Spectral Studies of Lactose Repressor Protein Modified with Nitrophenol Reporter Groups*

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The spectral characteristics of lactose repressor protein modified with two nitrophenol reporter groups have been determined. Reaction of 2-chloromercuri-4-nitrophenol with repressor increases the apparent pK of the nitrophenol from 6.75 to 8.3 and alters the wavelength of maximum absorption. Subsequent binding of inducer shifts the apparent pK toward slightly more acid pH. The pK values of the nitrophenol groups introduced into the repressor by reaction with 2-bromoacetamido-4-nitrophenol could not be determined, since they are below pH 7.0 where repressor is unstable. Titration of the nitrophenol chromophores indicates that the protein structure may undergo transitions as a function of pH.

The binding of inducer to the repressor protein results in conformational changes which are reflected in spectral alterations for the nitrophenol groups. The mercurinitrophensols undergo a shift in the wavelength of maximum absorption to shorter wavelength, interpreted as reflecting an environmental alteration which results in more polar surroundings. Conversely, the nitrophenol groups introduced by the alkylating agent have a shift of wavelength of maximum absorption to longer wavelength, apparently experiencing a less polar environment. The positions of the nitrophenol groups in the primary structure of the protein and these spectral observations are correlated. Anti-inducers and a neutral compound also bind to the repressor protein, eliciting similar spectral changes; these alterations are different from those observed for inducers, as predicted from the different effects of these ligands on the function of the protein. The rates of the spectral transitions in response to inducers have been measured and are found to be second order. The rates are similar to those observed previously for other chromophores/fluorophores and apparently reflect a concerted, rapid change in the structure of the protein on binding to inducers.

The lactose repressor protein regulates the synthesis of the enzymes for lactose metabolism in Escherichia coli (1) and has been isolated in amounts sufficient for chemical and physical studies (2, 3). Inducers destabilize the interaction of the repressor protein with operator DNA and elicit removal of the repressor required to initiate mRNA synthesis (4, 5). Anti-inducers compete with inducers for binding to repressor and when bound result in stabilization of the repressor operator complex (6). Previous spectral studies have demonstrated that the repressor protein undergoes a conformational change in response to binding inducers but not to binding anti-inducers (7, 8). This change in the structure of the protein with inducers is presumably responsible for the alteration in affinity for operator DNA.

McMurray and Trentham (9) recently reported a new class of chromophoric organomercurials and demonstrated the usefulness of one of these, 2-chloromercuri-4-nitrophenol, as a sulfhydryl reagent, as a probe for protein structure, and as a potential heavy atom isomorphous replacement. This mercu-}

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Spectral Studies of Chromophore-labeled Repressor

Materials and Methods

Repressor

Repressor was isolated from Escherichia coli M96 (obtained from David Jackson, Stanford University) by the method of Müller-Hill et al. (13) as modified by Platt et al. (14). Repressor in 1.0 M Tris/Cl pH as indicated, was reacted with 2-chloromercuri-4-nitrophenol (Eastman, recrystallized, m.p. 237 to 238°C, in 0.02 M NaOH). The amount of mercurial used was either 1 or 2 eq per repressor monomer. 3 Bromoaacetamido-4-nitrophenol (Sigma, 0.04 m in methanol, 100 μl/ml of protein solution, −200-fold total molar excess, either 1 addition or at least 2 (in mixed experiments) was reacted with repressor at pH 9.0 or 0.1 M Tris/Cl, 1.0 M NaCl. The excess reagent was removed by passage through a Sephadex G-25 column containing buffer at the desired pH. IPTG was added prior to gel filtration to all samples to ensure that differences observed between repressor reacted with and without inducer present were not due to residual amounts of sugar. Repressor concentrations range from 0.2 to 0.7 mg/ml. Inducer binding activity was determined by the ammonium sulfate precipitation assay (15).

