The modification of lac repressor protein with N-bromosuccinimide has been studied. Treatment with N-bromosuccinimide resulted in the oxidation of one of the two tryptophan residues in the repressor monomer, in the presence of inducer, isopropyl-1-thio-β-D-galactoside, the tryptophan was protected from oxidation. Anti-inducer, o-nitrophenyl-β-D-fucoside, did not protect the reactive residue. The tryptophan involved was identified as residue 209. Some loss of ability to bind inducer was observed upon treatment with N-bromosuccinimide; however, this loss was not correlated with the oxidation of any amino acid and appeared to be attributable to a general destabilization of the protein. The facts that (a) inducer protected the oxidation of tryptophan while anti-inducer did not, and (b) the oxidation of tryptophan did not alter the affinity of repressor for inducer imply that tryptophan 209 is affected by the conformational change upon inducer binding but is not in the inducer/anti-inducer binding site. This is consistent with conclusions drawn from previous spectral studies implicating tryptophan as participating in a conformational isomerization rather than interacting directly with ligand. Loss of operator binding activity was observed with low levels of N-bromosuccinimide. The presence of inducer or anti-inducer did not protect against this loss of operator binding activity. Methionine or cysteine (or both), which were each oxidized by N-bromosuccinimide, may be involved in the binding of operator DNA, and their oxidation may have directly affected DNA binding; more likely, their oxidation may have produced a secondary effect which elicited a change in the conformation of the protein rendering it incapable of binding operator DNA. At levels of N-bromosuccinimide where loss of ability to bind operator DNA occurred, ability to bind nonspecifically to DNA was maintained. This provides chemical evidence that the nonspecific DNA and the operator DNA binding sites are functionally separable and that operator binding can be altered without affecting the binding to nonspecific DNA.

N-Bromosuccinimide has been widely used as a modifying agent. It reacts preferentially with tryptophan resulting in the oxidation of the indole ring with an associated shift in the wavelength of maximum absorbance from 280 to 260 nm (6). The accessibility of tryptophan to reaction as a function of its exposure to solvent is well documented (7). Availability for reaction and rates of reaction have each been used as criteria for assignment of exposure of tryptophan. The use of N-bromosuccinimide as a modifying agent offers the possibility of determining tryptophan involvement in the conformational change of the lac repressor associated with inducer binding. Concurrently, the importance of an intact indole ring in the binding of inducer, nonspecific DNA, and operator DNA can be determined. N-Bromosuccinimide also reacts with tyrosine, methionine, and cysteine (6), and their involvement in the function of repressor can be simultaneously examined.

Lac repressor is a genetic control protein which functions by binding to a region of Escherichia coli DNA, the operator, thereby preventing transcription of the lac operon. Induction of the system occurs in response to inducer binding, apparently through a consequent conformational change in the repressor protein to a form with lower affinity for operator DNA (1). Elucidation of this process requires careful examination of the conformational change associated with inducer binding. Differences in both the ultraviolet and fluorescence spectra of repressor upon binding inducer have been examined. In the presence of IPTG, both Oshihina et al. (2) and Matthews et al. (3) have observed an ultraviolet spectral change corresponding to alterations in the environments of both tyrosine and tryptophan. In addition, the accessibility of the perturbant glycerol to both tryptophan and tyrosine was significantly decreased in the presence of inducer (3). Laiken et al. (4) found that the presence of IPTG produced a shift in the fluorescence emission spectrum corresponding to the movement of tryptophan to a more hydrophobic environment. Fluorometric solvent perturbation with iodide ion confirmed this conclusion; the susceptibility of tryptophan to quenching was significantly decreased in the presence of inducer (4). By the use of repressor with single amino acid substitutions, Sommer et al. (5) implicated tryptophan 209 as the residue changing in its environment in response to inducer binding.

Chemical modification provides a useful tool for examining the participation of individual residues in ligand binding or in conformational changes associated with ligand binding. It offers advantages over genetic methods in three respects: (a) the modification is performed after the tertiary and quaternary structure of the protein are formed; (b) more than one site can be observed simultaneously; and (c) increasing modification with greater excesses of reagent allows the correlation of changes in specific amino acids with changes in the activities of the protein.

