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Structural Analysis of the Carboxy Terminus of Bacteriophage Lambda Repressor Determined by Antipeptide Antibodies

RAQUEL SUSSMAN[1]* AND HANNAH BEN-ZEEV ALEXANDER[2]

Marine Biological Laboratory, Woods Hole, Massachusetts 02543,[1] and Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037[2]

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To analyze λ repressor function and structure, antibodies were generated with synthetic peptides corresponding to sequences believed to be involved in prophage induction. These site-directed antibodies seemed to recognize preferentially the primary sequence of repressor because they reacted better in competition experiments with the oligopeptide and with the partially denatured forms of repressor than with the native molecules. This information, together with the characteristic ability of the antibodies to immunoprecipitate or react with repressor in immunoblots, allowed us to infer some conformational properties of the specific regions that the antibodies recognized. The antibodies reacted less with some mutant repressors that had a single amino acid substitution within the cognitive sequences. RecA-catalyzed cleavage of repressor was inhibited to different extents in relation to the proportion of repressor that each antipeptide immunoglobulin G (IgG) was able to immunoprecipitate. The antipeptide IgGs did not affect specific binding of repressor to operator DNA, whereas the antirepressor IgG was inhibitory. The three different IgGs competed for binding to repressor in an enzyme-linked immunosorbent assay additivity test, which suggested that the three regions of conserved amino acids are probably located on the same side of the carboxyl domain of repressor and possibly close together in the tertiary structure.

The phenomenon known as SOS induction in *Escherichia coli* (32, 49) pertains to the coordinated control of a diverse set of genes that respond to chromosomal injury and are repressed by LexA protein. These genes include the cellular *din* genes as well as viral genes of radiation-inducible lysogens, such as lambda and P22 (31) (reviewed in references 26 and 47). Although the molecular mechanism responsible for SOS induction has been largely elucidated, there are still several unknown parameters. When cells suffer DNA damage (by interruption of chromosomal replication, irradiation, or in any other way), the repair machinery of the cell introduces nicks and single-stranded gaps as intermediates in the restitution of the chromosome. One of these intermediates constitutes the signal for SOS induction, which activates RecA protein to catalyze cleavage of LexA and prophage repressors. It is assumed that the signal is single-stranded DNA gaps on the basis of the facts that: (i) RecA protein (which is essential for in vivo inactivation of LexA and phage repressors) requires as cofactors a single-stranded polynucleotide and a nucleoside triphosphate to catalyze proteolytic cleavage (10) and (ii) lambda repressor has a higher affinity for duplex DNA containing single-stranded gaps than for the duplex, either intact or nicked, or for single-stranded DNA (44). Although lambda repressor does not seem to require DNA for in vitro cleavage, since the reaction can proceed in the presence of RecA complexed with oligo(dT_{16}) (9, 10), its affinity for gapped DNA may increase its local concentration in the cell, assisting in the induction.

Another unknown parameter is the composition of the complex that operates in the cell. Because cellular mutations in *ssb* impair SOS induction (3, 46) and the presence of SSB protein in vitro enhances the rate and extent of RecA-promoted cleavage of lambda repressor (33), we hypothesized that the active machinery for SOS induction in the cell is a quaternary complex composed of single-stranded gaps, dATP, RecA, and SSB (9). This complex, which we term inductisome (previously called activated RecA or RecA* [47]), inactivates repressors by catalyzing the hydrolysis of an Ala-Gly bond in the middle of the polypeptide chain (19, 29). The limiting factor in the cell is the available number and persistence of gaps, demonstrated by the correlation between the UV dose required to induce a lysogen and the concentration of repressor in the cell (4). Thus, in a lysogen this set of events leads to derepression of the prophage genes and lysis of the host by a burst of infective virus. In a sensitive bacterium, cleavage of LexA protein derepresses at least 17 *din* genes (47), some of which code for error-prone DNA repair enzymes, for an inhibition of cell division (SulA), and for RecA protein, which becomes greatly amplified.

LexA and phage repressors share two properties. First, they undergo autodigestion at alkaline pH (24). This proteolytic reaction is equal to RecA-mediated cleavage, occurring at the same Ala-Gly bond and proceeding at the same rate in each repressor. This activity is attributed to the repressor molecule itself because it does not require any of the components of the inductisome (42). The fact that a fragment of either repressor lacking the amino terminus can be autodigested (38, 42) indicates that the proteolytic active site resides in the COOH terminus of repressor. Second, LexA, phage repressors, and two other proteins involved in error-prone DNA repair (UmuD and MucA) have significant amino acid homology at the cleavage site as well as at the COOH domain (30, 39). It was recently established that UmuD and MucA proteins undergo RecA-dependent cleavage (6, 27, 40). In addition, mutations that affect induction also map at these specific regions of lambda repressor (12, 16, 23), which corroborates the location of the essential functions for induction in these areas.

This study was undertaken to gain further understanding of the structure-function relationships of the lambda repres-

* Corresponding author.

sor involved in induction, namely, proteolytic activity and the residues that interact with RecA. We prepared polyclonal antibodies against three synthetic peptides which correspond to sequences of the repressor that are highly conserved in radiation-inducible systems. We report the reactivity profiles of these antibodies, their effect on different repressor functions, and their contribution to elucidating the structure of these regions of the repressor molecule.

