarbonate resulted in decreased inducer binding activity. The maximum loss in activity determined by filter binding assays was observed at a 50–100-fold molar excess of DPC over *lac* monomer concentration (Figure 2). The repressor protein exhibits a shift in fluorescence emission maximum to shorter wavelength upon binding to inducer molecules (Figure 3). This shift allows the determination of inducer binding affinities by fluorescence titration. The modified repressor exhibited an approximately 10-fold decrease in affinity for IPTG compared to that of unmodified protein (Figure 3, inset) as determined by this method. This increase in the K_d accounts for the diminished binding observed in the standard filter binding inducer assay following modification. The inclusion of IPTG

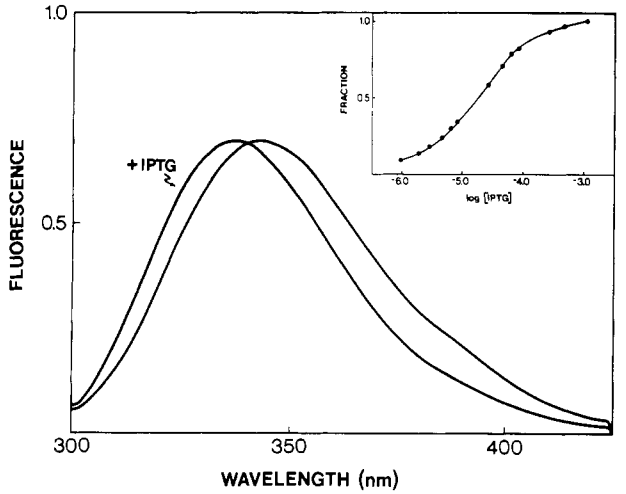

FIGURE 3: Fluorescence spectra of DPC-modified repressor. Repressor protein was modified with a 100-fold molar ratio of diethyl pyrocarbonate over monomer as described under Materials and Methods. Emission spectra were measured before and after addition of IPTG to 10^{-4} M. Excitation wavelength was 285 nm, and emission wavelength was scanned from 300 to 425 nm. The inset depicts the titration of DPC-modified repressor with IPTG. Modified repressor (2×10^{-6} M in 0.05 tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5, 0.5 M NaCl) was titrated with aliquots of IPTG, and the fluorescence was monitored above 350 nm by using a Corning 0-52 cutoff filter as described (Daly et al., 1986). Data are expressed as the fraction of the total fluorescence change vs the final IPTG concentration.

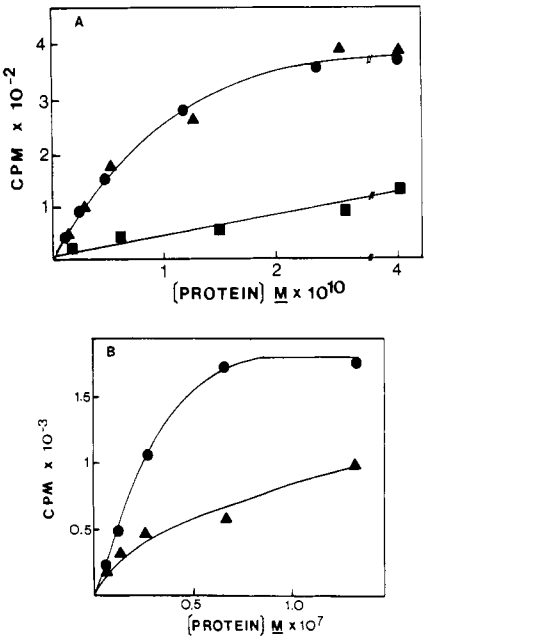

FIGURE 4: DNA binding of DPC-modified repressor. Reactions and assay were performed by using λp*lac* DNA (~3–5 × 10^{-11} M) as described under Materials and Methods. (Panel A) Operator binding curve for native repressor (●), repressor modified with a 25-fold molar ratio of DPC over monomer (■), and modified repressor after treatment with 0.1 M hydroxylamine, pH 7.5 (▲). (Panel B) Non-operator DNA binding activity of native repressor (●) and repressor modified with a 25-fold molar ratio of DPC (▲).