Measurements of Absorption Spectra

Mercurial – Repressor (4 to 8 mg/ml in 1.0 M Tris/Cl, pH 7.5) was diluted into 1.0 M Tris/Cl adjusted to various pH values; 2-chloromercuri-4-nitrophenol (1.0 mM in 0.02 M NaOH) was added in amounts corresponding to either 1 or 2 eq per monomer. The visible spectrum of the labeled repressor was determined using 3-mm cuvets (1 cm pathlength). To this protein solution, 1 to 5 μl of buffer solution containing 0.1 M IPTG was added. The spectrum was again recorded. Addition of 5 to 10 μl of water or buffer solution did not measurably affect the spectrum. Measurements of the pH of the solution were made after the spectral measurements using a Radiometer pHM26c pH meter. Duplicate spectral measurements were carried out for each determination. The absorbance values were determined independently for each measurement and averaged for plotting.

Alkylating Agent – Repressor treated with 2-bromoacetamido-4-nitrophenol was isolated as described above and dialyzed in 1.0 M Tris/Cl, 1.0 M NaCl, 3 × 10⁻⁵ M dithiobetrol, pH 7.5; this solution was diluted 1:1 with 1.0 M Tris/Cl buffer of varying pH. The absorbance for 2-bromoacetamido-4-nitrophenol per repressor monomer was approximately twice that observed for 1 eq. The point at which half-maximal absorbance is reached is pH 8.15 for induced repressor reacted with 2 eq of 2-chloromercuri-4-nitrophenol per repressor monomer is approximately twice that observed for 1 eq. The point at which half-maximal absorbance is reached is pH 8.3 for 1 eq and pH 8.35 for 2 eq of mercurial. Since the acetamidonitrophenol repressors modified with and without inducer present have pK values <7.0 and repressor is unstable at pH values below this region, it is not possible to determine the exact point of 50% absorbance. Although the pH value corresponding to 50% maximum absorbance may be used to determine the apparent pK for a chromophore, any changes in protein structure which alter the absorption properties (λmax, molar absorptivity) of the chromophore render a precise determination impossible. In this case, since the λmax shifts for the nitrophenols (see below), an exact pK determination cannot be made.

Effects of Inducers on Absorption Spectra – Addition of IPTG to the mercurial labeled repressor resulted in a shift in the wavelength of maximum absorption of the spectrum to shorter wavelengths and in a slight increase in the intensity of absorption (Fig. 3). A smaller increase is observed in acetamidonitrophenol-labeled protein, and the accompanying shift is to longer wavelength (Fig. 3). The visible spectra for modified repressors in the presence of inducer were determined at various pH values. The pattern was similar to that observed for modified repressor with no ligand present. Half-maximal absorbance is reached at pH 8.15 for induced repressor reacted with 1 eq of mercurial and at pH 8.3 for 2 eq. The values for acetamidonitrophenol repressors are again pH < 7.0 and therefore cannot be accurately determined.

λmax as Function of pH – The shift in wavelength of maximum absorption as a function of pH is observed for the nitrophenol repressors both in the presence and absence of inducer (Fig. 4). For the mercurial, near pH 12 the λmax approaches 410 nm, the absorption maximum for free 2-chloromercuri-4-nitrophenol in the Tris/Cl buffer system; this shift apparently corresponds to unfolding of the repressor structure to expose the nitrophenol groups to the solvent. In the presence of IPTG, the λmax for the mercurial-labeled protein is shifted ~4 nm toward shorter wavelength for both 1 and 2 eq. of mercurial; at
FIG. 1. Absorption spectra of nitrophenol-labeled repressors. A, repressor (0.25 mg/ml) modified with 1 eq of 2-chloromercuri-4-nitrophenol. B, repressor (0.5 mg/ml) modified with 2-bromoacetamido-4-nitrophenol. C, repressor (0.25 mg/ml) modified with 2 eq of 2-chloromercuri-4-nitrophenol. D, repressor (0.5 mg/ml) modified in the presence of inducer (IPTG) with 2-bromoacetamido-4-nitrophenol. The spectra were measured at the pH values indicated on the curve. The mercurial-labeled repressors were measured in 1.0 M Tris/Cl, and the alkylated repressors in 0.1 M Tris/Cl, 1.0 M NaCl.

