Kinetic Studies of Inducer Binding to Lactose Repressor Protein

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The kinetics of binding of the inducer, isopropyl-β-D-thiogalactoside to lactose repressor from Escherichia coli was studied by stopped flow rapid mixing techniques. Three different spectral probes for measuring changes in the conformation of the repressor were used: ultraviolet absorbance, fluorescence, and a reporter group, 2-mercuri-4-nitrophenol, in the visible region. Repressor can be reacted with this mercurial to modify two of the three free sulfhydryl groups per monomer without loss of inducer or operator binding activities. The observed first order rate constant for the reaction of repressor with 2-chloromercuri-4-nitrophenol at pH 7.5 and 20°C was found to be on the order of 0.1 s⁻¹, an unexpectedly slow rate for this type of reaction. Once bound to repressor, the nitrophenol serves as a chromophoric probe to monitor changes in the surrounding environment. The binding of inducer to repressor causes a change in the absorbance of the bound 2-mercuri-4-nitrophenol moiet and exhibits a second order rate of 3.2 × 10⁴ M⁻¹ s⁻¹. Similar rates are obtained when binding of inducer is monitored by changes in either the ultraviolet absorbance or fluorescence of tryptophan residues. Since the same rate of spectral perturbation is observed for different regions of the primary structure of the protein, the conformational change produced in response to inducer binding appears to be translated rapidly throughout the protein molecule.

Isolation of lactose repressor from Escherichia coli in quantities sufficient for physical studies makes possible an examination of the specific protein-sugar interactions which are responsible for the induction process (1). When a sugar which functions as an inducer is bound, the affinity of the repressor protein for operator DNA is greatly reduced (2), presumably due to a conformational isomerization of the protein molecule. The difference spectrum produced by inducer binding to repressor exhibits a characteristic decrease in absorbance at 289 nm (3), apparently as a result of alterations in the environment of at least one tryptophan residue, tryptophan-209. A shift in the λmax of the fluorescence emission spectrum has also been observed (4) which results from changes in the environment of tryptophan-209. The ultraviolet difference spectrum and altered fluorescence emission spectrum are not observed when sugars which function as anti-inducers (i.e. stabilize the repressor-operator interaction) are bound to repressor (3, 4). Thus, examination of the spectral properties of the tryptophan residues provides a probe for studying the kinetics of inducer-repressor interaction. Laiken et al. (4) observed a small, slow, apparently first order change in tryptophan fluorescence at 360 nm which followed a much longer, rapid second order change. These authors postulated that the slow first order process represents the physiologically important isomerization of the repressor molecule to a conformation which no longer exhibits a great affinity for operator DNA. The rapid second order change was correlated to IPTG binding.

Genetic evidence suggests that the tryptophan affected by inducer binding (tryptophan-209) is near the inducer/anti-inducer-binding site (5, 6), but spectral and chemical evidence indicates that it does not participate directly in binding to inducer (3). A second chromophoric probe at a location distinct from the tryptophan-209 region provides a useful method for corroborating the results observed using ultraviolet absorbance and fluorescence and a means for viewing structural changes at several points in the sequence of the repressor. 2-Chloromercuri-4-nitrophenol can be used to modify cysteine 107 and 268 without affecting either inducer- or operator-binding activities; cysteine-140 remains unreactive even in the presence of excess reagents. It is possible to modify differentially the two cysteines; that is, with one equivalent of reagent, only cysteine-268 is affected. This indicates that the two modifiable sulfhydryl groups exhibit different reactivities. Furthermore, variations are observed between the mercurinitrophenol difference spectrum produced by inducer binding to repressor modified at cysteine-268 and repressor modified at both cysteine-268 and cysteine-107. The bound mercurial, therefore, provides an experimental handle for examination of changes in the conformation of the molecule, and the results obtained can be correlated with similar measurements following tryptophan ultraviolet absorbance and fluorescence changes.

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‡ H. Sommer, P. Lu, and J. H. Miller, personal communication.