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1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactosido; ONPF, o-nitrophenyl-β-D-fucoside; NBS, N-bromosuccinimide; Dns, 5-dimethylaminonaphthalene-1-sulfonil.
Isolation of Repressor—Repressor was purified from Escherichia coli JM107. The protein was isolated according to the method of Muller-Hill et al. (8) with the modifications of Platt et al. (9). Cells were grown in 10-liter batches and stored frozen before use. The yield from 100 g of frozen cells was 100 mg of pure repressor protein. Repressor was frozen in 0.24 M potassium phosphate buffer, pH 7.6, 10 mM dithiothreitol, 5% glycerol following elution from the phosphocellulose column. Immediately before use, aliquots were thawed and dialyzed into either 0.2 M phosphate buffer, pH 7.6, 1.0 M Tris/Cl, pH 7.8, or 1.0 M Tris/Cl, pH 7.0. No sulfhydryl reagents were present. To prevent oxidation of cysteines during dialysis, buffer solutions were the race dialyzed with nitrogen. Final concentration of repressor was 2 to 4 mg/ml. The purified protein was subjected to sodium dodecyl sulfate-gel electrophoresis to assay its purity (10). The isolated repressor was fully active in binding IPTG and 40% active in binding to operator DNA. Repressor purified using the modifications of Rosenberg et al. yielded protein 100% active in operator binding. It was then stored and prepared for reaction in the same manner as described above.

Assay of Repressor—IPTG binding activity was determined by the ammonium sulfate precipitation method described by Bourgeois (11). The Kₜ for IPTG binding to repressor was obtained through equilibrium dialysis. The operator DNA binding assay was performed using nitrocellulose filters as described by Riggs et al. (12). The DNA was labeled with ¹³¹I-hytmidine and was obtained from a low thymine-requiring E. coli strain which carried a temperature-inducible lac pro gene (λ5-5, strain ABC 5, obtained from Mary Barkley, University of Kentucky; Preparation of thymine-phage and purification of DNA was carried out according to Wang et al. (13)). The ability of repressor to bind to nonspecific sequences of DNA was examined using a procedure developed in this lab using utilization of sedimentation of repressor-calf thymus DNA complexes in glycerol gradients. Repressor (0.1 mg/ml in 0.05 M Tris/Cl, pH 7.4) and fragmented calf thymus DNA (0.18 mg/ml) along with 10⁻¹ M [³²P]PTG were sedimented in a 5 to 20% glycerol gradient. The gradient was fractionated, and the relative concentrations of repressor were determined by utilizing the radioactive ligand. The ratio of native repressor sedimenting with the DNA to the total concentration of repressor was defined as unity. The percentage of nonspecific DNA binding activity for modified samples was determined by the comparison of the ratios of DNA-bound protein for modified and native repressor under identical conditions.

Chemicals—N-Bromosuccinimide was purchased from Sigma and was recrystallized from water immediately prior to use (m.p. 181-185°). Solutions were prepared fresh and maintained in the dark at 4°. 2-Chloromercuri-4-nitrophenol was obtained from Eastman and recrystallized before use. Methanesulfonic acid was obtained from Mallinckrodt and recrystallized from water immediately prior to use (m.p. 181-185°). 3-(2-Aminoethyl)indole/hydrochloride from Eastman; each was purified by column chromatography and eluted with water. Tryptically cleaved repressor was lyophilized for 12 to 15 h. The precipitated protein was dissolved in a solution of 20% potassium phosphate, 0.3% acetic acid, 0.3%). Bromphenol blue was used as the marker dye. Corresponding peptides from repressor, repressor modified with N-bromosuccinimide and repressor modified in the presence of inducer were electrophoresed simultaneously. Localization of peptides was carried out as follows. (a) To localize ninhydrin-positive peptides, the paper was sprayed with 0.02% ninhydrin in ethanol. Color development proceeded to fade over a period of hours. For the purpose of identification of the peptides, strips were cut from both sides of the electrophoresed gel. (b) For Ehrlich positive peptides, the paper was sprayed with Ehrlich reagent (1 part 10% p-dimethylaminobenzaldehyde in concentrated HCl and 4 parts acetic acid). The reaction proceeds to fade over a period of hours. For the purpose of identification of the peptides, strips were cut from both sides of the electrophoresed gel. (c) For tryptic fragments, the paper was treated with trypsin (1% by weight) for 3 h at 37°. An equivalent aliquot was added and incubation continued for an additional 3 h. Tryptically cleaved repressor was lyophilized for 12 to 15 h. The residue was applied in a total volume not exceeding 2 ml in 0.1 M NH₄HCO₃ to a Bio-Gel P-2 column and eluted using the same buffer. Reacted repressor samples were dialyzed against water for 22 h. The hydrolysate was partially neutralized by addition of an equal volume of 5.5 M sodium hydroxide. Amino acid analysis was performed using a Beckman 13C amino acid analyzer (16).

