Tetranitromethane Modification of the Tyrosine Residues of the Lactose Repressor*

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Repressor protein modified with N-ethylmaleimide has been used to determine the exclusive effects of tyrosine nitration by tetranitromethane. Since modification of proteins with tetranitromethane generally results in both cysteine oxidation and tyrosine nitration, N-ethylmaleimide has been used to protect the cysteines in the repressor against oxidation in subsequent tetranitromethane reactions. Nitration of tyrosine residues in repressor previously reacted with N-ethylmaleimide results in loss of both specific and nonspecific DNA-binding activities. Na2S2O4 reduction of tetranitromethane-modified protein restores partial operator DNA-binding and complete nonspecific DNA-binding capability. Residues primarily affected are tyrosines 7 and 17, which are both in the NH2 terminus. Inter- and intramolecular cross-links which are observed in the modified protein can be minimized by altering reaction conditions; the cross-links present occur between sites located in the NH2 termini. Modification of the core protein also results in loss of the operator DNA-binding capacity, and subsequent reduction restores partial operator-binding activity. Both operator and nonspecific DNA-binding capabilities of the repressor protein are protected by the presence of nonspecific DNA during the tetranitromethane modification, and simultaneously the extent of nitration is decreased.

The lactose repressor protein of Escherichia coli is one of the most intensively studied proteins involved in gene regulation. It binds specifically to the lactose operator and prevents the transcription of the lac structural genes by blocking initiation by RNA polymerase (1). Lactose repressor can bind nonspecifically to DNA sequences other than the operator sequence, although with lower affinity (1). Upon the addition of inducer, a conformational change occurs, and the affinity of repressor for operator, but not nonspecific DNA, is lowered (1, 2). The decreased affinity of the protein for the operator frees this region of the DNA, and thus transcription can begin.

The repressor is a tetramer of four identical subunits with no disulfide bonds present (1), and the amino acid sequence of the protein has been determined (3). Genetic studies indicate that most of the nonsense and missense mutants which affect operator binding map in the NH2-terminal region (4–6). The majority of these mutants are incapable of binding to nonspecific DNA (7). Mild proteolysis separates the NH2-terminal and core regions (8), and the isolated NH2 terminus binds to DNA both nonspecifically and specifically (9, 10). The NH2-terminal region has long been regarded as the site responsible for both nonspecific and specific DNA binding (1, 8, 10, 11); however, recent studies demonstrate that the core protein possesses not only normal inducer-binding activity (8), but also inducer-sensitive operator-binding capacity (12). Chemical modifications have been reported which result in repressor proteins which bind normally to nonspecific DNA and inducer but have lost operator-binding capacity (13, 14).

Each subunit of repressor tetramer contains eight tyrosines, four of which are found in the NH2 terminus (3). Several proposed models for NH2-terminal fragment interaction with operator DNA suggest that some or all of tyrosine residues 7, 12, and 17 are directly involved in binding (15–17). Suppression of nonsense mutants at the tyrosine positions 7, 17, 47, 273, and 282 results in repressors with altered phenotype; substitution at positions 7, 17, and 47 affects DNA binding; at position 273, it affects inducer binding; and at position 282, it affects the assembly of tetramers (4, 6). Ultraviolet spectral studies of repressor indicate that both tyrosine and tryptophan absorption are affected by the addition of inducer (18).

19F NMR also shows resonance spectral shifts of tyrosine residues upon the addition of isopropyl-β-D-thiogalactoside. Tyrosine residues have been studied previously by chemical modification (19, 20). However, oxidation of cysteine residues occurred in these studies, and oxidation of cysteine by N-bromosuccinimide was later shown to result in loss of operator DNA binding (21). Induction of the protein resulted in loss of operator binding; the residues affected were tyrosines 7, 12, and 17, and cysteine 107 (19). Tetranitromethane modification also produced the loss of operator DNA-binding capacity, and tyrosines 7 and 17 were identified as the residues modified (20); all 3 cysteine residues were oxidized. N-Ethylmaleimide, a reagent that modifies cysteines 107 and 140 of the repressor without the loss of any binding capabilities, can be utilized to protect all 3 cysteine residues from oxidation by tetranitromethane so that only the effects of tyrosine modification by TNM2 can be examined (22).

