Iodination of *Escherichia coli lac* Repressor. Effect of Tyrosine Modification on Repressor Activity†

T. G. Fanning

ABSTRACT: Treatment of *Escherichia coli lac* repressor with iodine (KI_3) at 4° and pH 7.5 resulted in the rapid loss of repressor DNA binding activity. At a 30-fold molar excess of iodine to repressor, inactivation was complete within 15 sec. Inducer binding under the same conditions was only slightly affected. Iodinated repressor remained tetrameric, indicating that no gross structural alteration of the protein had taken place. Control experiments demonstrated that side products of the reaction did not contribute to the observed activity loss. Moreover, no restoration of binding activity was observed when iodinated repressor was assayed under a variety of assay conditions. Incubation of repressor with *lac* operator containing λ p*lac* DNA during the iodination reaction resulted in approximately 50% protection of binding activity. This protective effect was only partially operator specific, as λDNA lacking the operator binding site afforded roughly 25% protection under the same conditions. Incubation with either inducer or anti-inducer molecules during the iodination reaction did not protect repressor DNA binding activity. Iodination with $K^{131}I_3$ demonstrated that at complete inactivation virtually all of the bound iodine was recovered as iodotyrosine. A minor amount of cysteine oxidation to cysteinesulfonic acid was also detected. This oxidation encompassed no more than 30% of a single cysteine residue (tentatively identified as cysteine-107). Unstable intermediate oxidation products of cysteine did not appear to be involved in the loss of DNA binding activity. Modification of amino acids other than tyrosine and cysteine was not observed. Tryptic digestion of ^{131}I-labeled repressor suggested that approximately 90% of the incorporated radioactivity was located in the repressor N-terminal tryptic peptide. Automated sequence analysis of iodinated repressor confirmed that at roughly 0.5 bound iodine atoms per repressor subunit (corresponding to approximately 5–10% activity loss) tyrosine residues 7, 12, and 17 were labeled in the ratios 1.0:0.5:0.8. Doubling the amount of bound iodine to 1.0 atom per subunit (corresponding to approximately 50–60% activity loss) did not significantly change the pattern of incorporation.

The *lac* repressor of *Escherichia coli* is encoded by a gene located near the operon it controls. This operon, the *lac* operon, consists of a genetically defined promoter region, three structural genes (for β-galactosidase, lactose permease, and transacetylase) and, between the promoter and the first structural gene, a second genetically defined region, the operator. Repressor binds to the operator element of the operon, preventing the movement of promotor-bound RNA polymerase through the operator region and, thus, preventing transcription of the distally located structural genes. Addition of inducer to the medium results in an inactivation of the repressor and a lifting of the transcriptional block (for a review of the *lac* system see Reznikoff, 1972). Although a number of compounds with inducing properties are known, it has recently been shown that an isomer of lactose, allolactose, is the natural inducer of the *lac* operon (Jobe and Bourgeois, 1972).

Within the past several years a great deal has been learned about the repressor molecule and its interactions with operator and inducer. Of particular interest in the present context is the following: *lac* repressor is a tetrameric protein of 1.5×10^4 daltons containing, in all likelihood, one DNA binding site and four independent inducer binding sites (Riggs and Bourgeois, 1968). In vitro studies with purified repressor have shown that the equilibrium constant for repressor–operator binding is 10^{-13} *M* and the half-life of the complex is approximately 45 min (Jobe et al., 1972). Repressor binding to nonoperator DNA has also been demonstrated. Here, the equilibrium constants vary with the source of the DNA and are generally six to seven orders of magnitude lower than with operator (Lin and Riggs, 1972). In the presence of the nonmetabolizable gratuitous inducer isopropyl β-D-thiogalactoside (IPTG),[1] a ternary complex is formed which dissociates with a half-life of less than 1 min into an inducer–repressor complex and free operator (Jobe et al., 1972). Other compounds (anti-inducers) which antagonize the effects of inducers in vivo strengthen the in vitro binding of repressor to operator (Müller-Hill et al., 1964; Jobe et al., 1972). A well-known example is *o*-nitrophenyl β-D-fucoside (ONPF) which increases the half-life of the repressor–operator complex from 45 to 105 min (Jobe et al., 1972). Both inducers and anti-inducers appear to act by inducing conformational alterations in the repressor molecule (Riggs et al., 1970; Laiken et al., 1972). Moreover, since ONPF acts as a competitive inhibitor of IPTG binding, the binding sites of both compounds are probably identical (Jobe et al., 1972).

At present the sequence of both the operator (Gilbert and Maxam, 1973) and the repressor (Beyreuther et al., 1973) are known. Also, electron microscopy has provided a rough

† From the Institut für Genetik, Universität zu Köln, 5 Köln 41, Germany. *Received December 16, 1974.* This work was supported by the Deutsche Forschungsgemeinschaft through SFB 74.

[1] Abbreviations used are: IPTG, isopropyl β-D-thiogalactoside; ONPF, *o*-nitrophenyl β-D-fucoside; MIT, monoiodotryrosine; DIT, diiodotyrosine; MIH, monoiodohistidine; DIH, diiodohistidine; TMS, 0.04 *M* Tris-HCl (pH 7.5), 0.01 *M* $Mg(OAc)_2$, 0.2 *M* KCl, 0.01 *M* mercaptoethanol, and 5% glycerol; BB buffer, 0.01 *M* Tris-HCl (pH 7.5), 0.01 *M* $Mg(OAc)_2$, 0.01 *M* KCl, 0.01 *M* mercaptoethanol, 10^{-4} *M* EDTA, and 0.05 μg/ml of bovine serum albumin; NEM, *N*-ethylmaleimide.

picture of the three-dimensional shape of the repressor molecule (Steitz et al., 1974). Based on electron micrographs, Steitz et al. (1974) have proposed a generalized model of repressor–operator binding in which an asymmetric (dumbbell shaped) repressor molecule binds with its long axis aligned along the length of the operator region. The repressor binding site is proposed to exhibit twofold symmetry, consistent with the known symmetry of the operator region (Gilbert and Maxam, 1973). Adler et al. (1972) have proposed a very detailed model based on genetic and sequence data. Here, not only is the repressor N-terminal region suggested to make contact with the operator, but the involvement of specific amino acids are also proposed. However, a critical evaluation of this model is hampered by a lack of biochemical evidence. Assuming that only a small number of repressor amino acids are involved in DNA binding, the task becomes one of determining their number and position in the primary structure experimentally.