MATERIALS AND METHODS

Buffers. PBS was 10 mM phosphate-buffered saline (pH 7.3). PBST was PBS plus 0.05% Tween-20. RIPA buffer was 0.15 M NaCl, 10 mM sodium phosphate buffer (pH 7.2), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). SB buffer, binding buffer, and washing buffer for DNA-binding assays were as described previously (20). IP buffer was 10 mM Tris hydrochloride (pH 7.4), 50 mM KCl, 0.1 mM EDTA, 2 mM $CaCl_2$, 1 mg of bovine serum albumin (BSA) per ml, and 1 mM phenylmethylsulfonyl fluoride. Papain buffer was 10 mM Tris hydrochloride (pH 8), 2 mM $CaCl_2$, 0.5 mM EDTA, 0.1 mM dithiothreitol, 2 mM cysteine, and 5% (vol/wt) glycerol.

Purification of proteins. Lambda repressor was isolated from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cultures of *E. coli* carrying *lacI*^{q} and plasmid pEA305 (2). Cells for labeling repressor were grown in low-sulfur medium (34). $H_2[^{35}S]O_4$ (10 μCi/ml) was added after IPTG induction, and growth was continued for 3 h. Cells were lysed by sonication or lysozyme treatment, and repressor was purified by a modification of the method of Johnson et al. (20). After ammonium sulfate precipitation of the extract and dialysis against SB buffer plus 50 mM NaCl, repressor was adsorbed to carboxymethyl-Sephadex (C50; Pharmacia Fine Chemicals, Piscataway, N.J.) and step eluted with SB buffer plus 250 mM NaCl. Further purification and concentration were achieved by adsorbtion of repressor to DEAE-cellulose (DE50; Whatman, Inc., Clifton, N.J.) at 50 mM NaCl and elution with SB buffer plus 200 mM NaCl. Repressor was estimated to be more than 95% pure by Coomassie blue staining of samples run in SDS-polyacrylamide gel electrophoresis (PAGE).

LexA protein was isolated from strain JL468(pJL59) (24) and also provided by J. W. Little. RecA protein was isolated from strain AB1157(pBEU14) (45) and purified according to published procedures (48).

Synthetic peptides. Oligopeptides were synthesized by R. Houghten of the Scripps Clinic (La Jolla, Calif.), and amino acid sequences were verified (5).

Antibodies. Antisera were produced in rabbits by injection of the synthetic peptides conjugated to keyhole limpet hemocyanin through the cysteine of the peptide according to the method of Lerner et al. (22). Antisera against native repressor were obtained in rabbits immunized with the purified protein emulsified in complete Freund adjuvant for the first injection and with incomplete adjuvant for secondary immunizations. Immunoglobulins were purified by chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) according to the directions of the manufacturer, precipitated with 45% saturated ammonium sulfate, and dialyzed against PBS or IP buffer.

ELISA determination of antibody activity. For enzyme-linked immunosorbent assays (ELISA), 96-well microdilution plates (Costar, Cambridge, Mass.) were treated with 1% glutaraldehyde for 1 h at room temperature, washed, and incubated with the antigen at 4°C overnight. Usually 5 pmol of protein or 50 pmol of synthetic peptide was used per well. After being washed and blocked with 1 M glycine for 1 h at room temperature, the wells were washed with PBST and reacted with the primary antibody. Incubation was carried out overnight at 4°C. Binding of specific immunoglobulin was assessed by using glucose oxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Organon Teknika, Malvern, Pa.), followed by washing and addition of developer [8 μg of horseradish peroxidase per ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 140 μg of 2,2′-azinodi(3-ethylbenzthiazoline sulfonic acid) per ml, 2% glucose, 0.1 M phosphate buffer (pH 6)]. A_{410} was measured.

Competition assay. Increasing concentrations of the competing protein or peptide were incubated with a limiting amount of antibody with shaking for 2 h at room temperature. Residual antibody activity was determined by transferring 25 μl of the mixture to the wells of a microdilution plate containing bound repressor as described above for ELISA. The plates were shaken for 1.5 h at room temperature before ELISA was performed.

Immunoprecipitation assay. Specific antibody binding to repressor in solution, under conditions used for testing repressor activities, was determined by precipitation of rabbit IgG with Pansorbin (Calbiochem-Behring, La Jolla, Calif.) (22). The reaction mixture (13 μl) contained 60 nM ^{35}S-labeled repressor (10 to 50 cpm/ng) in IP buffer and various concentrations of antibody. Preimmune sera was used as a control. After a 1-h incubation in ice, 100 μl of RIPA buffer and the amount of Pansorbin needed to bind all of the IgG present were added. The mixture was then vortexed, incubated in ice for 30 min, and centrifuged. The pellet was washed twice with 1 ml of 500 mM LiCl–100 mM Tris hydrochloride (pH 8.5), and radioactivity was measured in a scintillation counter.