Determination of Cysteine Content—Repressor reacted with N-bromosuccinimide was diazylated against glass-distilled water. The precipitated protein was resuspended in 8 M urea, 5 mM EDTA (0.5 mg/ml) and the absorbance at 280 and 410 nm was determined. The protein was titrated with aliquots of 2-chloromercuri-4-nitrophenol in 0.02 N NaOH (17). Yang et al. have found that denatured repressor exhibits an end point for the titration curve corresponding to the reaction of 3 cysteines/repressor monomer.

NH₂-terminal End Group Analysis—The NH₂-terminal residues of native and N-bromosuccinimide-modified repressor were determined by reaction with Dns-chloro(18).

Molecular Weight Determinations—The number of subunit species and their molecular weights were determined by the use of sodium dodecyl sulfate-gel electrophoresis (19). To determine the multimeric molecular weight, the reacted protein was chromatographed on Sephadex G-100 (2.5 × 60 cm). The protein was applied and eluted using 1 M Tris/Cl, pH 7.8, 10⁻⁴ M dithiothreitol. Standards were run to calibrate the column.

Circular Dichroism Spectrum of Lac Repressor—Repressor and N-bromosuccinimide-modified repressor were dialyzed against 1 M Tris/Cl, pH 7.8, and then diluted to a concentration of 0.5 mg/ml. Circular dichroism spectra were recorded on a Cary 61 recording spectrophotometer and the percentage of helical content determined (20).

Determination of Reactive Tryptophan—Samples were exhaustively diazylated against 0.1 M NH₄HCO₃. Prior to treatment with enzyme, the samples were boiled for 5 min to denature the protein. The presence of contaminating proteases in several repressor preparations had been noted; when this denaturation step was omitted, the protein weight, the reacted protein was chromatographed on Sephadex G-100 (2.5 × 60 cm). The protein was applied and eluted using 1 M Tris/Cl, pH 7.8, and then diluted to a concentration of 0.5 mg/ml. Circular dichroism spectra were recorded on a Cary 61 recording spectrophotometer and the percentage of helical content determined (20).

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N-Bromosuccinimide Modification of Lac Repressor Protein

RESULTS

Determination of Reaction Conditions—Treatment of repressor in phosphate buffer with N-bromosuccinimide resulted in the oxidation of one of the two tryptophan residues. Methionine and tyrosine were also susceptible to reaction. At low excesses of reagent, IPTG completely protected the oxidation of tryptophan; at higher excesses, tryptophan was reactive even in the presence of IPTG. However, at all levels, a differential was observed between the amount of tryptophan reacted in the absence and presence of IPTG. Anti-inducer, ONPF, did not provide corresponding protection. On exposure to denaturing conditions, 8 M urea or 6 M guanidine hydrochloride, the reaction of both tryptophan residues with N bromosuccinimide indicated that each can be exposed by the unfolding of the protein. Guanidine hydrochloride was more effective in exposing the unreactive tryptophan. At levels of N-bromosuccinimide where ~0.8 tryptophan/mer reacted in the native protein, both tryptophans were completely oxidized in the presence of denaturing agent.

N-Bromosuccinimide results in cleavage of tryptophyl peptide bonds in proteins under several conditions (6). To determine if any significant amount of cleavage was occurring in the reaction with repressor, the modified protein was reacted with Dns-chloride. The NH₂-terminal residue for repressor is methionine. If cleavage occurred at the tryptophans, either histidine or serine would also appear as NH₂-terminal residues. NH₂-terminal end group analysis gave Dns-methionine and e-Dns-lysine as the sole reaction products. Neither Dns-histidine nor Dns-serine was present. Sodium dodecyl sulfate-gel electrophoresis was performed on repressor, N bromosuccinimide-modified repressor, and repressor modified in the presence of IPTG. The gel patterns were identical, and the major band corresponded to the repressor monomeric molecular weight, 40,000.