MATERIALS AND METHODS

Isolation of Repressor—Repressor was isolated from E. coli CSH 46 according to the method described by Rosenberg et al. (23) with the modifications described by O’Gorman et al. (24). The purity of isolated protein was checked by sodium dodecyl sulfate gel electrophoresis (25, 26). The isolated repressor was >95% pure and 30 to 100% active in operator binding. Depending on the modification conditions to follow, aliquots of protein were thawed and dialyzed.

1 M. A. C. Jarema, P. Lu, and J. H. Miller, personal communication.

2 The abbreviations used are: TNM, tetranitromethane; IPTG, isopropyl-β-D-thiogalactoside; NEM, N-ethylmaleimide; SDS, sodium dodecyl sulfate.
Modification of the Tyrosine Residues of lac Repressor

into either 0.1 M Tris-HCl, pH 7.8; 0.1 M Tris-HCl, pH 8.0; 0.24 M potassium phosphate, 5% glucose, pH 7.6; or 0.24 M potassium phosphate, 5% glucose, pH 8.0, buffer immediately before use. No sulphydryl compounds were present in the buffers. Buffer was flushed with nitrogen, and the oxidation of the enzyme during the dialysis. The protein concentration was determined by either absorbance at 280 nm, Folin-Ciocalteau reaction (27), or Bio-Rad Coomassie blue assays (28). The final protein concentration ranged from 0.1 to 2.0 mg/ml.

Assay of Repressor—IPTG-binding activity was determined by the ammonium sulfate precipitation method described by Bourgeois (29). The 4× binding assay was performed by 1H-thymidine-labeled 8plac5 DNA isolated from E. coli MBC5 (obtained from Mary Barkley, University of Kentucky) by nick translation methods (30, 31). Per cent operator DNA-binding activity was determined by comparing the amount of modified protein required for 50% saturation binding of DNA to the same value for unmodified protein. Nonspecific DNA-binding assays were performed using nitrocellulose filters in a manner analogous to the operator-binding assay (24). A change (±20%) in the dissociation constant for the protein can be detected by this assay (24).

Chemicals—Tetranitromethane, sodium dithionite, N-ethylmaleimide, and IPTG were purchased from Sigma. [14C]IPTG was obtained from New England Products International. 2-Chloromercuri-4-nitrophenol was purchased from Eastman, recrystallized from water, and then stored in vacuum at −15 °C. Calf thymus DNA and poly(dA-T) were obtained from Sigma and fragmented by shearing to uniform size (15 × 106 daltons) prior to use. Reaction with Tetranitromethane—Appropriate aliquots of TNM in 100% ethanol were added to native or NEM-modified repressor in either Tris-HCl, pH 8.0, or 0.24 M potassium phosphate, 5% glucose, pH 8.0, buffer. The protein concentration in the reaction ranged from 0.1 to 1.0 mg/ml. Reaction was performed at room temperature for 90 min. Ten-fold dithiothreitol over the TNM concentration was added to stop the reaction. Excess TNM was also removed by passage through a Sephadex G-25 column equilibrated with sample buffer. The modified repressor was concentrated to 0.5 to 1.0 mg/ml by pressure dialysis. The extent of reaction was determined by either amino acid analysis or absorbance at 428 nm.

Reaction with N-Ethylmaleimide—N-Ethylmaleimide solution was made fresh in 0.24 M potassium phosphate, 5% glucose, pH 7.0, buffer and an appropriate aliquot (25-fold/monomer) of reagent was added to the protein. Reaction was carried out at room temperature for 4 h. The excess reagent was removed by either dialysis or passage through a Sephadex G-25 column. Free cysteine content was determined by 2-chloromercuri-4-nitrophenol titration (21).