RecA-dependent cleavage assay with repressor-IgG complexes. Reaction mixtures were prepared as described for immunoprecipitation: 13 μl contained 60 nM ^{35}S-repressor (10 to 50 cpm/ng) in IP buffer and excess antibody (determined by immunoprecipitation). After incubation for 1 h in ice, the necessary components for the standard RecA-dependent cleavage reaction were added to give final concentrations of 20 mM Tris hydrochloride (pH 8), 20 mM NaCl, 0.1 mM EDTA, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 5% (vol/wt) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 100 μg of BSA per ml, 1 mM ATPγS, 5 μg of oligodeoxythymidine pd(T_{16}) (Pharmacia) per ml, and 30 μg of RecA protein per ml. Incubation proceeded for 90 min at 37°C. Samples were analyzed by 15% SDS-PAGE, with subsequent autoradiography and scanning as previously described (33).

RecA-dependent cleavage in the presence of synthetic peptides. Increasing concentrations (up to 6 μM) of the oligopeptides were added directly to the standard cleavage assay containing 60 nM repressor and 30 μg of RecA protein per ml. The preparation was then incubated and analyzed as described above.

Immunoblots. Proteins and peptides were separated by SDS-PAGE and transferred to nitrocellulose membranes on a Bio-Rad Trans-Blot apparatus at 60 V for 3 h. The membranes were blocked with 3% BSA, washed, treated with the primary antibody, washed again, and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.). Enzymatic activity was visualized with 5-bromo-4-chloro-3-indolyl phosphate

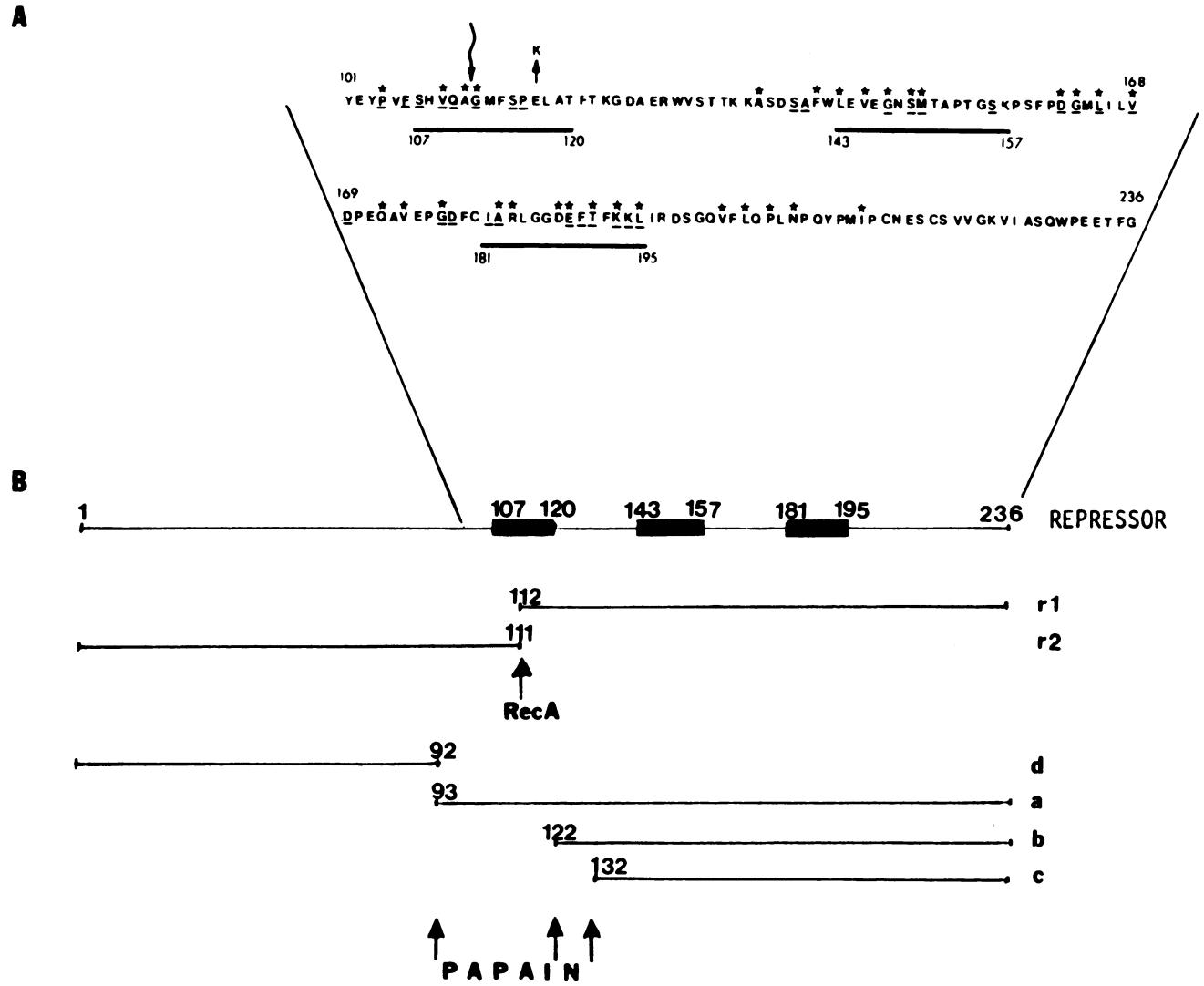

FIG. 1. Amino acid sequence of the COOH-terminal region of lambda repressor. (A) Amino acids are identified by single-letter code. Wavy arrow points to the site of cleavage between the Ala-111–Gly-112 bond; straight arrow identifies the *ind-1* mutation Glu-117 → Lys; asterisks represent identities between lambda repressor and LexA protein; underlined residues are those conserved among proteins UmuD and MucA; bold underlines indicate regions selected for peptide synthesis. (B) Schematic representation of lambda repressor indicating positions of synthetic peptide sequences and fragments generated by autodigestion (24) and by papain digestion (29). Numbers refer to positions of amino acids in the sequence of the intact repressor (36).