Fig. 2. Absorbance as a function of pH. A, repressor (0.25 mg/ml) modified with 2-chloromercuri-4-nitrophenol in 1.0 M Tris/Cl at the pH values indicated; absorbance was measured at 410 nm. •—•, 2 eq; ○—○, 1 eq. B, repressor (0.5 mg/ml) modified with 2-bromoacetamido-4-nitrophenol in 0.1 M Tris/Cl, 1.0 M NaCl at the pH values indicated. ○—○, modified in the presence of inducer, absorbance measured at 425 nm; •—•, modified with no ligand present, absorbance measured at 420 nm.

Fig. 3. Absorbance spectra for modified repressors with/without inducer. ---, modified repressors; -- --, modified repressors plus IPTG (10^-3 M). A, repressor (0.25 mg/ml) modified with 2 eq of 2-chloromercuri-4-nitrophenol in 1.0 M Tris/Cl, pH 9.4. B, repressor (0.5 mg/ml) modified with 2-bromoacetamido-4-nitrophenol with no ligand present in 0.1 M Tris/Cl, 1.0 M NaCl, pH 7.5.

PH 12 the \( \lambda_{\text{max}} \) is identical with free mercurial. The shift in \( \lambda_{\text{max}} \) is 50% complete at pH ~ 8.8, apparently reflecting a change in the repressor molecule at this pH resulting in alterations of the environment of mercurinitrophenol labeled...
residues. The transition occurs both for free repressor and repressor bound to inducer molecules. Studies in this laboratory have indicated a change in $K_r$ for inducer molecules occurring near pH 8.5. Similar shifts toward longer wavelength are observed in alkylated repressor as the pH is increased. Although the midpoint of the change cannot be accurately determined, it appears to be near pH 7.0. Wu et al. (16) showed that potentiometric titration of repressor indicated ionization of a group(s) between pH 7.0 to 7.5. These observations point to possible structural changes in the repressor at pH 7.0 to 7.5 and near 8.5, resulting from ionization or neutralization of a charged residue(s) in the protein structure. The repressor at pH 9.0 showed a decreased spectrum at pH 9.0, despite the greater amount of nitrophenol. If the nitrophenol moiety at cysteine 107 displays a spectrum similar to that of the mercurinitrophenol at that position (that is, the trough at 430 to 440 nm and peak at 390 to 400 nm), the intensity of the measured difference spectrum would be reduced by the combined spectra of the nitrophenols at cysteines 107 and 104. At pH 7.5 where the nitrophenol at cysteine 107 should be un-ionized by analogy with the mercurial, the difference spectrum of repressor is both decreased and altered in shape compared to alkylated repressor. The difference spectra of the mercurial and alkylated repressor were carried out in different buffer systems, since the reactions were carried out in these buffers. Measurement of the mercurial difference spectrum in the 0.1 M Tris, 1.0 M NaCl buffer system at pH 9.0 gave the spectrum shown in Fig. 6A. The maximum and minimum are at identical wavelengths to the 0.5 M Tris/Cl buffer, but the intensities are different. Using this spectrum and the pH 9.0 difference spectrum for alkylated repressor (Fig. 6A), a projected difference spectrum for the repressor alkylated in the presence of inducer can be calculated since the reactions were carried out in different buffer systems, since the reactions were carried out in these buffers.
tions observed result from conformational changes in the protein structure which may reflect the changes responsible for the induction process. The environmental shift experienced by the mercurinitrophenol cysteine residues (107 and 268) appears to be toward more polar conditions, since the $\lambda_{\text{max}}$ is shifted to shorter wavelength (blue shift), while the acetamidonitrophenol cysteine residue (140) experiences less polar surroundings (red shift) (18).

**Difference Spectra with Anti-inducers**—The anti-inducer o-nitrophenyl-β-D-fucoside produced spectral changes on binding to mercurial-labeled repressor (Fig. 7). The decrease in absorbance at the $\lambda_{\text{max}}$ indicates a decrease in the extent of ionization or a decrease in molar absorptivity. Difference spectra for the alkylated protein in response to anti-inducers were also measured (Fig. 7). The difference spectra for both types of alkylated repressor indicate a decrease in absorbance and a shift in the $\lambda_{\text{max}}$, since the trough does not correspond to the $\lambda_{\text{max}}$.