The abbreviations used are: IPTG, isopropyl-β-D-thiogalactoside; ONPF, o-nitrophenyl-β-D-fucoside.
† Yang, D. S., and Matthews, K. S. (1976) J. Mol. Biol., in press.
‡ C. F. Sams, B. E. Friedman, D. S. Yang, and K. S. Matthews, manuscript submitted for publication.
Materials and Methods

Lactose repressor was isolated according to the procedure of Müller-Hill et al. (7) modified by Platt et al. (8) from frozen cells of Escherichia coli M36. The purified repressor was frozen immediately after the phosphocellulose column in 0.2 M potassium phosphate, pH 7.6, or in 1.0 M Tris-Cl buffer, pH 8.1 (20°), 10^{-4} M dithiothreitol, and stored at a concentration between 1 and 2.5 mg/ml. In preparation for all experiments, the protein was concentrated by an ammonium sulfate precipitation and dialyzed into the appropriate buffer. Prior to reaction with 2-chloromercuri-4-nitrophenol, the protein was dialyzed into buffer of the appropriate pH without dithiothreitol and under a nitrogen atmosphere to prevent oxidation of the free sulfhydryls. Dialysis to experimental concentrations was performed after the time of use. Assays of IPTG binding activity were according to Bourgeois (9) using the ammonium sulfate precipitation method. IPTG binding activity was checked immediately prior to each experiment. IPTG and ONPF were obtained from Sigma.

A Gibson-Durrum stopped flow spectrometer equipped with a 1.6-cm path length fluorescence cuvette or a 2.0-cm path length absorbance cuvette was employed to carry out the stopped flow measurements. The absorbance, fluorescence, or transmittance changes were displayed on an oscilloscope and analyzed photographically by enlarging 35-mm negatives of the various time courses.

Results

Mercural Binding to Repressor—The binding of 2-chloromercuri-4-nitrophenol to lactose repressor was examined to characterize the reaction prior to use as a kinetic chromophoric reporter group. The decrease in absorbance of the chromophore was followed at 405 nm, where 2-mercuri-4-nitrophenol bound to repressor exhibits half-maximal absorbance at pH 8.3 (approximate pH). Free 2-chloromercuri-4-nitrophenol in solution has a pH of 6.75. Therefore, at pH 7.5, the absorbance changes associated with the decreased ionization produced by binding to repressor were easily measured. Various concentrations of the thiol reagent were reacted with a fixed concentration of reporter group. The decrease in ionization produced by binding was followed at 405 nm, where 2-mercuri-4-nitrophenol bound to repressor exhibits half-maximal absorbance at pH 8.3 (approximate pK). Free 2-chloromercuri-4-nitrophenol in solution has a pH of 6.75. Therefore, at pH 7.5, the absorbance changes associated with the decreased ionization produced by binding to repressor were easily measured. Various concentrations of the thiol reagent were reacted with a fixed concentration of repressor, 1.64 x 10^{-4} M tetramer before mixing, in 1 M Tris-Cl buffer adjusted to pH 7.5. The data were analyzed as the log of absorbance change versus time in order to determine the apparent rate of the reaction. As shown in Fig. 1A, the reaction of 2-mercuri-4-nitrophenol with repressor is purely first order, slow (0.098 s^{-1}), and relatively independent of the 2-mercuri-4-nitrophenol concentration over a 20-fold range (0.5 to 10.0 eq/monomer). Similar first order behavior was also obtained when the reaction was conducted at elevated concentrations of repressor, 8.2 x 10^{-6} M, with 1 or 2 eq of 2-mercuri-4-nitrophenol. The reaction of 2-mercuri-4-nitrophenol with repressor containing bound effector molecules was also observed at various 2-mercuri-4-nitrophenol concentrations, ranging from 0.5 to 5.0 eq. The inducer used was isopropyl-β-d-thiogalactoside and the anti-inducer was α-nitrophenyl-β-d-thioside. The results presented in Fig. 1B show that the presence of either inducer or anti-inducer has little influence on either the first order behavior or absolute rate of the mercurinitrophenol reaction. The rate constant for the binding of 2-chloromercuri-4-nitrophenol to repressor in 0.17 M potassium phosphate buffer at the same pH (0.107 s^{-1}) was similar to that measured in Tris-Cl buffer, whereas the reaction appears to be slightly faster when conducted at pH 9.25 in 0.1 M glycine-NaOH buffer/0.2 M NaCl (0.154 s^{-1}). The decrease in absorbance is significantly less at higher pH, making the measurements more difficult.