and Nitro Blue Tetrazolium according to the technical manual of Promega Biotec.

Papain digestion. Papain (Worthington Diagnostics, Freehold, N.J.) was activated by incubation in 0.1 M acetate buffer (pH 5.5), 50 mM cysteine, and 3 mM EDTA at 37°C for 30 min and filtered through a 22-μm-pore-size membrane (Millipore Corp., Bedford, Mass.). Repressor (1 mg/ml) in papain buffer was digested with 5 μg of papain per ml at 20 to 21°C for 30 min. The enzyme was inhibited with 10 mM iodoacetamide.

Thermolysin treatment. Thermolysin (2 μl; 0.3 mM) was added to 8 μl of repressor in SB buffer plus 200 mM NaCl (400 μg/ml), and the mixture was incubated at 37°C for 30 min. Immediately after treatment, the reaction was terminated by addition of 1 μl of EDTA (100 μM).

Other assays. The autodigestion assay was carried out as described by Little (24) at pH 10.5 and 45°C. The operator-binding assay was performed according to the method of Johnson et al. (20).

RESULTS

Selection of peptides used to elicit antibodies against specific epitopes. Three peptides with amino acid sequences corresponding to three regions of the lambda repressor considered to be involved in prophage induction functions were synthesized. One region comprised the Ala-111–Gly-112 bond that undergoes cleavage during this process (Fig. 1). The other two regions were chosen because they shared common amino acids with other inducible repressors, such as LexA and imm^{434} (39), and with proteins UmuD and MucA (30) (Fig. 1). Moreover, most of the mutations affecting prophage induction occur in these regions (12, 16, 23). Peptides were also selected with regard to the secondary structure of the

TABLE 1. Synthetic peptides and titers of antipeptide antibodies elicited in two rabbits

Peptide designation	Peptide sequence	Titer[a] of antipeptide antibody (rabbit 1/rabbit 2) versus peptide:		
		(107–120)	(143–154)	(181–195)
(107–120)	Ser His Val Glu Ala Gly Met Phe Ser Pro Glu Leu Arg Thr Cys	1,280/640–1,280	<20	<20
(143–157)	Leu Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Cys	<20	1,280/>1,280	<20
(181–195)	Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe Lys Phe Leu Ile Cys	<20	<20	1,280/1,280

[a] Titer refers to antibody activity determined in ELISA, expressed as the last dilution that gave an optical density higher than did the negative control.

repressor molecule, analyzed according to Chou and Fasman (7), and the hydrophilicity profile obtained with the program of Hopp and Woods (18). Both parameters are important in immunogenesis (21). Table 1 shows the amino acid sequences of the peptides. A cysteine was added at the carboxyl end of each peptide to facilitate conjugation to the carrier protein. Two rabbits were immunized with each peptide, and high antipeptide antibody titers were obtained. No cross-reactivity was observed with the other two peptides (Table 1).

Reaction of antibodies with native and denatured repressor. To characterize the antibodies according to activity toward native and denatured repressor, we performed ELISA competition experiments. A limiting concentration of IgG was incubated with increasing concentrations of (i) native repressor, (ii) denatured repressor (treated with 8 M urea or heated at 75°C), and (iii) specific peptide. The residual antibody activity was measured by ELISA. The controls were lysozyme and lysozyme denatured with 8 M urea. The data from two such experiments are averaged in Fig. 2. Other competition experiments with different concentrations of antibodies gave comparable results. The antirepressor IgG reacted equally well with native or denatured repressor within the error of the method, whereas it did not react significantly with peptide (107-120) (peptide extending from amino acids 107 through 120) or with lysozyme (Fig. 2A). All antipeptide IgGs displayed higher reactivity toward denatured than toward native repressor, whereas the highest competition occurred with the immunogenic peptide (Fig. 2B through D). These results clearly indicate that the antipeptide IgGs recognized the primary structure of repressor better than they recognized all of the other structures present in solution. Anti-peptide (107-120) IgG required 4 times more heat-denatured, 14 times more urea-treated, and 28 times more native repressor to reduce activity by 50% (Fig. 2B). These observations suggest that this sequence is probably folded, becoming more accessible after denaturation. Antipeptide (143-157) IgG reacted very poorly with native repressor below a concentration of 0.5 μM and reached a plateau at 50% inhibition (Fig. 2C). In other experiments, even less inhibition was observed with excess IgG. Denatured repressor was four times more effective in competition and also formed a plateau at 60% inhibition. These results are in agreement with the limited reactivity displayed by this IgG in immunoprecipitation and indicate that the (143-157) region is not easily accessible to this probe in a high proportion of the molecules. The anti-peptide (181-195) IgG required 30 times more native repressor and 15 times more urea-denatured repressor than heat-denatured repressor to decrease activity by 50% (Fig. 2D). We deduce from these results that the (181-195) region must be folded but flexible in the native molecule and becomes more exposed upon denaturation.