Precipitation of the repressor protein occurred upon addition of N-bromosuccinimide in phosphate buffer. Exposure to light enhanced the precipitation, which may be attributable to light-catalyzed bromine free radical formation and subsequent random bromination of repressor. The instability of the protein prohibited the use of spectral techniques to determine the extent of modification. The problem of precipitation also precluded spectral studies of modified protein. To increase the stability of the reacted protein, 1 M Tris/Cl was examined as a possible buffer system. The modified protein, although still less stable than native repressor, was significantly more stable in Tris/Cl buffer than in phosphate. Concurrently, lower levels of N bromosuccinimide were found to be adequate to achieve the same extent of tryptophan oxidation. In an attempt to further increase the reactivity of the tryptophan, the pH of the buffer system was lowered from pH 7.8 to pH 7.0. However, at this pH, inducer did not appear to protect to the same extent observed at pH 7.0.

Both recrystallized and non-recrystallized N-bromosuccinimide were used in the initial experiments. No difference was observed in the residues which reacted or the associated effects of reaction; however, there was a significant difference in the amount of N-bromosuccinimide required for reaction to occur. The recrystallized N-bromosuccinimide was more effective in oxidizing the protein. Hydrolysis with p-toluensulfonic acid was performed on N-bromosuccinimide-modified repressor to determine the extent of reaction. Preparation of the acid involved crystallization from hydrochloric acid, and some variation in the quantitation of tryptophan using this acid apparently resulted from the trace amounts of HCl. Methanesulfonic acid, which is a liquid, was also used; more consistent tryptophan determination by amino acid analysis was found using this acid.

Reaction with N-Bromosuccinimide in Tris/Cl Buffer—Titration of repressor with N-bromosuccinimide in 1 M Tris/Cl was performed as described under "Materials and Methods" (Fig. 1). After addition of initial aliquots of N-bromosuccinimide to repressor, the absorbance at 280 nm began to decrease with subsequent addition of N-bromosuccinimide. A minimum was reached with approximately a 5-fold excess of N-bromosuccinimide and corresponded to the oxidation of ~0.5 tryptophan. When inducer was present, no decrease in the absorbance at 280 nm was observed. For denatured protein, in the presence of 6 M guanidine hydrochloride, the decrease in absorbance at 280 nm occurred at a more rapid rate and the minimum corresponded to ~0.7 tryptophan. The spectral titration indicated that the native protein required approximately twice the molar excess of N-bromosuccinimide per tryptophan oxidized compared to denatured repressor. Since there was little oxidation of tyrosines with N-bromosuccinimide at low excesses, the change in absorbance at 280 nm corresponded to the oxidation of tryptophan. Excellent correlation could be drawn between the decrease in absorbance and the loss of tryptophan as measured by amino acid analysis. However, the spectral end point occurred before the end point determined by amino acid analysis was reached. According to the N-bromosuccinimide spectral titration, only ~0.5 tryptophan was available for reaction with the native repressor and ~0.7 tryptophan was available in denatured repressor. However, amino acid analysis indicated that ~1 tryptophan was oxidized by N-bromosuccinimide in the native protein and ~2 tryptophans were oxidized in the denatured repressor.

![Graph showing absorbance change at 280 nm upon addition of NBS](http://www.jbc.org/)
Table I contains the ratios of the amount of each amino acid found in modified repressor to that found in native repressor for repressor, repressor plus IPTG, and repressor plus ONPF modified with a 4.2-fold excess of N-bromosuccinimide. Tryptophan, tyrosine, methionine, and cysteine were the only amino acids affected by reaction with N-bromosuccinimide; Fig. 2 represents the reaction of these amino acids with increasing excesses of N-bromosuccinimide. At the greatest extent of N-bromosuccinimide examined (Fig. 2), ~1 tryptophan, ~3 cysteines, ~3 methionines, and ~3 tyrosines were available for reaction with N-bromosuccinimide. IPTG protected tryptophan from oxidation at all excesses; however, at increased excesses of N-bromosuccinimide, tryptophan was oxidized even in the presence of inducer, although to a lesser extent than in its absence. At various excesses, a differential extent of oxidation of cysteine, but inducer did protect the oxidation of tyrosine and methionine to some degree. Amino acid analysis also indicated that the tryptophans exposed through the use of denaturing agents, urea or guanidine hydrochloride, were more reactive than the tryptophan in the native repressor. Both tryptophans in denatured repressor were oxidized with excesses of N-bromosuccinimide sufficient to react only ~0.8 tryptophan in the native protein. Approximately 18 mol of N-bromosuccinimide/mol of tryptophan oxidized were required by the native protein, whereas only 6 mol of NBS/mol of tryptophan oxidized were required by denatured repressor.