Reduction with Sodium Dithionite—An appropriate amount of sodium dithionite (70-fold/monomer, either in solid form or dissolved in deoxygenated buffer) was added to the protein. Reaction was carried out at room temperature for 10 min, and the excess reagent was removed either by passage through a Sephadex G-25 column or dialysis. The extent of reaction was followed both by the disappearance of NO2-tyrosine peaks in amino acid analysis and loss of absorbance at 428 nm.

Isolation of Core Protein—The core repressor was purified according to the methods described by Matthews (12) except that 5% glucose was added to the buffers and 1.0 to 1.5% (w/w, Sigma) trypsin was utilized. The core purity was checked by SDS-gel electrophoresis. The core protein exhibits inducer-sensitive operator DNA binding which is minimally affected by the addition of poly(dA-T). The purified core concentration ranged from 0.5 to 1.0 mg/ml.

Amino Acid Analysis—Protein samples were hydrolyzed in 6 N HCl for 20 h at 110 °C (32). Analysis was carried out on a Beckman 120C amino acid analyzer. NO2-tyrosine elutes 14 min after phenylalanine, and NH2-tyrosine elutes before the basic amino acids from the column for the basic amino acids (1 × 7 cm).

Peptide Mapping of Modified Protein—Native or modified repressor (6 to 10 mg) was dialyzed into 0.1 M ammonium bicarbonate, pH 7.8, buffer and 1% trypsin (w/w) was added for 20 min at room temperature. The NH2-terminal and core protein were separated by passing the treated protein through a Sephadex G-75 column (2.5 × 16 cm) equilibrated with the same buffer. The protein fractions were monitored by absorbance at 230 nm and the two peaks pooled separately. The NH2-terminal portion was further digested by both trypsin and chymotrypsin, and the core protein was further digested by trypsin only. The peptides were mapped by electrophoresis/chromatography as described previously (20). Tyrosine-containing peptides were located by nitrrosonaphthol reagent (33). NO2-tyrosine-containing peptides were located by yellow color when exposed to NH3 fumes; the appropriate regions were cut from the paper and eluted by 10% acetic acid or 0.1 M NH4HCO3, pH 7.8, overnight with several changes of solvent. The eluted peptides were subjected to amino acid analysis to quantitate the specific NO2-tyrosine residues.

TNM Modification in the Presence of Nonspecific DNA—Repressor protein was dialyzed into 0.048 M potassium phosphate, 5% glucose, pH 8.0, buffer; the protein was then added to a solution of calf thymus DNA to a ratio of 0.1 mg of DNA/mg of protein. After 10 min incubation at room temperature, the TNM modification was carried out as described above. Calf thymus DNA can be partially removed by ammonium sulfate precipitation of modified protein. Nonspecific and operator DNA-binding assays were carried out either in the presence or with partial removal of calf thymus DNA. Native and SDS-polyacrylamide gel electrophoresis were used to monitor any cross-linking formed during the modification.

RESULTS

Reaction of Repressor with TNM—Modification of repressor with TNM results in both oxidation of cysteine residues and nitration of tyrosine residues. The amount of cysteine oxidation is shown in Fig. 1. At an 8-fold molar excess of TNM/monomer, only 20% of the cysteine residues remain unoxidized (i.e. of the 3 cysteines/monomer, less than one remains unreacted). At this reagent level, amino acid analysis indicates that less than 10% of the 8 tyrosine residues/monomer have been nitrated. Since cysteine oxidation has been shown to cause loss of operator-binding activity (21), it is necessary to protect against cysteine oxidation during the TNM modification by reaction with N-ethylmaleimide (22) in order to monitor the effects of nitration of tyrosine residues alone. The operator-binding activity of native and NEM-modified repressor after exposure to 4-fold TNM/monomer is shown in Fig. 2. No difference is found between the native and NEM-modified repressor. In contrast, with a 4-fold TNM molar excess, the NEM-modified repressor retains most of its operator DNA-binding activity as compared to the unmodified repressor. Although cysteine 281 is not affected by NEM modification, minimal oxidation of this residue was found after TNM modification of the NEM-reacted protein (see Fig. 3A). All studies cited below used NEM-modified protein.