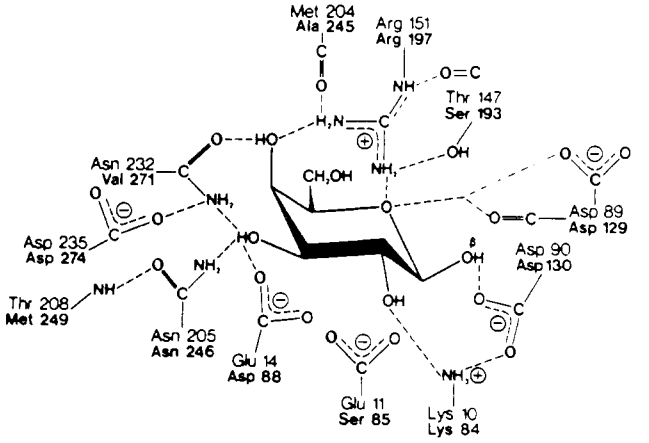

FIGURE 5: Diagram of the sugar binding site of the arabinose binding protein with indicated amino acid substitutions to form a hypothetical *lac* repressor sugar site (Sams et al., 1984). Amino acids of arabinose binding protein are indicated in normal lettering, while the *lac* repressor amino acids are indicated by bold lettering. The site is drawn with the β-anomer of D-galactose present. Note the close association of Lys-84 and Asp-130.

(10^{-3} M) during the modification reaction afforded protection against the loss of sugar binding activity (Table I; Figure 2, inset). In contrast, no protection of inducer binding was afforded by calf thymus DNA in the reaction mix. The extent and rate of modification of the histidine residues were unaltered by the presence of inducer, and removal of the carbethoxy groups from the histidines by treatment with hydroxylamine did not restore activity (Table I). Although the extent of inducer binding loss and histidine modification appeared to parallel the reagent excess, the time course of histidine reaction did not correlate with the loss in inducer activity. By use of a 100-fold molar ratio of DPC over monomer, the observed rate for inducer loss was 1.2×10^{-3} s^{-1} ($t_{1/2}$ = 590 s), while that for histidine reaction was 6.3×10^{-3} s^{-1} ($t_{1/2}$ = 110 s); both time courses were first order. This 5-fold difference in rate indicated that carbethoxylation of histidine was not involved in the loss of inducer binding activity. The modification of one lysine residue was observed concomitant with the decrease in inducer binding activity (Table I). The presence of IPTG during the reaction with DPC prevented the modification of lysine as well as protected repressor from the loss in sugar binding activity (Figure 2, inset; Table I). The inability to recover sugar binding activity upon treatment with hydroxylamine is consistent with the modification of a lysine residue since carbethoxylation of the ε-amino group is not reversible with hydroxylamine.

Effects of DPC Reaction on DNA Binding Activities. Carbethoxylation of repressor by DPC resulted in the loss of operator and nonspecific DNA binding functions (Figure 4). The decrease in DNA binding correlated with the carbethoxylation of three histidines in the repressor protein. The presence of inducer during the DPC reaction had no effect on the loss of DNA binding activity or on the modification of histidine residues. Treatment of the modified repressor with hydroxylamine restored operator DNA binding, indicating that the activity loss was a result of the modification of histidine residues (Figure 4; Table I). The removal of the carbethoxy groups from all histidines was confirmed by the elimination of the 240-nm peak in the ultraviolet difference spectrum.

Quantitation of Incorporation of Carbethoxy Groups. Measurements of the degree of incorporation of carbethoxy groups using ^{14}C-labeled DPC indicated no difference in the presence or absence of IPTG; the ratio between incorporation with inducer to that without inducer present was 1.09 ± 0.14. The extent of labeling was consistent with the modification of three residues. Thus, the modification of lysine observed in the absence of inducer did not involve the addition of a carbethoxy group from the DPC reagent. This initially puzzling result suggested the possibility that the reagent was acting as a catalyst. DPC has been reported to catalyze the formation of an amide bond between a lysine residue and an adjacent carboxylic acid (Wold, 1972; Wolf et al., 1970). The possible close association of a lysine residue and a carboxylic