Chemical modification has proven to be a useful tool in the investigation of protein structure–function relationships. The present study was undertaken in an attempt to define the roles of individual amino acids in repressor–DNA complex formation.

Materials and Methods

Preparation of Repressor. *Lac* repressor was prepared from a strain of *E. coli* carrying a temperature-inducible *lac* prophage (strain BMH 593 obtained from B. Müller-Hill). The *lac* promoter element on this prophage contains the i^{SQ} mutation, a mutation resulting in an overproduction of repressor (Miller et al., 1970; Platt et al., 1973). The procedure used to purify the repressor was similar to that described by Müller-Hill et al. (1971) as modified by Platt et al. (1973). Yields of 80–90 mg of purified repressor/100 g of frozen cells were normally obtained and stored in small aliquots at −20° in TSM buffer (0.04 *M* Tris-HCl (pH 7.5), 0.01 *M* $Mg(OAc)_2$, 0.2 *M* KCl, 0.01 *M* mercaptoethanol, and 5% glycerol). Purified repressor showed a major band in sodium dodecyl sulfate polyacrylamide gels (90–95% purity) and exhibited approximately 10% of the theoretical DNA binding activity.

Iodination. Stock iodine solutions (0.01 *M* I_2, 0.04 *M* KI; both analytical grade) were made up in distilled water. In a typical iodination experiment concentrated solutions of repressor were diluted to 0.6–0.8 mg/ml and dialyzed at 4° for 5–6 hr against TSM buffer without mercaptoethanol. This step was necessitated by the fact that mercaptoethanol, a component of the buffer system used to stabilize repressor, reacts readily with iodine. Aliquots containing 30–40 μg of the dialyzed repressor were distributed into small plastic culture tubes and allowed to react with an equal volume of iodine solution for 1 min at 4°. The reactions were stopped by the addition of a large excess of mercaptoethanol. Iodine solutions were made up by diluting a stock solution to the appropriate concentration with TSM buffer (without mercaptoethanol) shortly before the iodination reaction. Following the reaction aliquots of the iodinated repressor solutions were removed, diluted if necessary, and tested for activity. Iodination with $K^{131}I_3$ was performed in an essentially identical manner. To a stock iodine solution was added enough ^{131}I ($Na^{131}I$, 200 mCi/ml, Amersham Buchler) to give a specific activity of approximately 7×10^{12} cpm/mol of iodine. At the end of the incubation period the samples were dialyzed exhaustively to remove unbound ^{131}I and either dried in vacuo or counted for radioactivity.

Assay of Repressor. [^{14}C]IPTG binding to repressor was assayed either by the filter binding procedure described by Riggs and Bourgeois (1968) or by the equilibrium dialysis method of Gilbert and Müller-Hill (1966). The DNA binding activity of the repressor was measured by trapping repressor–DNA complexes on nitrocellulose filters essentially following the method of Riggs et al. (1968). Binding assays were carried out in BB buffer (0.01 *M* Tris-HCl (pH 7.5), 0.01 *M* $Mg(OAc)_2$, 0.01 *M* KCl, 10^{-4} *M* EDTA, 0.01 *M* mercaptoethanol, and 0.5 μg/ml of bovine serum albumin). The DNA used in the present study was obtained from an *E. coli* strain carrying a nondefective temperature-inducible *lac* prophage (strain BMH 782, originally obtained from J. Shapiro). Preparation of phage and purification of phage DNA were performed essentially by the method of Riggs et al. (1970) except that no helper phage was used. Preparations of purified repressor were tested for their ability to bind DNA and the concentration of repressor necessary to obtain a plateau level of binding was determined. Iodinated repressor was routinely assayed at concentrations of one-half to two-thirds plateau level.

Zone Sedimentation. Zone sedimentation of repressor was done in 5-ml block gradients of 17.5–30% glycerol made up in BB buffer. Each gradient contained: 0.5 ml of 30%, 1.0 ml of 25%, 2.0 ml of 20%, and 1.5 ml of 17.5% glycerol. A sample of 100–200 μl was gently layered on top of the gradient and the tubes were centrifuged at 4° for 16 hr at 45,000 rpm in the SW 50.1 rotor. Marker proteins were γ-globulin (7 S) and β-lactoglobulin (~3 S) which correspond to the size of the repressor tetramer and monomer, respectively.

Identification of ^{131}I-Labeled Amino Acids. Samples of ^{131}I-labeled repressor were treated with pepsin and Pronase as described by Roholt and Pressman (1972). After digestion and drying, the residue was dissolved in 5 μl of 0.5 *M* formic acid and spotted on a 20 × 20 cm thin-layer plate (G 1440 cellulose plate, Schleicher and Schüll). Electrophoresis was carried out for 1 hr at 800 V in a pH 2.1 buffer (8% acetic acid, 2% formic acid, and 90% water). The dried plate was sprayed with ninhydrin and the area of the plate through which the sample had passed was partitioned into rectangular fractions with a pencil and ruler, taking care not to gouge the cellulose (see Figure 6). With a metal spatula the fractions were scraped from the plate and the cellulose powder collected in glass tubes. Elution of the amino acids was accomplished by gently swirling the powder in 40% acetic acid for at least 30 min. At the end of this time an aliquot was removed, dissolved in Bray's solution (Bray, 1960), and counted in a liquid scintillation counter. Recoveries were normally 85–90% of the input radioactivity. In a control experiment repressor was iodinated in 8 *M* urea. Following enzymatic digestion and thin-layer electrophoresis four peaks of radioactivity were detected and assumed to correspond to the mono and diiodo derivatives of tyrosine and histidine. The mobilities of the iodinated amino acids, relative to diiodotyrosine, were: DIT (1.0), MIT (1.3), DIH (1.9), and MIH (3.7). This is in excellent agreement with the values obtained by Roholt and Pressman (1972) with the exception of MIH which, in their system, has a relative mobility of 2.7.