**Difference Spectra with Neutral Compound**—The neutral compound o-nitrophenyl-β-D-galactoside, which binds repressor but does not function as an inducer or an anti-inducer, produces a difference spectrum for the nitrophenols which is similar to that observed for the anti-inducer o-nitrophenyl-β-D-fucoside (Fig. 7). Apparently these two types of compounds affect the reporter groups similarly despite their different effects on the function of the protein. Although genetic information indicates that inducer and anti-inducer (and presumably neutral compounds) bind at the same site on the protein (19), these molecules differ in the effects of their binding on the protein structure.

**Rates of Spectral Changes**—The kinetics of the mercurinitrophenol spectral change in response to inducer have been measured. IPTG (5 x $10^{-4}$ M before mixing) and repressor (0.9 mg/ml before mixing) in 0.1 M Tris/Cl, 1.0 M NaCl, pH 9.0, were reacted in the stopped flow spectrophotometer. Increase in absorbance was monitored at 453 nm. The spectral band width was 3 nm and a time constant of 0.1 ms was used.
previously measured (20). The rate of the spectral change is second order and was measured to be $3.2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ at pH 9.25, 0.1 M glycyl/NaOH, 0.2 M NaCl. Similar experiments were carried out at pH 9.0, 0.1 M Tris/Cl, 1.0 M NaCl, for the alkylated repressor and a sample oscilloscope trace is shown in Fig. 8. The second order rate was calculated to be $5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$. Experiments in this laboratory indicate a pH and salt dependence for the rates of spectral alterations observed; the rate for the alkylated repressor is consistent with other rate measurements of inducer binding at similar conditions of salt and pH.

**Glycyl Perturbation of Nitrophenol-labeled Repressor**—
Glycyl perturbation has been used to study exposure of chromophores in proteins (21). If a chromophoric group is exposed to solvent, the presence of glycyl (and other compounds) in the solvent will affect the absorption characteristics, resulting in an increase in the molar absorptivity and a shift to longer wavelength of the $\lambda_{\text{max}}$ (21). Studies on the repressor protein had previously indicated the exposure of tyrosine and tryptophan residues and demonstrated effects on this exposure upon addition of inducer (8). Therefore, the glycyl perturbation spectra of the repressor protein reacted with nitrophenol-containing chromophores were explored to determine the exposure of the chromophore under various conditions. The perturbation spectra indicated little perturbation of the nitrophenol groups in repressor compared to free nitrophenols. It appears that the nitrophenol groups bound to the modified cysteine residues are not fully accessible to solvent molecules.

**DISCUSSION**

Introduction of two nitrophenol reporter groups into the structure of the repressor at three different sites without altering the inducer or operator binding activities provides a probe of the protein structure in regions not previously accessible spectrophotometrically. Two equivalents of the mercivial introduced nitrophenol groups onto cysteines 107 ($\approx 70\%$), 140 ($\approx 40\%$), and 268 ($\approx 80\%$), while 1 eq affects primarily cysteine 268 ($\approx 75\%$). This allows observation of cysteines 107 and 268 with little contribution from cysteine 140 in 2 eq and cysteine 268 with small interference for 1 eq of reagent. The alkylation probe on the other hand affects primarily cysteine 140 (~85%); cysteine 107 reacts ~35% under these conditions, but if reaction is carried out in the presence of inducer, reaction of 107 is increased to near 80%. Thus, cysteine 140 can be observed almost exclusively by reaction in the absence of inducer, and cysteines 107 and 140 together by reaction in the presence of inducer. The similarity of the nitrophenol reporter groups allows comparison of the spectra observed.

The reaction of the mercivial chromophore, 2-chloromercury-4-nitrophenol, with repressor results in an increase in its apparent $pK$ from 6.75 to 8.3, and a shift in the wavelength of maximum absorption. On binding of inducer to the mercivial-labeled repressor, the apparent $pK$ values of the nitrophenol groups shift to slightly more acid $pH$. Only small differences were observed between protein modified with 1 and 2 eq of mercurinitrophenol. It was not possible to determine the $pK$ of the nitrophenol in repressor alkylated with 2-bromoacetylaminophenol, since the ionization occurred at $pH$ values below 7.0, and the alkylated repressor is not stable in this $pH$ range; however, the apparent $pK$ for repressor alkylated both in the presence and absence of inducer was less than $pH$ 7.0. The shift to higher $pK$ for the mercivial nitrophenols on reaction with inducer indicates that cysteines 107 and 268 are in regions that are hydrophobic, consistent with the inability to perturb these nitrophenols by glycyl.