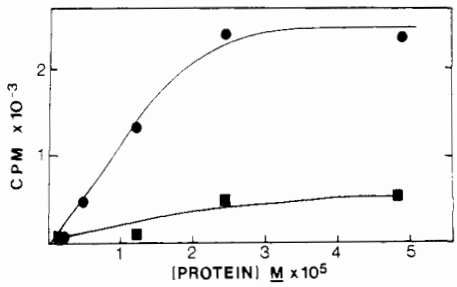

FIGURE 6: DNA binding of DPC-modified NH_2-terminal fragment. NH_2-terminal fragment of *lac* repressor was reacted with a 25-fold molar ratio of DPC in 0.06 M potassium phosphate, pH 7.5, and 5% glycerol. DNA binding activities of NH_2 terminus (●) and DPC-modified amino terminus (■) with ^{32}P-labeled 40-bp operator fragment ($\sim 10^{-11}$ M) are depicted.

acid moiety was previously suggested in a model for the three-dimensional structure of the sugar binding site of the *lac* repressor reported by our laboratory (Figure 5). Lysine 84 is quite close to aspartic acid 130 in the model presented. It is conceivable that these residues become cross-linked through amide bond formation catalyzed by DPC. Since these groups are located within the sugar binding cleft of the repressor, a cross-link of this type would presumably diminish inducer binding.

DPC Reaction of Amino Terminus and Core Protein. Limited tryptic digestion of native repressor protein yields two major products, an amino-terminal fragment (amino acids 1–59) and a tetrameric core protein (amino acids 60–360). The amino-terminal region has been implicated in DNA binding (Platt et al., 1973; Geisler & Weber, 1977; Jovin et al., 1977; Ogata & Gilbert, 1978, 1979), and the core protein contains the inducer binding region as well as some elements required for recognition of operator DNA sequences (Platt et al., 1973; Butler et al., 1977; Matthews, 1979). Treatment of isolated amino terminus with DPC resulted in complete modification of the single histidine residue. No other amino acids were modified by the reagent. Thus, carbethoxylation of histidine 29 yielded total loss of DNA binding by the modified amino termini (Figure 6), and hydroxylamine treatment removed the carbethoxy group and restored full binding activity. DPC reaction of core protein produced modification of the expected two (1.9) histidine residues and one (0.9) lysine per core monomer. As anticipated on the basis of reaction with intact repressor, inclusion of IPTG in the reaction mix provided protection of the inducer binding and prevented the modification of the lysine residue. Modification of histidines was not affected by inducer presence during reaction, and removal of the carbethoxy groups from the modified core by hydroxylamine did not restore inducer binding activity.

Discussion

Diethyl pyrocarbonate has been typically used as a relatively specific reagent for the modification of histidine residues in proteins. While histidines are readily carbethoxylated by DPC, several other nucleophilic amino acids can be modified as well. Reaction of *lac* repressor with DPC resulted in the modification of approximately three histidines and one lysine per monomer. No modification of cysteine residues was observed following DPC reaction; MNP titration indicated three cysteines were present whether or not the repressor had been reacted with DPC. The absence of alterations in either the absorbance at 280 nm or the fluorescence emission spectrum indicated tyrosine and tryptophan residues were unaffected by exposure to DPC. The presence of inducer prevented the modification of the lysine residue and protected the repressor from the loss of inducer binding function. The time course of histidine modification did not parallel the decrease in affinity for inducing sugars, and the presence of IPTG had no effect on the rate or extent of modification of histidine residues. Since IPTG protects the repressor from the loss in inducer binding activity, the histidine residues must not be directly involved in inducer binding.

Extensive genetic studies have implicated amino acid residues between 90 and 273 as involved in inducer binding activity (Miller, 1979). No direct chemical evidence for the presence of any specific amino acids in the sugar binding site has been presented. Recently, we have published a hypothetical inducer site (Sams et al., 1984) based on sequence homologies between *lac* repressor and arabinose binding protein (Müller-Hill, 1984). Insertion of the homologous amino acids of repressor into the known three-dimensional structure of ABP was used to generate the site (Figure 5) and, where multiple amino acid substitutions were possible, genetic data were used to establish the most likely residues. One feature of the proposed site is the presence of a lysine residue (Lys-84) adjacent to an aspartic acid residue (Asp-130). Amide bond formation between these two amino acids catalyzed by DPC would result in a modified repressor with reduced affinity for sugar ligands. The formation of amide bonds catalyzed by DPC has been reported in the literature (Wold, 1972; Wolf et al., 1970).