**Table I**

Amino acid composition of N-bromosuccinimide-modified lac repressor

| Amino acid | Ratio of amino acid unmodified | NBS-repressor | NBS-repressor + ONPF | NBS-repressor + IPTG |
|------------|-------------------------------|---------------|---------------------|---------------------|
| Aspartic acid | 1.03                          | 1.01          | 1.05                |
| Threonine   | 0.98                          | 0.98          | 1.00                |
| Serine      | 0.99                          | 0.99          | 1.00                |
| Glutamic acid| 1.00                          | 1.02          | 1.01                |
| Proline     | 1.03                          | 1.12          | 1.02                |
| Glycine     | 1.00                          | 0.98          | 0.98                |
| Alanine     | 1.00                          | 0.91          | 1.00                |
| Cysteine†   | 0.29                          | 0.48          | 0.32                |
| Valine      | 1.01                          | 0.99          | 0.99                |
| Methionine  | 0.92                          | 0.86          | 0.99                |
| Isoleucine  | 1.01                          | 0.90          | 1.00                |
| Leucine     | 1.01                          | 1.02          | 1.03                |
| Tyrosine    | 1.03                          | 0.96          | 1.05                |
| Phenylalanine| 1.12                          | 0.99          | 0.99                |
| Tryptophan  | 0.83                          | 0.76          | 0.97                |
| Lysine      | 0.96                          | 0.99          | 0.98                |
| Histidine   | 1.01                          | 0.98          | 0.97                |
| Arginine    | 1.04                          | 1.01          | 1.01                |

† Ratio of the amount of amino acid found in modified repressor relative to that observed for native repressor.

* Repressor modified with a 4.2-fold molar excess of NBS.

+ Repressor modified with a 4.2-fold molar excess of NBS in the presence of ONPF.

IPTG binding activity of repressor reacted with NBS. IPTG binding assays were conducted as described under "Materials and Methods."

- Repressor reacted with NBS; ■—■, repressor reacted in the presence of IPTG; ▲—▲, repressor reacted in the presence of ONPF.

**FIG. 2.** Moles of amino acid reacted with NBS per monomer of repressor. The amount of tryptophan, tyrosine, and methionine were determined from amino acid analysis (see "Materials and Methods"). Cysteine was quantitated by titration with 2-chloromercuri-4-nitrophenol as described under "Materials and Methods." Cysteine was determined from amino acid analysis (see "Materials and Methods"). Cysteine was quantitated by titration with 2-chloromercuri-4-nitrophenol as described under "Materials and Methods." Cysteine was quantitated by titration with 2-chloromercuri-4-nitrophenol as described under "Materials and Methods."
IPTG (Kᵣ = 3 ± 1 × 10⁻⁶, in 0.5 M Tris/Cl, pH 7.0), one portion of the repressor population retained unaltered IPTG binding while another portion did not bind IPTG to an extent detectable by equilibrium dialysis. Since no change was found in the Kᵣ for IPTG (Kᵣ = 3 ± 1 × 10⁻⁶, in 0.5 M Tris/Cl, pH 7.0), one portion of the repressor population retained unaltered IPTG binding activity while another portion did not bind IPTG to an extent detectable by equilibrium dialysis.

Measurement of operator binding activity reflected a loss of 80% of the ability to bind operator in repressor modified with less than a 5-fold excess of N-bromosuccinimide per monomer (Fig. 4). This loss was not protected by the presence of IPTG or ONPF in the reaction mixture. The largest drop in the operator binding activity occurred between a 2-fold and 3-fold excess of N-bromosuccinimide per repressor monomer. The percentage of loss of operator binding activity was the same for repressor which was 40 or 100% active in binding before modification. As previously determined, oxidation with N-bromosuccinimide did not yield cleavage of the repressor protein. However, the two methods of examination were restricted to observing the monomeric molecular characteristics. A possible explanation for the loss of operator activity is that the quaternary structure of the protein was altered by the modification. It has been observed that mutants which yield repressor monomers that will not aggregate are also deficient in operator binding (20). To ensure that the modified protein was still a tetramer, chromatography on Sephadex G-100 was performed. The modified protein eluted in a single peak corresponding to the elution volume of lac repressor protein (Mᵦ = 150,000). Circular dichroism spectroscopy was used to determine if the backbone structure of the protein was altered. There was no detectable alteration of the circular dichroism spectrum for repressor modified with a 4-fold excess of N-bromosuccinimide (helicity = 39%). For repressor reacted with a 16-fold excess of N-bromosuccinimide there was a substantial alteration in the conformation of the protein as evidenced by a decrease in the helicity to 30%. This decrease in structural integrity for at least a portion of the molecules may account for the loss of inducer binding activity with higher excesses of N-bromosuccinimide.