Alkylation of Sulfhydryl Residues. The cysteine residues of iodinated repressor were labeled with [^{14}C]-*N*-ethylmaleimide ([^{14}C]NEM). Following iodination the protein solution was dialyzed extensively against 10% acetic acid and dried in vacuo. The dried protein was dissolved in 8 *M*

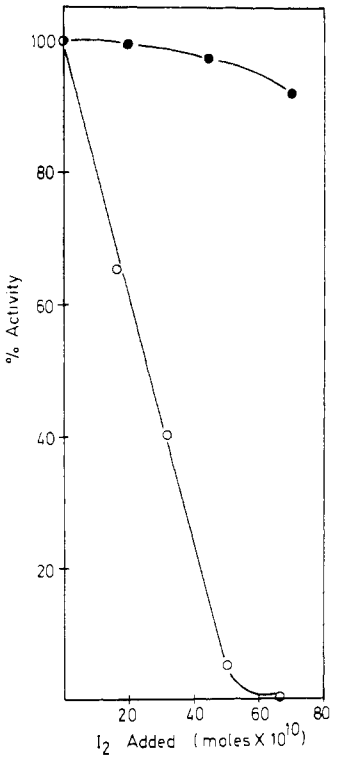

FIGURE 1: Effect of increasing iodine concentration on repressor activity. Samples containing approximately 1.5×10^{-10} mol of repressor were incubated with the indicated amounts of iodine for 1 min at 4°. The reactions were terminated with mercaptoethanol and aliquots from each sample were assayed for either ^{32}P-λ *plac* DNA binding (○), or [^{14}C]IPTG binding by the equilibrium dialysis method (●). The curve for DNA binding is the average of two, that for [^{14}C]IPTG binding the average of four, independent experiments.

urea, 0.02 *M* Tris-HCl (pH 8.0), and 0.01 *M* mercaptoethanol and incubated for 4 hr at 37°. An aliquot of the solution was then removed and the sulfhydryl content determined by the method of Ellman (1959). The solution was made 0.15 *M* in Tris-HCl (pH 6.9) and [^{14}C]NEM in 8 *M* urea, 0.01 *M* Tris-HCl (pH 6.9) was added to give 3–5-fold excess over sulfhydryl groups. The [^{14}C]NEM solution was prepared by diluting a stock [^{14}C]NEM solution (5.4 Ci/mol, Schwarz/Mann) with nonradioactive NEM to give a final specific activity of approximately 35 μCi/mmol. After a 1-hr incubation at room temperature the reaction was stopped by the addition of a large excess of mercaptoethanol. The solution was dialyzed exhaustively against 10% acetic acid to remove unbound reagent and dried in vacuo.

Digestion with Trypsin. Tryptic digestion of ^{131}I- or [^{14}C]NEM-labeled repressor was carried out in 2 *M* urea. The dried protein was dissolved in 8 *M* urea and the solution diluted to 2 *M* urea with 0.1 *M* Tris-HCl (pH 8.0). Stirring was begun and an aliquot of trypsin (treated with TPCK by the method of Carpenter (1967)) in 10^{-3} *M* HCl was added to give a concentration of 1:20 (w/w) with respect to repressor. The solution was allowed to incubate for 4 hr at room temperature, after which a second aliquot of trypsin was added. After an additional 16-hr incubation the solution was acidified to pH 2.5 with glacial acetic acid. The trypsin treated sample was adjusted to 4 *M* urea with a concentrated urea solution and an aliquot was subjected to gel chromatography on a "double-decker" Sephadex column. The lower layer of the column contained 1 × 27 cm of G-50 and the upper layer 1 × 27 cm of G-25. The column was equilibrated with 4 *M* urea (pH 9.5) and was developed at room temperature. Fractions of approximately 0.5 ml were collected, dissolved in Bray's solution, and counted in a liquid scintillation counter. Recovery of input radioactivity was normally greater than 95%.

Amino acid analyses were carried out according to the procedures of Spackman et al. (1958). Samples were hydrolyzed in vacuo with 5.7 *M* HCl for 20 hr at 110°. Tryptophan was determined after 20-hr hydrolyses in vacuo at 110° with 4 *M* methanesulfonic acid and 0.2% 3-(2-aminoethyl)indole as suggested by Liu and Chang (1971). Alkaline hydrolyses with $Ba(OH)_2$ were carried out for 8 hr at 110° as described by Knox et al. (1970).

Automated sequence analyses were performed with the Beckman Sequencer using a program similar to that described by Edman and Begg (1967). Sequence analyses were routinely carried out through 20 cycles, after which the remaining core protein was washed from the machine and collected in 0.1 *M* NaOH. The amino acid samples were dried in vacuo and dissolved in absolute methanol and aliquots were removed for scintillation counting. A portion of the core protein was also counted to determine the amount of radioactivity not released during the run. Sequencer runs were kindly performed by Dr. Konrad Beyreuther.

Results

Iodination of Repressor. Repressor was routinely assayed in vitro by one of two procedures: the binding to ^{32}P-λ *plac* DNA or the binding of the gratuitous inducer [^{14}C]isopropyl β-D-thiogalactoside ([^{14}C]IPTG). Incubation of repressor with increasing concentrations of iodine resulted in the progressive loss of the DNA binding activity. IPTG binding activity, under the same conditions, was only marginally affected (Figure 1). Thus, at an iodine concentration sufficient to cause a greater than 95% reduction in DNA binding, less than 5% of the IPTG binding activity was lost. This slight drop in IPTG binding activity may be insignificant since in several experiments IPTG binding activity has either remained constant or, in fact, shown a slight increase after iodination.

The iodination reactions were carried out at 4° in a pH 7.5 buffer containing 0.2 *M* KCl to ensure maximal repressor stability. Although activity loss was complete within seconds (see below), a reaction time of 1 min was chosen for convenience. Even under these very mild conditions only moderate amounts of iodine were required to produce complete activity loss. In the experiments depicted in Figure 1 each incubation mixture contained approximately 1.5×10^{-10} mol of repressor. A 95% activity loss required 50×10^{-10} mol of iodine, corresponding to no more than an eightfold molar excess over repressor subunits.

Zone Sedimentation. Although inducer (IPTG) binds equally well to both tetrameric repressor and repressor subunits, only the tetrameric form of repressor is capable of binding to DNA (Gilbert and Müller-Hill, 1967; Riggs et al., 1970). Thus, a trivial explanation for the differential loss of repressor activity is the dissociation of iodinated repressor into its component subunits. Zone sedimentation, however, indicated that the tetrameric form predominated after the reaction (Figure 2).