Inter-/intramolecular cross-linking of proteins in response to TNM reaction has been previously observed (34-36). The pattern produced by native polyacrylamide gel electrophoresis of TNM-modified repressor is shown in Fig. 4A. With 0.5 mg/
ml of protein in the reaction mixture, intertetramer cross-linking is observed at a 2-fold and, more extensively, at a 10-fold molar excess of TNM. By reducing the protein concentration to 0.1 mg/ml, this intertetramer cross-linking is eliminated in the TNM reaction even using a 50-fold molar excess of reagent. Bands other than the monomer are observed on SDS-gel electrophoresis (Fig. 4B). At 0.5 mg/ml, bands corresponding to dimers, tetramers, and higher oligomers are found. Cross-linking is still observed at 0.1 mg/ml, but to a significantly lesser extent. This result suggests that small amounts of intertetramer (intermonomer) cross-links, which are independent of the protein concentration, are formed. Mild tryptic digestion of repressor yields NH₂ terminus and core protein as products. SDS-gel electrophoresis of this digest of TNM-modified protein shows the core protein migrating faster than the repressor monomer as expected and the simultaneous disappearance of the dimer subunit band. Thus, the cross-linking in repressor to produce dimer must occur between two NH₂-terminal regions. Electrophoresis of core protein modified with TNM does not demonstrate any cross-linked protein in either native or SDS-polyacrylamide gels. The modifications discussed below were carried out at 0.1 mg/ml of protein concentration to eliminate intertetramer cross-links and to minimize intertetramer cross-links (<5%).

Effects of TNM Modification on Protein Activities—The effects of TNM modification at increasing molar excesses are shown in Fig. 3. Minimal oxidation of the remaining cysteine, 281, occurs in NEM-modified repressor with up to 50-fold molar excess of TNM over monomer. Inducer-binding activity is decreased only slightly by the modification reaction at all excesses. However, the operator-binding activity decreases with increasing TNM molar excess, with essentially no activity remaining at a 50-fold TNM excess/monomer. Surprisingly, an increase in nonspecific DNA-binding activity is observed at a 10-fold excess of TNM, while significant loss of this activity occurs at higher molar excesses of reagent.

Amino Acid Analysis and Identification of Nitrated Tyrosine Residues—Acid hydrolysis of the protein modified with a 50-fold molar excess of TNM followed by amino acid analysis reveals that ~10% of the tyrosine residues are nitrated to NO₂-tyrosine. The NH₂ terminus and core protein were separated by mild tryptic digestion followed by passage through a Sephadex G-75 column. More than 80% of the nitrotyrosine formed is found in the NH₂ terminus. The four tyrosine-containing peptides found in the NH₂-terminal region can be separated

FIG. 2. Operator DNA-binding curves for modified repressors. TNM modification was carried out in 0.24 M potassium phosphate, 5% glucose, pH 7.8, buffer with a protein concentration of 1.0 mg/ml. NEM modification was performed as described under “Materials and Methods” using 25-fold molar excess of reagent. ●, native repressor; ■, NEM-modified repressor; ▲, native repressor modified with a 4-fold molar excess of TNM per monomer; ▼, NEM-modified repressor modified with a 4-fold molar excess of TNM per monomer.

FIG. 3. Characterization of TNM-NEM modified repressors. TNM modification was carried out at a protein concentration of 0.1 mg/ml in 0.24 M potassium phosphate, 5% glucose, pH 8.0, buffer. The protein was previously modified with NEM as described under “Materials and Methods.” The TNM reaction was monitored at increasing molar excesses of reagent up to 50-fold. A, determination of unoxidized cysteine residues; B, determination of IPTG-binding activity; C, determination of operator DNA-binding activity; D, determination of nonspecific DNA-binding activity. Values represent mean ± standard deviation from at least three different samples.
Modification of the Tyrosine Residues of lac Repressor

Amino acid analysis indicated that ~70% of the NO$_2$-tyrosine present is at position 17, ~20% at position 7, and less than 10% at positions 12 and 47.