The presence of a cross-link as opposed to the direct modification of the lysine residue was suggested by several lines of evidence. First, the inclusion of IPTG in the reaction mix prevented the decrease in sugar binding activity, although the incorporation of labeled DPC into repressor was unaltered. Since lysine was protected from reaction by the presence of inducer while the histidine reaction remained constant, a decrease in DPC incorporation in the presence of IPTG would be expected. The absence of this decrease indicates reaction of the lysine residue did not involve the insertion of a carbethoxy group from the labeled DPC. If the reagent functioned as a catalyst, no label would be incorporated but the lysine would still be unavailable for reaction with TNBS in the quantitation procedure. Additionally, cross-linking Lys and Asp in the proposed inducer site would not be expected to eliminate binding, since the formation of such an amide bond between closely spaced residues would not completely disrupt the site. Conversely, the presence of a carbethoxy group on a lysine in the binding site would introduce severe steric hindrance and would presumably elicit more significant loss of inducer binding. We observed a decrease of $\sim$10-fold in IPTG binding affinity upon DPC reaction, a value that appears to be inconsistent with the introduction of a carbethoxy group into the proposed sugar binding site. The observed decrease in inducer affinity correlates to a loss in binding energy ($\delta\Delta G$) of approximately 1.4 kcal/mol. This decrease can be ascribed to the loss of hydrogen bonding between the affected residues and the binding ligand. Although loss of polar interactions is also a possibility, the absence of salt effects on inducer binding at neutral pH argues against this explanation (Friedman et al., 1977).

In contrast to inducer binding, the decrease in DNA binding correlated with histidine modification, and hydroxylamine treatment significantly restored the capacity of the modified protein to bind to operator and nonspecific DNA. The presence of inducer during reaction did not alter the extent of DNA activity loss. The role of histidine 29 in the DNA binding loss can be inferred from the effects of modification

on the isolated NH_2 terminus. This domain of the repressor contains a single histidine, which is completely modified by reaction with DPC; the activity loss accompanying modification is reversed by hydroxylamine treatment similar to that of the intact protein. Müller-Hill (1975) has reported that mutation of His-29 to Tyr yields an i^{-d} phenotype indicative of operator binding loss. Furthermore, Boelens et al. (1987) have concluded, on the basis of NMR measurements of the NH_2-terminal domain complexed with operator fragments, that His-29 is close to the ribose proton of an adenine in the operator sequence and makes an ionic contact with the phosphate backbone. Carbethoxylation of this residue would introduce considerable steric hindrance and alters the ionization properties of the imidazole ring. The data from diethyl pyrocarbonate modification of the protein are consistent with His-29 participation in DNA binding function in the repressor, as indicated by the genetic and NMR data and as postulated on the basis of homology to other DNA binding proteins (Matthews et al., 1982).

In summary, modification of the *lac* repressor protein with diethyl pyrocarbonate results in the loss of both inducer and DNA binding activities. The reagent carbethoxylates three histidine residues and may catalyze the formation of an amide bond between a lysine and an adjacent carboxylic acid group in the inducer binding site of the repressor. The amide bond formation is responsible for the reduction in inducer binding affinity, while the modification of histidine 29 appears primarily responsible for the decrease in DNA binding. The reaction of the histidine residues has no apparent effect on the binding of sugar molecules. These data provide the first chemical evidence for the involvement of specific amino acid residues in the inducer binding site of the *lac* repressor protein and supply supporting evidence for the involvement of His-29 in DNA binding function.

ACKNOWLEDGMENTS

The initial experiments of Francis Kwok in this laboratory suggested that reaction of repressor with diethyl pyrocarbonate was worthy of detailed investigation. Technical support and scientific discussion with Peggy Whitson are also gratefully acknowledged.

Registry No. IPTG, 367-93-1; DPC, 1609-47-8; His, 71-00-1; Lys, 56-87-1.

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