Binding of Inducers to the Lactose Repressor—Interaction of inducer molecules with the repressor protein results in conformational changes which alter the environment of tryptophan-209 and the 2-mercuri-4-nitrophenol moieties on cysteine-268 and -107 (3). The time course of these environmental changes can be followed by observing the spectral changes which accompany them. Using the bound mercurial as a reporter group, the interaction of the repressor with an inducer was examined by following spectral changes in the visible region. It was necessary to carry out these measurements at pH values above 9 in order to maximize the spectral perturbations. Protein at a concentration of 9.7 x 10^{-4} M in 0.1 M glycine-NaOH, 0.2 M NaCl buffer solution, pH 9.25, was first mixed with an appropriate concentration of 2-chloromercuri-4-nitrophenol; this solution was subsequently mixed with a solution of isopropyl-β-d-thiogalactoside in the stopped flow spectrometer. The changes in absorbance at 405 nm were then recorded on an oscilloscope and photographed (Fig. 2A). A second order rate equal to 3.2 x 10^{-5} M^{-1} s^{-1} was determined from a plot of the observed pseudo-first order rate constant versus inducer concentration (Fig. 3). It appears that the observed rate is directly dependent on IPTG concentration, since no limiting rate is approached at this pH, even at the upper limits of inducer concentration. In addition, no significant buffer effects were observed for the rate of the spectral changes.

Examination of the rate of change of tryptophan spectral properties gave results similar to those obtained using 2-mercuri-4-nitrophenol-repressor. The repressor at a concentration of 1.25 x 10^{-5} M in 1 M Tris-Cl, pH 9.25/10^{-4} M dithiothreitol, was mixed with various concentrations of IPTG, and the decrease in absorbance at 289 nm (shown as an increase in transmittance in Fig. 2B) was recorded. The small magnitude of the absorbance change necessitated the use of protein solutions of relatively high concentrations and with correspondingly large background absorbances. It was possible to make measurements using these concentrations, but the noise level was significantly large.

To examine the changes in tryptophan fluorescence, protein in 0.1 M glycine-NaOH buffer/0.2 M NaCl/10^{-4} M dithiothreitol, pH 9.25, was diluted to a concentration of 6.13 x 10^{-7} M and mixed in the stopped flow spectrometer with IPTG solutions of varying concentration. The buffer was identical with that used for the 2-mercuri-4-nitrophenol-repressor measurements. An excitation wavelength of 290 nm was used, and all fluorescent emission at wavelengths longer than 350 nm was re...
corded. The spectral band width was 3 nm and a time constant of 0.5 ms was used. The volts fully reacted were 0.35. C, repressor (6.13 x 10^{-5} M) in 0.1 M glycine-NaOH/0.2 M NaCl, pH 9.25. Excitation was at 290 nm using a 75-watt xenon lamp and a band width of 6 nm. Emission at wavelengths longer than 350 nm was recorded using an appropriate glass cut-off filter. A time constant of 0.1 ms was used.

recorded (Fig. 2C). A plot of the observed first order rate constants versus IPTG concentration gave a second order rate equivalent to that obtained with 2-mercuri-4-nitrophenol-repressor. Again, no limiting rate for the spectral change was observed at this pH (Fig. 3). When fluorescence changes were measured using protein and sugar in 0.17 M potassium phosphate/10^{-4} M dithiothreitol at pH 7.5, the rate of spectral change associated with inducer binding was increased to ~1.2 x 10^{-5} m^{-1} s^{-1}. This increase correlates well with the observed decrease in the dissociation constant for inducer binding. The $K_d$ for IPTG decreases more than 1 order of magnitude between pH 9 and pH 7.5; this increase in affinity of repressor for inducer appears to be due primarily to an increase in the second order association rate of inducer binding. Similar increases in rate of spectral change were obtained when measurements were made using ultraviolet absorbance at neutral pH. 2-Mercuri-4-nitrophenol cannot be used as a reporter group at pH values below 8.5, since the magnitude of the nitrophenol absorbance change decreases markedly in going from pH 9.0 to pH 7.5.