Reduction of TNM-modified Protein with Sodium Dithionite—NO$_2$-tyrosine can be reduced to NH$_2$-tyrosine by sodium dithionite. The effect of this reduction on the operator and nonspecific DNA-binding activities is shown in Fig. 5 and Table I. Reduction of the 50-fold TNM/monomer-NEM-modified repressor restores ~35% operator DNA binding and all (or more) nonspecific DNA-binding activity. Minimal effects are observed on any binding activities following treatment of NEM-modified repressor. Amino acid analysis indicates the NO$_2$-tyrosine peak disappears upon reduction. No absorbance at 428 nm is found in the reduced, TNM-NEM-modified repressor. After reduction, NH$_2$-tyrosine can be detected by amino acid analysis, eluting after the neutral amino acids but prior to elution of the basic amino acids on the short column.

Nitrification of Core Protein—The trypsin-resistant core protein of repressor exhibits lower affinity for both operator DNA and nonspecific DNA than repressor (12). NEM protection is carried out prior to nitrification because all 3 cysteines are located in this region. No further cysteine oxidation by TNM is observed under the conditions used. At a 30-fold molar excess of TNM, only 20% of the operator DNA binding is retained. After reduction with sodium dithionite, the binding activity is restored to 50%. No quantitatable NO$_2$-tyrosine peak is ob-

![Fig. 4. Electrophoresis of modified repressors. A, native polyacrylamide gel (5%) electrophoresis demonstrates the cross-linking of tetramers: a, NEM-modified repressor; b, 10-fold TNM/monomer-NEM-modified repressor; c, 2-fold TNM/monomer-NEM-modified repressor. The protein concentration used in these reactions was 0.5 mg/ml. d, 50-fold TNM/monomer-NEM-modified repressor; the protein concentration used in this reaction was 0.1 mg/ml. B, SDS-polyacrylamide gel (7.5%) electrophoresis demonstrates subunit cross-linking: a, NEM-modified repressor; b, NEM-modified repressor in the presence of calf thymus DNA; c, 2-fold TNM/monomer-NEM-modified repressor; d, 2-fold TNM/monomer-NEM-modified repressor with mild digestion by trypsin (1%, w/w for 20 min); e, 2-fold TNM/monomer-NEM-modified repressor in the presence of calf thymus DNA; f, 2-fold TNM/monomer-NEM-modified repressor in the presence of calf thymus DNA and digested by trypsin (1%, w/w for 20 min); g, 10-fold TNM/monomer-NEM-modified repressor; h, 10-fold TNM/monomer-NEM-modified repressor in the presence of calf thymus DNA; i, 10-fold TNM/monomer-NEM-modified repressor with mild digestion by trypsin (1%, w/w for 20 min); j, 10-fold TNM/monomer-NEM-modified repressor in the presence of calf thymus DNA and digested by trypsin (1%, w/w for 20 min). The protein concentration used in these reactions was 0.5 mg/ml. The tetramer band observed in the channel of NEM-modified repressor in the presence of calf thymus DNA may be due to the incomplete dissociation of tetramer by SDS.

![Fig. 5. Effects of reduction on the operator DNA binding of TNM-NEM-modified repressors. TNM modification was carried out at a protein concentration of 0.1 mg/ml in 0.24 M potassium phosphate, 5% glucose, pH 8.0, buffer. The molar excess used was 50-fold. All the modification and binding assays were performed as described under “Materials and Methods.” A, operator DNA binding curves for modified repressors. ▲—▲, NEM-modified repressor; ●—●, TNM-NEM-modified repressor; ■—■, TNM-NEM-modified repressor followed by reduction with a 70-fold molar excess of Na$_2$S$_2$O$_4$. ▼—▼, NEM-modified repressor in the presence of IPTG to measure the nonspecific DNA binding background. B, nonspecific DNA binding curves for modified repressors. ●—●, NEM-modified repressor; ▲—▲, TNM-NEM-modified repressor; ■—■, Na$_2$S$_2$O$_4$-reduced, TNM-NEM-modified repressor.](http://www.jbc.org/late/)
served in amino acid analysis utilizing the usual amount of protein (3 μmol); however, a NO₂-tyrosine peak can be detected by using a larger amount of protein. Paper electrophoresis does not reveal any visible yellow spots corresponding to NO₂-tyrosine-containing peptides. Inducer-binding activity is not affected by either nitration or reduction; no cross-linking of the modified protein can be observed by either native or SDS-gel electrophoresis.