In order to detect iodine modified repressor by zone sedimentation it was necessary to use concentrated repressor solutions (50–100 μg/ml). DNA binding tests, on the other hand, were performed at repressor concentrations between 10^{-2} and 2×10^{-2} μg/ml. The possibility that iodinated re-

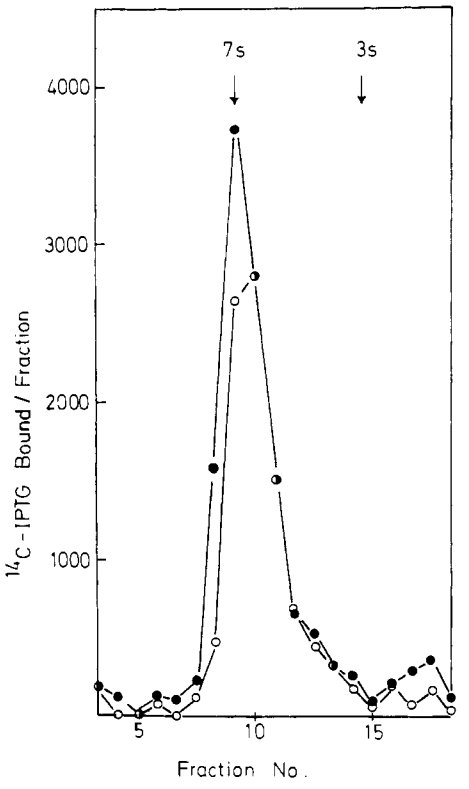

FIGURE 2: Glycerol gradient analysis of iodinated repressor. Repressor was treated with a concentration of iodine chosen to give 100% inactivation. Following the reaction samples (85 μg) of untreated and treated repressor were sedimented in 17.5–30% glycerol gradients, and fractions were collected and assayed for [^{14}C]IPTG binding using the nitrocellulose filter technique. (●) Iodine-treated repressor; (○) untreated repressor.

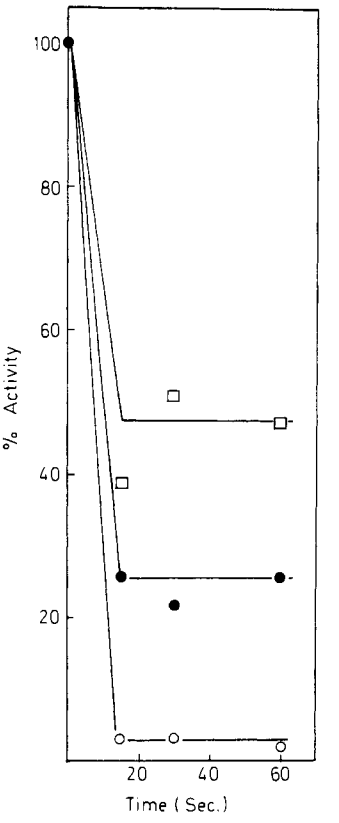

FIGURE 3: Kinetics of repressor inactivation. Three separate experiments were performed at iodine concentrations chosen to give 100, 75, and 50% inactivation. Repressor and iodine solutions were rapidly mixed at 4°. At the indicated times an aliquot was removed and the reaction terminated with excess mercaptoethanol. The samples were then diluted and tested for ^{32}P-λ p*lac* DNA binding.

pressor remained tetrameric in concentrated solutions, but dissociated upon extreme dilution, was tested with ^{131}I-labeled repressor. Repressor was labeled to 4×10^5 cpm/μg with sufficient iodine to ensure complete activity loss. After extensive dialysis the solution was diluted to 0.85 μg/ml in BB buffer (the buffer routinely used for binding tests) and an aliquot was centrifuged in a 17.5–30% glycerol gradient made up in BB buffer. The final concentration of repressor in the gradient was no greater than 5×10^{-2} μg/ml. A single peak of ^{131}I-labeled repressor was seen at 7 S (no slower sedimenting material was observed), indicating that iodine-treated repressor, even upon extensive dilution, remained tetrameric (data not shown).

Alteration of Assay Conditions. It is known that the binding of native repressor to DNA is relatively insensitive to the composition of the binding mixture (Riggs et al., 1970). Chemical modification of the protein might, however, alter this situation. Iodinated repressor normally retained a small amount (1–10%) of residual DNA binding activity. This residual activity could conceivably reflect a dependence of the modified repressor on one or more components in the binding mixture. If this were the case, then binding activity might be restored to its original level by, for example, doubling or tripling the concentration of a particular buffer component. Systematic changes in the concentrations of Mg^{2+} (10^{-3}–4×10^{-2} *M*), Tris (3×10^{-3}–4.5×10^{-2} *M*), and KCl (3×10^{-3}–4.5×10^{-2}*M*) and pH (6.4–8.5) of the assay buffer, however, had no effect on the DNA binding activity of iodinated repressor.

Another conceivable, though unlikely, possibility was that iodinated repressor was capable of binding to DNA, but bound much more slowly than the unmodified repressor. However, time course experiments with iodinated repressor carried out to 1 hr failed to detect any significant DNA binding activity.

Kinetics of Activity Loss. The loss of repressor DNA binding activity during the iodination reaction was very rapid; inactivation was complete, even at intermediate iodine concentrations, within 15 sec (Figure 3). In other systems, for example, lysozyme (Covelli and Wolff, 1966) and fructose 1,6-diphosphatase (Rosen and Rosen, 1967) iodination has also been shown to be complete within minutes. The extremely rapid inactivation of repressor, however, raised the question of a possible involvement of reaction side products in the activity loss.

Mercaptoethanol is known to react almost instantaneously with iodine and one of the products of this reaction, diethanol disulfide (oxidized mercaptoethanol), has been shown to inhibit phenylalanyl-tRNA binding to *E. coli* ribosomes (Furano, 1968). Since mercaptoethanol was used to stop the iodination reactions, the possible inhibitory effects of reaction side products were tested. A solution of untreated repressor was dialyzed against TSM buffer without mercaptoethanol. An aliquot of the diffusate was removed and incubated with iodine, and excess mercaptoethanol followed by untreated repressor were added to the mixture after 1 min. The DNA binding activity of this sample was no different than that of an untreated control sample, indicating that side products of the iodination reaction do not contribute to the observed activity loss.