Denaturation of repressor assessed by susceptibility to proteolysis. The fact that the procedure for denaturing repressor was critical for binding of repressor to the antipeptide IgGs (Fig. 2) suggested either that we were dealing with preparations that contained different proportions of unfolded molecules or that different regions of the molecule were destabilized by these treatments or both. There is evidence that repressor denatured by SDS regains its proper tertiary structure after prolonged dialysis against urea and then buffer, becoming competent to bind operator DNA (43). We wished to determine the extent to which the repressor was refolding and whether we could detect a difference in structures formed after the different treatments. Figure 3 shows an SDS-PAGE analysis of the fragments formed by treatment with thermolysin of repressor that had been subjected to the denaturing regimens used in the ELISA competition experiments. When repressor was treated with thermolysin at 80°C, it was completely hydrolyzed (Fig. 3, lane 3); therefore, it must have been completely unfolded. If heated repressor was allowed to reach room temperature and then tested with thermolysin at 37°C, some fragments became resistant to thermolysin attack (Fig. 3, lane 4), which indicated that refolding of specific areas of the molecule had occurred. The presence of urea during the thermolysin treatment produced a different set of fragments than were produced by proteolysis in the absence of urea (Fig. 3, compare lanes 6 and 8 with lane 2). Since we used urea at room temperature, we surmise that the repressor was partially denatured, although we cannot assess the proportion of molecules completely denatured or how disordered certain regions became with this treatment.

Reactivity of antibodies with mutant repressors. ELISA was performed, using as antigens a set of *ind* mutant repressors that had been isolated, sequenced, and provided by R. Sauer (16). These mutants confer a noninducible phenotype to the lysogens. In vitro, some of these altered repressors fail to undergo autodigestion, whereas others are cleaved less efficiently or not at all by RecA protein (17). These proteins were attached to the wells of a microdilution plate at 6 μg/ml in PBS, and the antibodies were titrated