Repressor protein will bind to nonspecific sequences as well as to operator DNA. It was important to determine if the loss of ability to bind operator DNA was accompanied by a loss in the ability to bind nonspecific DNA. For this purpose, modified repressor protein was sedimented in a glycerol gradient with fragmented calf thymus DNA. The percentage of nonspecific DNA activity was defined as the fraction of N-bromosuccinimide-modified protein bound to DNA divided by the fraction of unmodified repressor bound using identical concentrations of repressor and DNA in the same buffer system. Modification of repressor with N-bromosuccinimide resulted in the loss of nonspecific DNA binding activity (Fig. 5); however, the excess at which this occurred was significantly greater than that at which operator activity was lost. In the region where the ability to bind to operator DNA was lost, no loss in nonspecific binding activity was observed. The nonspecific binding activity could not be determined for protein modified in the presence of IPTG or ONPF since radioactive IPTG was used to determine the concentration of repressor at various points in the glycerol gradient, and small amounts of inducer or anti-inducer competed with the labeled IPTG.

Identification of Reactive Residue—Application of tryptically cleaved repressor to a Bio-Gel P-2 column resulted in the separation of the two tryptophan-containing peptides. Fig. 6a presents the elution pattern obtained. Subsequent electrophoresis (Peak I after chymotryptic cleavage), at pH 6.5, resulted in the separation of each tryptophan-containing peptide from the peptides co-eluting from the Bio-Gel P-2 column (Fig. 7). The tryptophan-containing peptides were identified using Ehrlich reagent. After elution from electrophoresis paper, hydrolysis and amino acid analysis were performed. By comparison with the amino acid sequence of lac repressor (21), the tryptophan-containing peptide eluting in Peak II was identified as amino acids 187 through 192, including tryptophan 190. Although the other tryptophan-containing peptide was not positively identified due to overlap of other peptides, it did exhibit the expected electrophoretic mobility for amino acids 197 through 209, including tryptophan 209. This peptide would arise from the chymotryptic digestion of the peptide containing amino acids 197 through 242 which should elute in the first peak (the void volume).

As a control, N-bromosuccinimide-treated N-acetyltrypto-
N-Bromosuccinimide Modification of Lac Repressor Protein

**DISCUSSION**

Tryptophan 209 of the lac repressor protein reacts with N-bromosuccinimide; the presence of inducer, but not anti-inducer, protected this residue from oxidation. Since tryptophan 190 was unreactive in the presence or absence of ligand, it is apparently not exposed to reagent in either conformation. After reaction, the two tryptophans were separated chromatographically. Identification of the modified tryptophan was based on the spectral properties of the indole, the product of N-bromosuccinimide oxidation, and on its reactivity with a stain specific for tryptophan after electrophoretic separation of the peptides. The pattern of reaction of tryptophan with N-bromosuccinimide is consistent with results obtained from ultraviolet and fluorescence spectral studies on repressor (2-5). Amino acid analysis indicated that tyrosine, methionine, and cysteine also reacted with N-bromosuccinimide. The reaction of cysteine was not protected by the presence of inducer or anti-inducer, while tyrosine and methionine oxidation were reduced to some extent in the presence of IPTG. The lack of effect of IPTG or ONPF on the reactivity of cysteine with N-bromosuccinimide paralleled that previously observed using 2-chloromercuri-4-nitrophenol (22).