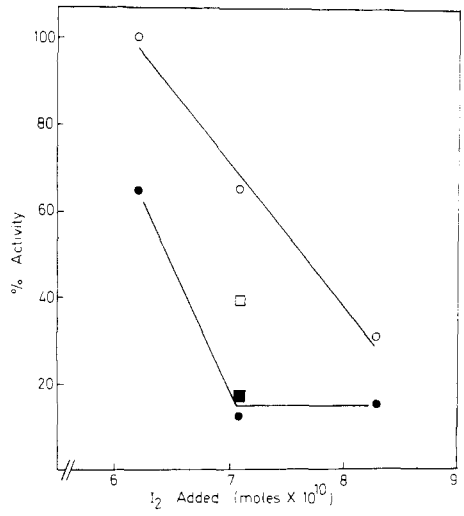

FIGURE 4: Effect of bound DNA on repressor inactivation. Repressor (6.6×10^{-12} mol/reaction) was treated with the indicated amounts of iodine for 1 min at 4° in the presence (○) and absence (●) of saturating concentrations of ^{32}P-λ *plac* DNA. To determine whether the effect was specific for operator containing DNA, experiments were also conducted in the presence (□) and absence (■) of λDNA. Three independent experiments with λDNA were done at 7×10^{-10} mol of I_2 and the data shown here are representative. For additional details see text.

Protection by Bound DNA. Amino acid residues in the binding site of a protein would be expected to remain unmodified, or undergo modification more slowly, when complexed with substrate (Means and Feeney, 1971). Thus, repressor bound to DNA should show an inactivation curve different from that of uncomplexed repressor. To test this prediction a solution of ^{32}P-λ *plac* DNA in 0.01 *M* Tris-HCl (pH 7.5)–0.01 *M* KCl–10^{-3} *M* EDTA was first treated with a large excess of iodine at 4° to remove any iodine binding sites due to the presence of contaminating proteins. Following the reaction the DNA solution was dialyzed exhaustively to remove reaction side products. Under the conditions used no reaction between iodine and DNA was expected (Commerford, 1971) and iodine-treated DNA exhibited no loss of binding activity.

Protection experiments were conducted in the following manner. Three identical repressor solutions were made up in BB buffer without mercaptoethanol or bovine serum albumin. To two of the solutions were added saturating amounts of iodine-treated ^{32}P-λ *plac* DNA. The third solution received buffer. The mixtures were allowed to incubate at 4° for 20 min and then iodine was added to the mixture which had received buffer and to one of the mixtures that had received DNA. After a 1 min incubation at 4° the iodination reactions were stopped with excess mercaptoethanol. Buffer was then added to the two solutions that had received DNA and DNA was added to the solution that had received buffer. After 10 min at room temperature the solutions were diluted with BB buffer and aliquots were passed through nitrocellulose filters. DNA binding activity was then calculated for the iodine-treated samples using the untreated sample as standard. Similar experiments were repeated at three different iodine concentrations and in each case significant DNA-dependent protection of repressor activity was observed (Figure 4). Under optimal conditions approximately 50% of the repressor activity was protected by λ *plac* DNA.

To determine whether the protective effect was specific for DNA containing the *lac* operator, a protection experiment was carried out using equivalent amounts of iodine-treated λDNA lacking the *lac* operator element. The result (from three independent experiments) indicated that approximately one-half of the observed protection under optimal conditions was not dependent upon operator binding (Figure 4).

Table I: Amino Acid Analysis of Iodinated Repressor.

Amino Acid[a]	I-R/R[b]	Amino Acid[a]	I-R/R[b]
Asp	1.00	Met	0.98[d]
Thr	1.04	Ile	0.99
Ser	1.05	Leu	1.03
Glu	1.03	Phe	1.01
Pro	0.96	Lys	1.02
Gly	1.09	Trp	1.50[e]
Ala	1.00[c]	Arg	1.07
Val	1.03		

[a] Hydrolyses, except for Met and Trp, were conducted in 5.7 *M* HCl at 110° for 20 hr. [b] Ratio (iodinated repressor/untreated repressor) of recovered amino acids. [c] The relative concentrations of amino acids in the two samples were determined by normalizing the amount of recovered Ala to unity. [d] Determined after 8-hr hydrolyses at 110° with $Ba(OH)_2$ after Knox et al. (1970). [e] Determined after 20-hr hydrolyses at 110° with methanesulfonic acid after Liu and Chang (1971).

Incubation with IPTG and ONPF. Since the binding of inducer and anti-inducer molecules appears to cause conformational alterations in the repressor molecule (Riggs et al., 1970; Laiken et al., 1972), it was of interest to determine if DNA binding activity could be protected by the presence of these compounds in the reaction mixture. Repressor was incubated with either a 2×10^4-fold molar excess of inducer (IPTG) or a 10^3-fold molar excess of the anti-inducer *o*-nitrophenyl β-D-fucoside (ONPF) for 20 min prior to iodination. The iodination reactions were performed at 4° for 1 min as usual, after which the mixtures were dialyzed exhaustively to remove IPTG or ONPF. The dialyzed solutions were then tested for DNA binding. Neither IPTG nor ONPF at the above indicated concentrations afforded protection of repressor DNA binding activity during the iodination reaction.

Amino Acid Analysis. The amino acid analysis of untreated repressor and repressor iodinated to greater than 95% activity loss is shown in Table I. The data are presented for each amino acid as the ratio (nanomoles present in iodinated repressor)/(nanomoles present in untreated repressor) as suggested by Koshland et al. (1963). In all cases, with the exception of tryptophan, excellent agreement between treated and untreated preparations was observed. In the case of tryptophan somewhat higher recovery was obtained with the iodinated sample. *Lac* repressor contains two tryptophan residues per subunit (Beyreuther et al., 1973). Based on the number of alanine residues per repressor subunit (44), the number of tryptophan residues recovered were approximately 2 and 3 for untreated and iodinated samples, respectively. The cause of this discrepancy is unknown, but probably reflects differential rates of tryptophan destruction between the two samples.