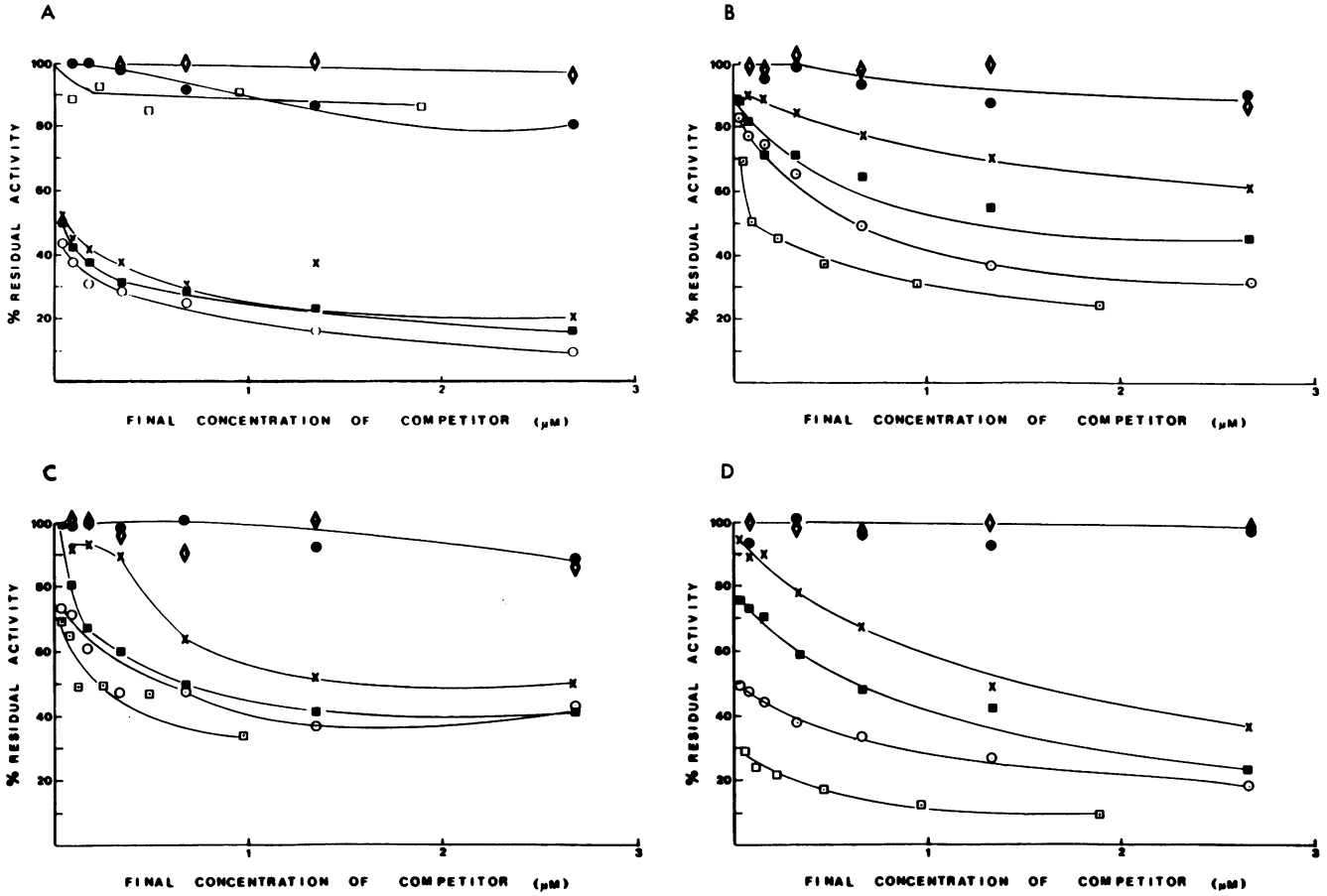

FIG. 2. Comparative reactivity of IgGs toward native repressor, denatured repressor, and peptide as determined by competition ELISA. A 25-μl amount of each purified IgG in PBS containing 0.1% BSA at a concentration that gave half-maximal titer was mixed with 10 μl of different concentrations of the competing protein or peptide. After incubation for 2 h at room temperature, 25 μl of the mixture was transferred to a 96-well microdilution plate that had been coated with repressor (6 μg/ml) and blocked with glycine. The plates were shaken at room temperature for 1.5 h, washed, reacted with oxidase-conjugated goat anti-rabbit IgG, and developed as described in Materials and Methods. Values represent the means of two experiments, expressed as percentage of enzyme activity obtained in control wells with no competing protein. (A) Antirepressor IgG; (B) anti-peptide (107-120) IgG; (C) anti-peptide (143-157) IgG; (D) anti-peptide (181-195) IgG. Competing peptides: ●, lysozyme; ◇, lysozyme treated with 8 M urea; ×, native repressor; ■, repressor treated with 8 M urea; ○, repressor heated at 75°C for 15 min and cooled; ⊡, immunogenic peptide. Peptide (107-120) was used in panel A.

simultaneously with each altered repressor, using wild-type repressor as a control. Figure 4 shows the optical densities obtained at maximum reaction in ELISA, which is a measure of the amount of antibody bound. All but a very few of the repressors reacted with the IgGs as much as did the wild-type repressor. Those that did not react were repressors that had the altered amino acid within the segment of the sequence recognized by the antipeptide antibody to which they had significantly less reactivity. The control antirepressor IgG was retained equally by all proteins (data not shown). It is evident that the antipeptide IgGs were able to distinguish among residue changes within their cognitive epitopes, although not all replacements were equally effective. For example, *ind-111*, *ind-117* (known as *ind-1*) (36), and *ind-143* proteins did not behave significantly different from the wild-type repressor (Fig. 4). Equally indistinguishable was the superinducible mutant *ind*^{s}*-233* (8, 16) (data not shown). This finding is understandable, considering that not all residues within an epitope bind to the same extent, some being more critical than others. To confirm these results, we performed competition ELISA experiments with the mutant repressors in solution. The same mutants that reacted less with a given IgG in direct ELISA competed to a lesser extent than did the wild type but only with the corresponding antipeptide IgG (data not shown).

Reactivity of antibodies with repressor fragments. Repressor was autodigested, giving rise to r1 and r2 fragments (34), and also proteolytically cleaved with papain into four fragments, a, b, c, and d (29) (Fig. 1). The fragments were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were developed by using each of the antisera and processed as described in Materials and Methods. The results obtained with these immunoblots (Fig. 5) can be summarized as follows. Anti-peptide (107-120) IgG did not bind to fragment r2, although it shared five amino acids with its immunogenic peptide (Fig. 1). On the other hand, it had high reactivity with r1, with which it had nine residues in common (Fig. 5, lane 2). The other two antipeptide antibodies also reacted with r1 only, as expected from their positions in the molecule (Fig. 5, lanes 3 and 4). All three antipeptide sera reacted with the whole molecule, with anti-peptide (143-157) IgG showing a much weaker reaction (Fig. 5, lanes 3 and 11); this finding confirmed the low reactivity displayed in ELISA (Fig. 3). The antirepressor