**TNM Modification in the Presence of Nonspecific DNA**—Because low ionic strength buffer is required for the binding of repressor to nonspecific DNA, 0.048 M potassium phosphate replaced 0.24 M potassium phosphate. Calf thymus DNA was used as a nonspecific DNA and was sheared to small size prior to use. The protein concentration used in this reaction was 0.5 mg/ml. Both operator DNA-binding and nonspecific DNA-binding activities were monitored. At a 10-fold molar excess of TNM, significant loss of operator binding is found in the TNM-NEM-modified repressor, but in the presence of nonspecific DNA, essentially all the operator DNA-binding activity is retained (Fig. 6). Instead of a loss of nonspecific DNA binding, we observed an increase in this binding activity (Fig. 7). Although exact Kd values cannot be measured by this method, the differences observed suggest an increase in the affinity of these modified proteins for nonspecific DNA.

![Fig. 6. Operator DNA binding curves for repressor modified with TNM in the presence of nonspecific DNA.](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>Operator DNA-binding activity</th>
<th>% Non-specific DNA-binding activity</th>
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<tbody>
<tr>
<td>NEM-modified repressor</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50-fold TNM-NEM-modified repressor</td>
<td>&lt;5</td>
<td>22</td>
</tr>
<tr>
<td>Na₂S₂O₄-reduced, 50-fold TNM-NEM-modified repressor</td>
<td>35</td>
<td>140</td>
</tr>
</tbody>
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* Under stoichiometric conditions, i.e. [0] > K_d.
* Under conditions to measure K_d, i.e. [DNA] ≈ K_d.
* TNM modification was carried out at a protein concentration of 0.1 mg/ml in 0.24 M potassium phosphate, 5% glucose, pH 8.0, buffer as described under “Materials and Methods.”
* Na₂S₂O₄ reaction was conducted as described under “Materials and Methods.”

**DISCUSSION**

In previous studies of TNM and iodine reaction with repressor, all 3 cysteine residues were oxidized; cysteine oxidation was later shown to cause loss of operator DNA binding (21). Thus, the effects of tyrosine modification alone were not resolved in these studies. In this paper, we have measured the operator DNA-binding activities following nitration of repressor in which the cysteines have been protected by NEM (22). Our results demonstrate that cysteine oxidation obscured the effects of nitration in the previous TNM studies, and care must be exercised to monitor the exclusive effects of tyrosine modification. Intersubunit cross-linking of repressor was found in the TNM-modification studies of repressor by Alexander et al. (20). In the present study, we have observed both inter- and intratetramer cross-linking. Intertetramer cross-linking can be reduced by lowering the protein concentration in the reaction mixture, but a low level of intratetramer cross-linking is observed independent of protein concentration. By determining conditions which minimize both cysteine oxidation and cross-linking of the proteins, it has been possible to focus on the effects of modifying tyrosine residues alone.

The maintenance of inducer binding following reaction with TNM indicates that the conformation of the region responsible for this activity is not significantly altered in the modified protein. In contrast, both operator and nonspecific DNA-binding capacities are diminished by modification. Reduction of NO₂-tyrosine to NH₂-tyrosine in the modified repressor restores the nonspecific binding completely and the operator-binding activity partially. The tyrosine residues nitroated are located in the NH₂-terminal region, primarily at tyrosines 7 and 17; no other amino acids are detectably affected by the modification. Introduction of the NO₂-group into the phenyl ring of a tyrosine residue decreases the pK of the phenol and results in a charged group at neutral pH. This charge may block the interactions of repressor with the DNA by interfering with either hydrogen bonding (37, 38) or intercalation (39) or by charge repulsion of the phosphate backbone of DNA.
Reduction of the NO2-tyrosine to NH2-tyrosine restores the pK’ of the phenol to a value similar to that for tyrosine (40). The recovery of repressor binding to nonspecific DNA after reduction suggests involvement of tyrosines via an electrostatic or hydrogen-bonding component in interacting with nonspecific DNA, while the lack of complete recovery of operator DNA-binding activity suggests that the presence of the NH2-group may offer some steric hindrance to binding.