Cysteine residues in proteins are known to react readily with iodine to give a stable final product, cysteinesulfonic acid (Koshland et al., 1963). The presence of cysteinesulfonic acid residues in iodinated repressor was determined by alkylation with *N*-ethylmaleimide (NEM). Repressor was

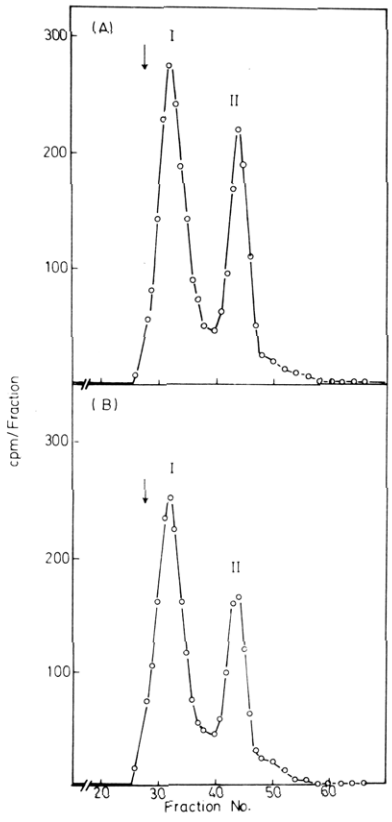

FIGURE 5: Sephadex chromatography of [^{14}C]NEM-treated repressor following trypsin digestion. Samples were alkylated, digested with trypsin, and chromatographed as described under Materials and Methods. (A) Untreated repressor; (B) repressor iodinated to greater than 98% activity loss. The total cpm under peaks I and II are: (A) 1660 and 925, and (B) 1650 and 670. The arrow indicates the void volume of the column.

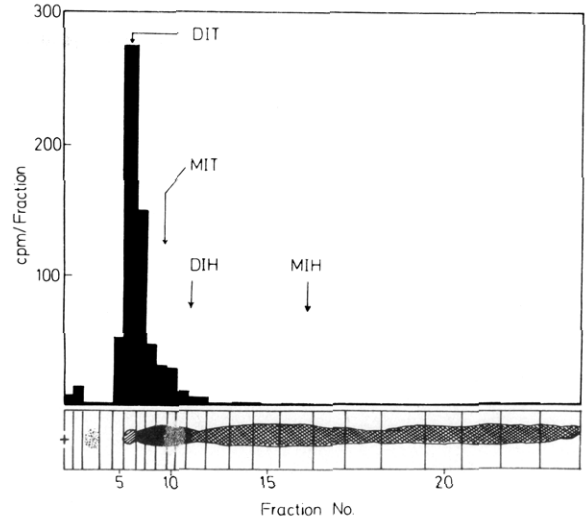

FIGURE 6: Identification of ^{131}I-labeled amino acids. ^{131}I-repressor was digested with pepsin and Pronase and the residue subjected to thin-layer electrophoresis at pH 2.1. The chromatogram was divided into fractions which were scraped from the plate, extracted with acetic acid, and counted for radioactivity. The lower illustration represents the ninhydrin reactive material and the upper represents the amount of radioactivity per fraction. The + in the lower illustration is the origin. The positions of the various residues were determined as described under Materials and Methods.

iodinated to greater than 98% activity loss, dialyzed, dried, and alkylated as described under Materials and Methods. Under the conditions used all oxidation intermediates would be expected to be reduced to cysteine and, hence, should react with the alkylating agent (Trundle and Cunningham, 1969). Cysteinesulfonic acid, a stable oxidation product, would not be expected to react. The results of a typical alkylation experiment are depicted in Figure 5. Two peaks of radioactivity following Sephadex chromatography of trypsin-digested [^{14}C]NEM-treated repressor were detected. The first peak represents two cysteine-containing peptides of 27 and 39 amino acids (Beyreuther et al., 1973), which were not resolved on the column. On the basis of bound [^{14}C]NEM neither residue appears to have undergone oxidation to the sulfonic acid stage during the iodination reaction. The second peak probably represents a cysteine-containing peptide of 17 amino acids. Following iodination this residue bound only 70–75% as much [^{14}C]NEM as control samples, indicating that the complete oxidation of this residue to the sulfonic acid stage had occurred in approximately one-third of the repressor subunits during the iodination reaction and/or subsequent handling of the sample. Based on elution behavior this residue has been tentatively identified as cysteine-107 (see below).

No attempt has been made to identify unstable intermediate oxidation products of cysteine. However, the possibility that intermediates such as sulfenyl iodine, sulfenic acid, and sulfinic acid are involved in repressor activity loss has been tested. Iodinated repressor was incubated for 14 hr at 37° in the presence of 0.01 *M* mercaptoethanol. Under these conditions intermediate oxidation products of cysteine would be expected to undergo reduction, regenerating the original cysteine residue (Trundle and Cunningham, 1969). The only observable effect of this treatment was a slight reduction in the binding activity of the control sample: no recovery of binding activity with the iodinated sample was detected.

Tyrosine and histidine react with iodine giving rise to relatively stable mono and diiodo derivatives (Koshland et al., 1963). Repressor was allowed to react with an amount of $K^{131}I_3$ double that needed for complete inactivation. The iodinated sample was then digested with pepsin and Pronase and subjected to thin-layer electrophoresis (Figure 6). Greater than 96% of the bound iodine was recovered as mono- and diiodotyrosine (MIT and DIT). No monoiodohistidine (MIH) was observed, although some material migrating at the position of diiodohistidine (DIH) was seen. Based on the extent of incorporation this material would represent no more than 0.14 residue of DIH per repressor subunit. It is quite probable, however, that this material actually represents MIT since the formation of minor amounts of DIH with no corresponding MIH is unlikely (Covelli and Wolff, 1966, 1967; Wolff and Covelli, 1969). As little or no iodohistidine was present in this sample of heavily iodinated repressor the possibility of iodohistidine formation and subsequent deiodination during the proteolytic and/or electrophoretic procedure seems remote.