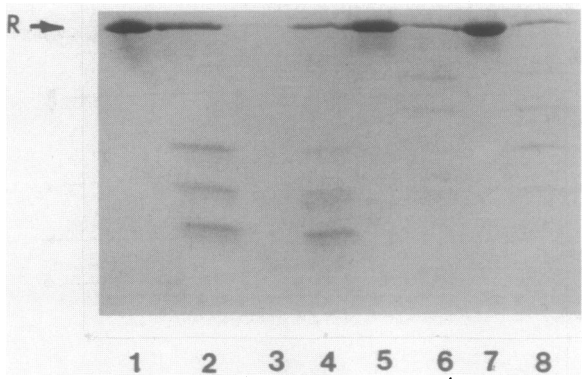

FIG. 3. Denaturation of repressor assessed by proteolysis with thermolysin. Repressor (400 μg/ml) was subjected to the indicated treatments followed by addition of thermolysin to a final concentration of 75 μM. After incubation at 37°C for 30 min (unless specified otherwise), the reaction was terminated by addition of EDTA to 10 μM. Equal portions were analyzed by SDS-PAGE and staining with Coomassie blue. Lanes: 1, repressor, no thermolysin; 2, repressor treated with thermolysin at 37°C; 3, repressor treated with thermolysin for 20 min at 80°C; 4, repressor incubated at 80°C for 20 min and cooled at room temperature before thermolysin treatment; 5, repressor in 8 M urea, no thermolysin; 6, repressor in 8 M urea treated with thermolysin; 7, repressor in 6 M urea, no thermolysin; 8, repressor in 6 M urea treated with thermolysin. R, Repressor band.

IgG reacted strongly with all three bands (Fig. 5, lane 1). Binding of the antibodies to the papain fragments on the immunoblot was again as predicted from their positions in the molecule. Anti-peptide (107-120) IgG reacted only with fragment a (Fig. 5, lane 6), and the other two bound to fragments a, b, and c, as expected (Fig. 5, lanes 7 and 8). None of the antipeptide IgGs were expected to recognize the amino terminus of the repressor; indeed, they failed to bind to fragment d, in contrast to the control antirepressor IgG (Fig. 5, lanes 5 through 8).

When repressor was more extensively degraded by papain because of the presence of a small amount of SDS, the fragments transferred to nitrocellulose were differentially recognized by the antipeptide sera (Fig. 5, lanes 9 through 12). This result exemplifies the convenience of using antibodies against known epitopes to classify unknown fragments and to determine whether their structures are still intact.

Immunoprecipitation of repressor. To carry out experiments designed to measure the ability of the antibodies to affect repressor functions, it was first necessary to establish the equivalent concentrations of sera that would bind the required amount of antigen. This was accomplished by immunoprecipitation of 60 nM ^{35}S-labeled repressor, in IP buffer, with increasing amounts of antibody. The maximal proportions of repressor precipitated with purified IgG at plateau were 100% with antirepressor IgG, 92% with anti-peptide (107-120) IgG, 31% with anti-peptide (143-165) IgG, and 89% with anti-peptide (181-195) IgG. Thus, the antipeptide IgGs were unable to precipitate all repressor present, even when exposed to a large excess. The anti-peptide (143-157) IgG in particular had very low reactivity.

RecA-dependent cleavage of repressor in the presence of antibodies. ^{35}S-labeled repressor was first incubated with antibody for 1 h in ice, under the conditions used for the immunoprecipitation assay, and then the necessary buffer and factors required for the RecA-dependent cleavage reaction were added as described in Materials and Methods. The reactions were stopped after 90 min of incubation at 37°C, and the extent of cleavage was determined by SDS-PAGE and autoradiography. With preimmune sera, the cleavage reaction proceeded as well as in the control experiment without serum. The antirepressor serum inhibited the reaction completely whereas the antipeptide serum was partially inhibitory (data not shown). The extent of inhibition paralleled the proportion of repressor that each antibody reacted with in the immunoprecipitation assay. The least inhibitory was anti-peptide (143-157) IgG.

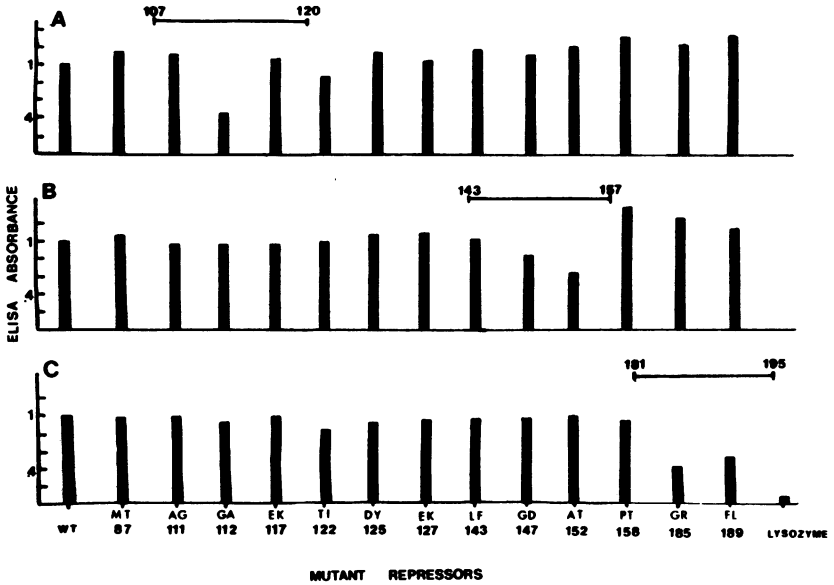

FIG. 4. Reactivities of mutant repressors toward antipeptide antibodies. ELISA was performed by attaching the wild-type and mutant repressors and lysozyme to the wells of microdilution plates at 6 μg/ml in PBS. Dilutions of each antibody in PBS were reacted with all antigens simultaneously, and the assay was continued as described in Materials and Methods. Values represent the highest optical densities obtained in these titrations. (A) Anti-peptide (107-120) IgG; (B) anti-peptide (143-157) IgG; (C) anti-peptide (181-195) IgG.