Replacement of tyrosines 273 and 282 in the core region with other amino acids by suppression of nonsense mutants alters the characteristics of the repressor, while no effect is observed on replacing tyrosines 126 and 204 (4, 6). Modification of the core protein with TNM results in loss of operator binding, and the reduction of NO2-tyrosine to NH2-tyrosine partially restores the binding capacity. The actual decrease observed in operator-binding affinity for the core region following TNM modification is significantly less than for native repressor, since the concentrations of components in the assay are near the values for the dissociation constant as opposed ~100-fold higher as for repressor (i.e. the repressor assay conditions are at stoichiometric binding concentrations). While failure to restore all the operator-binding activity for the core protein parallels the results for native repressor and suggests that some of the effects observed for native repressor may be attributed to modification in the core region, the differences between the patterns for core and repressor binding loss/restoration indicate involvement of tyrosines in the NH2-terminal region as well as the core region in the decreased affinity observed for nitrated NEM repressor.

Calf thymus DNA was used to determine the effects of nonspecific DNA on the modification reaction. Nonspecific DNA presence has been reported previously to protect against the loss of operator-binding activity on iodination of tyrosine residues, although this effect may be due to protection of the cysteines from oxidation (19, 21). Operator-binding activity and nitration of tyrosines were both protected by the presence of nonspecific DNA during the TNM modification of NEM-modified repressor. A similar protection was observed for oxidation of cysteine with N-bromosuccinimide (21). This effect of nonspecific DNA on nitration may be primarily due to steric hindrance to reagent access. The difference observed in nitration pattern for the modified protein is in the NH2 terminus, and this region has been previously suggested to be a part of the site for nonspecific and operator DNA binding (1, 8, 10, 11). This result demonstrates that the nonspecific DNA protection against nitration of the tyrosine residues in the NH2 terminus affects operator DNA binding directly. This effect indicates, as anticipated, that a portion of the operator binding site involves this region; the effects of nitration on core-operator binding indicate that this region also is involved in binding to the operator (12, 41).

The NEM-reacted protein exposed to TNM in the presence of nonspecific DNA yielded products with higher nonspecific DNA binding affinity than control protein. Gel electrophoresis demonstrates that a small degree of inter- and intrateramer cross-linking occurs in these proteins. At higher molar excesses of TNM, decreased nonspecific DNA-binding activity was observed, and the presence of calf thymus DNA in the reaction mixture protected against the loss of nonspecific binding. We propose that two opposing effects in terms of nonspecific DNA binding are involved in the TNM modification: (i) the nitration of tyrosine residues decreases the pK’ of the tyrosine residues, which results in loss of nonspecific DNA binding, and (ii) the cross-linking of monomers, which in some fashion results in a higher nonspecific DNA binding affinity for repressor. In our studies, we found the inter-/intramolecular cross-linking occurred between NH2-terminal regions of the monomers, the site implicated in nonspecific binding (1, 8, 10, 11, 41). No cross-linking was observed in the core region whether isolated or in the intact repressor.

In summary, the residues affected by nitration are primarily tyrosines 7 and 17 in the NH2 terminus, and the presence of nonspecific DNA protects against nitration of these residues. These 2 residues must be involved in or close enough to portions of the binding site(s) for both operator and nonspecific DNA binding for nitration to influence these interactions. Reduction of nitrated NEM-repressor eliminates all the NO2-tyrosine residues formed and restores complete nonspecific DNA binding and partial operator DNA binding. These different effects observed on operator and nonspecific binding in response to reduction indicate that the tyrosine residues affected contribute differently to the two types of binding. Since nitration of NEM-modified core protein alters the operator DNA binding, tyrosine residues in this region are also implicated in operator DNA binding.

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