**DISCUSSION**

The lactose repressor protein functions by responding to signals in the environment which elicit its dissociation from the operator DNA so that mRNA corresponding to the lac enzymes can be synthesized (1). This dissociation process is apparently mediated by a conformational isomerization in the protein in response to binding sugar molecules (3, 4). The rate of the sugar binding process has been measured by three different methods and found to be similar in all three cases. The first method utilized the reporter group, 2-mercuri-4-nitrophenol. The preliminary experiments on the rate of 2-chloromercuri-4-nitrophenol reaction with repressor provided some surprising observations. The reaction exhibits first order behavior, in apparent conflict with conclusions drawn from the results of static experiments, i.e. that the two sulfhydryl groups affected can be differentially modified with this reagent. However, of particular significance in providing an explanation for the first order nature of the reaction is the fact that the observed rate is unusually slow ($t_m \approx 5$ s) in comparison with rates that have been obtained when this mercurial is reacted with sulfhydryl groups which are readily accessible to solute molecules ($t_m \approx 5$ ms) (10). Furthermore, the sulfhydryl groups in untreated repressor are not reactive with either iodoacetate or iodoacetamide, and are unresponsive to dye-catalyzed oxidation, and the 2-mercuri-4-nitrophenol groups in modified repressor are not perturbed by glycerol. Based on these observations, a probable explanation for the first order nature of the 2-mercuri-4-nitrophenol absorbance change involves the assumption that the rate of the reaction is determined by a slow, rate-limiting step, perhaps involving diffusion of the mercurial through the protein or the slow formation of a particular conformation in the immediate environment of the sulfhydryl groups which is required for the covalent reaction.

It has been concluded that the location of the reactive cysteine residues is distinct from the binding site of the sugar molecule, because (a) binding of inducer or anti-inducer to the repressor neither blocks the reaction of the free sulfhydryl with the mercurial nor alters the rate of this reaction; and (b) bound 2-mercuri-4-nitrophenol elicits no effects on either DNA- or IPTG-binding activities. This conclusion is supported by genetic mapping studies on the
inducer binding region (5, 6), which indicate that this binding site covers residues approximately 200 to 250, whereas 
2-mercuri-4-nitrophenol binds to cysteine-107 and cysteine-268. However, any changes in the overall conformation of the protein may generate alterations of the local environments of the 2-mercuri-4-nitrophenol sulphydryl groups, affecting the ionization state as well as absorbance properties of the reporter group. These types of 2-mercuri-4-nitrophenol spectral changes are produced in response to inducer binding, and their rates have been measured and found to be identical with those measured by tryptophan spectral changes.

The rates of the ultraviolet and fluorescence changes produced by inducer binding are identical, as was expected, since the same tryptophan residue (209) appears to be involved. As a result of fluorescence-quenching experiments, Sommer et al. concluded that tryptophan-209 has only limited access to solvent, and although its environment is affected by inducer binding, it does not come in contact with IPTG directly, despite its location in the region implicated genetically to be the IPTG-binding site (5, 6). Furthermore, the absence of effects of anti-inducer on the spectral characteristics of both the modified and unmodified repressor indicates that both the cysteine and tryptophan residues do not occupy sites in the inducer binding region (3, 4). The similarity of the tryptophan-209 and 2-mercuri-4-nitrophenol rates indicates that the structural transition which takes place in the repressor molecule in response to inducer binding is reflected simultaneously in at least three regions of the primary structure, tryptophan-209, cysteine-107, and cysteine-268.

Laiken et al. observed a slow, apparently first order change in tryptophan fluorescence which followed a rapid second order phase attributed to binding of IPTG. A slow phase was not observed in these studies either at pH 7.5 or pH 9.25. The similarity in the rates of inducer binding determined by the three spectral changes shown in Fig. 2 implies that there is a substantial conformational change which is translated rapidly throughout the protein molecule as soon as inducer is bound. This result implies that inducer binding produces a rapid, long range conformational change in the repressor molecule, which could be of sufficient magnitude to affect operator binding.

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