Tryptic Digestion of ^{131}I-Repressor. *Lac* repressor contains eight tyrosine residues in tryptic peptides of 39 amino acids (Tyr-126), 27 amino acids (Tyr-260, Tyr-269), 22 amino acids (Tyr-7, Tyr-12, Tyr-17), 14 amino acids (Tyr-47), and 4 amino acids (Tyr-193) (Beyreuther et al., 1973). Repressor was incubated with sufficient $K^{131}I_3$ to give an activity loss of approximately 50%. The sample was then treated with trypsin and allowed to pass through a Sephadex column containing G-25 and G-50 (see Materials and

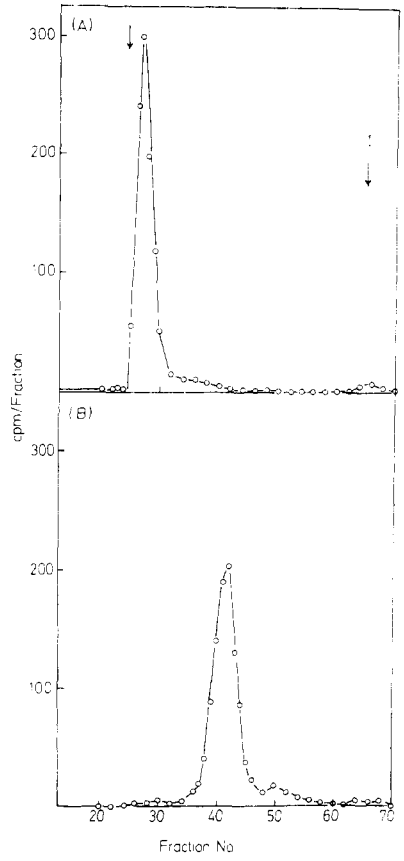

FIGURE 7: Sephadex chromatography of iodinated repressor before and after trypsin digestion. A sample of repressor was iodinated to approximately 50% activity loss. After extensive dialysis the sample was divided into two aliquots. (A) The sample was passed untreated through a "double-decker" Sephadex column containing G-25 and G-50 in 4 *M* urea. (B) The sample was digested for 20 hr with a 1:10 (w/w) amount of trypsin prior to chromatography. Recoveries of input radioactivity in both cases were greater than 98%. Arrows represent the void volume and salt front of the column.

Table II: Correlation of Repressor-Bound Iodine and Activity.[a]

Iodine Atoms Bound per Repressor Subunit	% DNA Binding Activity
0.2	95
0.8	76
1.2	40
2.5	19
3.5	1

[a] 43-μg aliquots of repressor were treated with either $K^{131}I_3$ or an equivalent amount of KI_3. The nonradioactive samples were tested for ^{32}P-λ *plac* DNA binding as described under Materials and Methods. The radioactive samples were dialyzed exhaustively and the bound ^{131}I cpm were used to calculate the iodine content per repressor subunit. The data are the average of two independent experiments.

Table III: Automated Sequence Analysis of Iodinated Repressor.

Iodine Atoms per Repressor Subunit	Tyrosine Residue	% cpm in N-terminal[a]
0.45	7	44.5
	12	20.5
	17	35.0
1.0	7	48.0
	12	23.5
	17	28.5

[a] Cpm in the N-terminal represent approximately 90% of the total bound radioactivity.

Methods). The elution profile indicated the presence of a single radioactive tryptic peptide (Figure 7).

The possibility that the ^{131}I-tryptic peptide was a mixture of several radioactive peptides could be discounted on the basis of additional data. First, when repressor was labeled with $K^{131}I_3$ in 8 *M* urea, treated with trypsin and allowed to pass through the Sephadex column, three predominant peaks were seen at fractions 35, 41, and 58. Since proteolytic digestion indicated that 90% of the radioactivity was in tyrosine, the peak at 58 could be assigned to the 4 amino acid residue peptides containing tyrosine-193. The assignment of the other two peaks was done in the following way. Repressor sulfhydryl residues were labeled with [^{14}C]-*N*-ethylmaleimide. The protein was then digested with trypsin and passed through the Sephadex column. Two peaks were observed, one eluting at fraction 33 and presumably containing the two large tryosine-containing tryptic peptides of 39 amino acids (Tyr-126, Cys-140) and 27 amino acids (Tyr-260, Tyr-269, Cys-268) and a second peak at fraction 44 (Figure 5). The third cyteine residue of repressor, Cys 107, is part of a tryptic peptide of 6 amino acids and would be expected to elute near the salt front of the column. That this peptide elutes at fraction 44, however, is consistent with the fact that Cys-107 is adjacent to a lysine residue and, following alkylation, would be expected to inhibit trypsin activity. The size of the peptide eluting at fraction 44 should, therefore, be the sum of two peptides, or 17 amino acids in length (Beyreuther et al., 1973). Of the two remaining tyrosine-containing peptides (14 and 22 amino acids) the 14 amino acid residue peptide would be expected to elute even later than fraction 44. Thus, the only remaining candidate for the peptide depicted in Figure 7 is the 22 amino acid residue peptide, the N-terminal tryptic peptide.

Sequence Analysis of Iodinated Repressor. The previous evidence suggested that the modified residues were located in the repressor N-terminal. The three N-terminal tyrosine residues at positions 7, 12, and 17 should be easily resolvable by automated sequence analysis. Using a modified procedure of Edman and Begg (1967) with the Beckman sequencer, the iodinated tyrosine residues were easily identified and the extent of reaction at each quantitatively determined.

Repressor was labeled with $K^{131}I_3$ to 0.45 and approximately 1.0 bound I atom per subunit, corresponding to approximately 10 and 50% activity loss, respectively (Table II). Automated sequence analysis indicated that in both cases all three N-terminal tyrosine residues had reacted (Table III). At 0.45 I atom per subunit the three residues had reacted in the ratios: Tyr-7 (1.00), Tyr-12 (0.46), Tyr-17 (0.79). At 1.0 atom per repressor subunit the only differences were slight (relative) increase in the incorporation into residue 7 and 12 at the expense of incorporation into residue 17. Thus, the loss of repressor DNA binding activity coincides with the incorporation of iodine into all three repressor N-terminal tyrosine residues.