The titration of the nitrophenol chromophores also indicates that there are shifts in the protein structure as the $pH$ is altered. Both chromophores shift in their wavelength of maximum absorption for all the conditions studied. These shifts are consistently toward longer wavelength, indicating a less polar environment for the chromophore as $pH$ increases. The nitrophenols from the alkylation reaction undergo this transition at lower $pH$ than the mercurial nitrophenols, although for repressor alkylated in the presence of inducer, the transition continues at higher $pH$ values. Transitions in the protein molecule have been indicated for the regions of $pH$ where these spectral transitions occur. Wu et al. (16) have shown by potentiometric titration of the repressor that protonation of residues occurs in the region between $pH$ 7.0 and 7.5. Any accompanying structural changes in the protein may be observed through the nitrophenol spectral probes. Similarly a change in the $K_a$ of repressor and inducer occurs between $pH$ 8.0 and 8.5. Again such transitions are likely conformational and would affect the spectral characteristics of the reporter groups in the protein as observed.

A conformation change in the repressor on binding to inducer has been demonstrated spectrally (7, 8) for tryptophan and tyrosine. The introduction of nitrophenol reporter groups at specific sites allows observation of these residues in response to binding inducer. The difference spectra produced by inducer binding vary for the reporter groups at different sites and $pH$ values. The mercurial nitrophenols at cysteines 107 and 268 exhibit a shift in the wavelength of maximum absorption to shorter wavelength, implying a transition to a more polar environment (18) for both cysteines. The increased absorbance in addition to the shift appears to be due to the decreased $pK$ for the nitrophenols in the presence of inducer, resulting in greater ionization. For the alkylated repressors, the intensity of the difference spectra at $pH$ 7.5 are similar, despite the increased amount of reporter group present for the protein reacted in the presence of inducer. At $pH$ 9.0, the spectrum for alkylated repressor is increased, but that for inducer-alkylated repressor is decreased and altered in shape. This altered spectrum is due to the differences in the absorbance for the nitrophenols at different parts of the molecule. Apparently cysteine 140 undergoes a shift to a more hydrophobic environment (i.e., shift in $\lambda_{\text{max}}$ to longer wavelength, red shift) on binding to inducer, while cysteine 107 (and cysteine 268) shift to more polar environments (blue shift). For alkylated repressor, the spectrum observed is primarily contributed by the nitrophenol at cysteine 140, while for repressor alkylated with inducer present, the spectrum is a combination of the nitrophenols at cysteines 140 and 107. Since the shifts on binding inducer are in opposite directions for these two nitrophenols, for repressor modified significantly at both 140 and 107, the sum of these two spectra will decrease and alter in shape, resulting with maxima and minima at wavelengths different from the parent spectra (see Fig. 6). Presuming by analogy to the mercivial that the $pK$ for the nitrophenol at cysteine 107 is $pH > 7.5$, the contribution of 107 to the spectrum should increase with increasing $pH$. Therefore, the difference spectrum should change most significantly at high $pH$, as observed. Apparently cysteines 107 and 268 are in similar environments and shift to more polar regions when inducer is bound, while cysteine 140 undergoes a change to less polar surroundings. These observed shifts giving rise to the difference spectra are presumably the result of the overall confor
In conclusion, nitrophenol chromophores have been introduced into the structure of the repressor protein at three different sites and several combinations of these sites. The spectral changes occurring with pH and on addition of ligands indicate conformational transitions in the protein. The changes observed are different for inducers and anti-inducers/neutral compounds which implies that these have different effects on the structure of the protein, consistent with their observed effects on repressor function. The rates of the spectral transitions in response to inducer are second order and apparently reflect a rapid, concerted change in the structure on binding this ligand.

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