Reaction with large excesses of N-bromosuccinimide resulted in a partial loss of the ability of the protein to bind

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**Fig. 6.** Chromatography of tryptically cleaved repressor peptides. (a), elution pattern of tryptically cleaved repressor peptides from Bio-Gel P-2 column. ○○○○, absorbance at 280 nm; ●●●●, absorbance at 250 nm. The column used was 1.5 x 35 cm. The procedure used was that described under "Materials and Methods." (b), 280/250 nm absorbance ratio for tryptically cleaved peptide elution from Bio-Gel P-2 column. ○○○○, repressor; ■■■■, modified repressor; □□□□, repressor modified with NBS; ○●●●●●●●●, repressor modified in the presence of inducer.

**Fig. 7.** High voltage electrophoresis of peptides obtained from Bio-Gel P-2 column. Electrophoresis was carried out at 1000 V for 27/2 h at pH 6.5 (pyridine, 10% and acetic acid, 0.3%). Enclosed areas are ninhydrin-positive; shaded areas indicate Ehrlich-positive regions; dotted lines enclosed areas that stain weakly with ninhydrin. Peak I represents the electrophoresis pattern of the peptides which eluted in the void volume from the Bio-Gel P-2 column (see Fig. 6). The peptides were treated with chymotrypsin and lyophilized before electrophoresis. Peak II represents the electrophoresis of the peptides which include the second tryptophan-containing peptide eluted from the Bio-Gel P-2 column (see Fig. 6). The Ehrlich-positive region in the electrophoresis pattern for Peak I included the peptide containing tryptophan 190. Peptides were identified by elution and subsequent amino acid analysis. The procedure used was that described under "Materials and Methods."
IPTG, but the decreased ability to bind inducer could not be correlated to the oxidation of a specific amino acid. This loss in activity was confined to only a fraction of the protein, since equilibrium dialysis yielded a dissociation constant equivalent to that obtained for repressor in the same buffer system. The observation that exposure to light in the presence of N-bromosuccinimide resulted in the loss of IPTG binding activity implies that inactivation was probably due to secondary reactions. With a 16-fold excess of N-bromosuccinimide, the loss in IPTG binding activity was also accompanied by a change in the circular dichroism spectrum of the protein; this circular dichroism change reflected partial unfolding of the repressor protein. The fact that even with high excesses of N-bromosuccinimide, at least one-half of the population of repressor retained full inducer binding activity implies that tryptophan 209 is not part of the inducer binding site. This conclusion is in agreement with spectral studies which have indicated that tryptophan is perturbed by the conformational change upon inducer binding rather than by the ligand itself (2–5).

In contrast to inducer binding activity, the ability of the protein to bind operator DNA was markedly affected by N-bromosuccinimide modification. With less than a 5-fold excess of reagent, the protein lost 80% operator binding activity while retaining 95% of its ability to bind IPTG. The loss in operator binding activity could not be directly correlated to the oxidation of any single amino acid. It is possible that the loss in activity was due to the oxidation of cysteine or methionine (or both) with a subsequent alteration of the secondary or tertiary structure of the surrounding region of the protein. The oxidation of tryptophan 209 was probably not responsible for the loss in operator binding activity since its reaction was protected by the presence of inducer and operator activity was not. Chromatography on Sephadex G-100 indicated that the decreased binding to operator DNA was not due to an alteration of the oligomeric structure of the protein. Likewise, circular dichroism spectroscopy indicated no detectable change in the helical backbone structure of the protein. However, given the size of the operator binding site and its lability, it is not inconsistent that changes undetectable by these methods could still result in alterations in the active structure of the protein.

Previous chemical studies have indicated a concomitant loss of both operator and nonoperator DNA binding activities of the lac repressor protein. Treatment of the protein with heat or trypsin, or the presence of actinomycin, affected the two DNA binding activities identically (23). However, Schlotmann et al. (24) and Beyreuther (25) have isolated a small class of mutants which will bind to DNA cellulose but have lost the ability to bind operator DNA. Modification of repressor with N-bromosuccinimide provides the first chemical evidence that the operator and nonoperator binding sites are functionally separable. With low excesses of N-bromosuccinimide, 80% of the operator binding activity was lost, while no alteration in the ability to bind nonoperator DNA was observed.

This study of the reaction of lac repressor with N-bromosuccinimide yields two conclusions regarding the function of the protein molecule. (a) Tryptophan 209 is affected by the conformational change which accompanies inducer binding but does not appear to be located directly in the inducer binding site. (b) The operator DNA and nonspecific DNA binding sites are functionally separable; alteration of the operator DNA site does not necessarily affect alteration of the nonspecific DNA site.

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