Iodine incorporated into the three N-terminal repressor tyrosine residues represents approximately 90% of the bound iodine. The remaining 10% was not lost, but was recovered in the "core" material remaining in the sequencer at the end of the run. Possibilities to explain the failure to

obtain complete recovery of the bound iodine in the first three repressor tyrosine residues include the following. (a) A fourth repressor tyrosine residue is labeled to a minor extent. The small peak at fraction 50 (Figure 7B) could represent, for example, the 14 amino acid residue peptide containing tyrosine-47. (b) 5–15% of the repressor subunits contain nondegradable N-termini (K. Beyreuther, personal communication). (c) Minor impurities in the preparation bind iodine. Note, for example, the radioactive material trailing behind the main peak in Figure 7A.

Discussion

Iodination, especially at high molar excess, is known to result in modification of the tyrosine, histidine, cysteine, tryptophan, and methionine residues of proteins (Koshland et al., 1963). The conditions used in the present study were chosen to reduce the extent of modification as much as possible. Iodination of *lac* repressor at 4° with low molar excesses of iodine resulted in the rapid and irreversible inactivation of repressor DNA binding activity (Figures 1 and 3). Analysis of the iodinated protein suggested that the reaction was confined almost exclusively to tyrosine residues (Table I and Figure 6). No evidence for reactions with histidine, tryptophan, or methionine was obtained, and only a minor amount of cysteine oxidation was detected. The possible occurrence of intermediate oxidation products of cysteine was not examined, but under conditions known to result in the reduction of these intermediates to cysteine, no restoration of repressor activity was detected. This indicates that such intermediates, if formed, played no role in the observed activity loss. Of the eight repressor tyrosine residues, three were found to be modified. These three residues are all located in the first 17 amino acids of the N-terminus of the repressor molecule.

Recent evidence has focussed attention on the N-terminal region of the repressor as the site of DNA recognition. First, a class of repressor mutations, i^{-d} (d = trans dominant) mutations, which are unable to bind DNA, but still bind inducer molecules, have been mapped in the operator proximal region of the gene map (Pfahl, 1972; Pfahl et al., 1974). The operator proximal region of the gene map corresponds to the N-terminal region of the protein (Miller et al., 1968). Secondly, studies with an early amber mutation in the repressor gene have shown that a "repressor" molecule can be synthesized in this strain by a reinitiation event at methionine-42 (Platt et al., 1972). The reinitiation protein isolated from this strain (which lacks the amino acid sequence 1–42) has been shown to be, like native repressor, tetrameric and able to bind inducer molecules. The protein is, however, inactive in DNA binding (Platt et al., 1972). Platt et al. (1972) suggest that the N-terminal region of the repressor is either involved directly in DNA binding or is important for assuming the DNA binding conformation.

The present study suggests that the iodination of one or more repressor N-terminal tyrosine residues results in the complete loss of DNA binding activity. The recent demonstration that aromatic amino acids interact with DNA may explain this effect (Gabbay et al., 1972; Dimicoli and Hélène, 1974). Hélène and his collaborators have demonstrated that the tyrosine residues of small peptides are capable of intercalating in single-stranded DNA and forming hydrogen bonds with double-stranded DNA (Dimicoli and Hélène, 1974). Both forms of interaction could be drastically altered by iodination. For example, the hydrogen bonding potential of tyrosine is a function of the phenoxyl p*K*. The apparent p*K* of MIT has been shown to be reduced, relative to tyrosine, by as much as 2 units for both the free amino acid (Edelhoch, 1962) as well as for MIT in proteins (Covelli and Wolff, 1966). The apparent p*K* for DIT is, as would be expected, correspondingly lower. Similarly, since iodine has a van der Waals radius approximating that of an aromatic nucleus, steric effects might be expected to inhibit any intercalation process. These two mechanisms, disruption of hydrogen bonding potential and steric hindrance, need not, of course, be mutually exclusive.

By the classical definition of "exposed" and "buried" residues all three N-terminal tyrosine residues must be considered exposed. The extremely rapid reaction of these residues (Figure 3) would seem to indicate, moreover, that they are not involved in interactions with neighboring amino acids since H-bonding renders tyrosine residues refractive to iodination (Hayashi et al., 1968). Thus, the three N-terminal tyrosine residues appear neither to be buried within a crevice of the protein nor to be involved in interactions with other parts of the protein. These residues could, therefore, participate in the types of interactions demonstrated for small tyrosine peptide–DNA interactions, namely, H-bonding and/or intercalation (Dimicoli and Hélène, 1974). Whether such interactions occur through a Gierer-like structure (Gierer, 1966) or penetration of the N-terminal region into the major groove of the (B form) DNA molecule (Sung and Dixon, 1970; Adler et al., 1972) is unknown; however, recent evidence suggests that *lac* operator DNA may be in a conformation other than the B form (Chan and Wells, 1975) and is untwisted by repressor binding (Wang et al., 1974).

That the altered tyrosine residues are actually situated in or near the DNA binding site is also suggested by the observation that prebound DNA protected repressor activity (Figure 4). The protective effect was exhibited both in the presence of *lac* operator containing DNA and DNA lacking the operator binding site, although in the former case approximately twice as much protection was observed. The protective effect of nonoperator-containing DNA may indicate that as much as 50% of the observed protection is an artifact. There exists, on the other hand, ample evidence for repressor binding to nonoperator DNA (Lin and Riggs, 1972). This binding, although orders of magnitude lower than binding to operator DNA, could conceivably be tight enough to result in the observed protective effect.

A major objection to a proposal that the three repressor N-terminal tyrosine residues are in or near the DNA binding site is the lack of information concerning possible conformational changes following iodination. The fact that IPTG binding is not lost shows that, at least near the inducer binding site, no denaturation occurs. Also, the highly exposed nature of the three tyrosine residues, along with the demonstration that no breakdown in repressor structure takes place during the reaction, argues against a conformational alteration as the cause of activity loss. The use of more sophisticated techniques, e.g., optical rotatory dispersion or circular dichroism, may give more pertinant information in this respect.

Added in Proof

Using a novel approach developed by K. Beyreuther it is now possible to specifically cleave the repressor molecule in the N-terminal region between amino acids 56 and 57 (Beyreuther, Böhmer, and Raufuss, manuscript in preparation). Using this method it can be shown that ^{131}I-labeled

repressor (ca. 3.5 I atoms/repressor subunit) contains no detectable label beyond the first 56 amino acids.

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