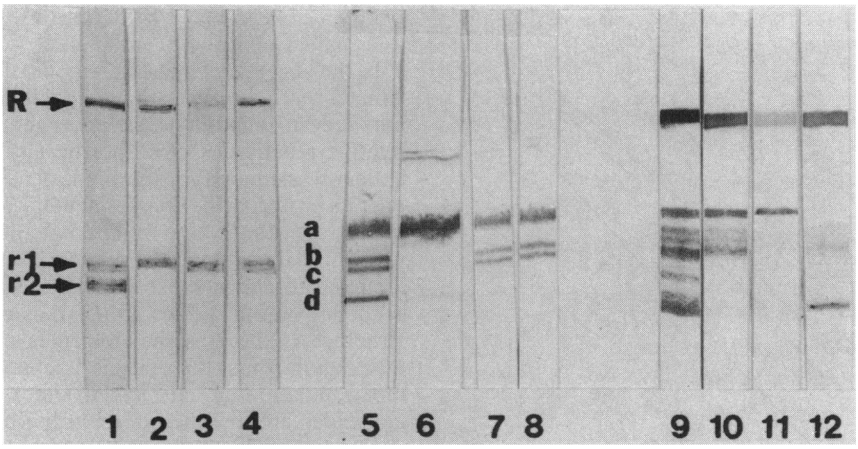

FIG. 5. Immunoblots showing reactivity of antipeptide and antirepressor antibodies with repressor and repressor fragments. Lanes: 1 through 4, repressor (R) and autodigestion fragments (r1) and (r2); 5 through 8, papain-generated fragments a through d; 9 through 12, repressor and unidentified fragments obtained by treatment of repressor with papain in the presence of 0.1% SDS. Incubation was as follows: antirepressor IgG (lanes 1, 5, and 9); anti-peptide (107-120) IgG (lanes 2, 6, and 10); anti-peptide (143-157) IgG (lanes 3, 7, and 11); anti-peptide (181-195) IgG (lanes 4, 8, and 12). Repressor was autodigested at pH 10.5 and 45°C according to the method of Little (24). Papain digestion (29) of repressor (1 mg/ml) in 10 mM Tris hydrochloride (pH 8), 2 mM CaCl, 0.5 mM EDTA, 0.1 mM dithiothreitol, 2 mM cysteine, 5% (vol/wt) glycerol, and 5 μg of activated papain per ml was performed at 21 to 22°C for 30 min. The enzyme was inhibited with 10 mM iodoacetamide. Repressor and fragments were separated by SDS-PAGE, transferred to nitrocellulose membranes, and processed as described in Materials and Methods.

To ascertain whether the antipeptide antibodies were binding at the same site in the tertiary structure or close enough to block by steric hindrance, we performed the ELISA additivity test (13), which measures whether two antibodies that recognize different epitopes can bind simultaneously to the antigen. This assay requires that the antigen be saturated with each antibody tested. Accordingly, we determined the lowest concentration of each IgG at which saturation was achieved and used those concentrations to perform ELISA with single and with mixed pairs of antibodies. The results of several experiments with sera as well as with purified IgGs and one Fab preparation are summarized in Table 2. An additivity index of 1 indicates complete competition, whereas an index of 2 means that there was no competition; thus, the epitopes did not overlap. Since the indices obtained were closer to 1, there was some competition between these antibodies for a site (or adjacent sites) in the molecule, most likely as a result of steric hindrance of the IgGs.

RecA-dependent cleavage in the presence of synthetic peptides. To determine whether the synthetic peptides could compete with repressor for a site on the RecA protein, we performed the cleavage reaction in the presence of up to 100 times the molar concentration of each peptide. We were unable to detect any inhibition of the cleavage reaction under these conditions (data not shown).

Repressor autodigestion in the presence of antibodies or peptides. Radiolabeled repressor was incubated with purified antibody for 1 h in ice, under the conditions used for immunoprecipitation assay, followed by addition of concentrated autodigestion buffer, bringing the final concentration and pH to that of the standard reaction. After incubation at 45°C for 90 min, the extent of autodigestion was determined by SDS-PAGE and autoradiography. No inhibition was observed with any sera, including the control antirepressor IgG (data not shown). The failure to observe inhibition could be attributed to the extreme conditions necessary to induce

TABLE 2. ELISA additivity test

Antibody prepn	IgGs and dilutions[a]	Optical density		Additivity index[c]
		Observed	Expected[b]	
Sera	(107–120) 1:320 + (143–157) 1:160	1.03	1.33	1.5
	(107–120) 1:320 + (181–195) 1:320	1.03	1.47	1.4
	(143–157) 1:160 + (181–195) 1:320	0.53	0.74	1.4
Different-batch sera	(107–120) 1:200 + (143–157) 1:50	0.74	1.26	1.17
	(107–120) 1:200 + (181–195) 1:400	0.63	1.11	1.13
	(143–157) 1:50 + (181–195) 1:400	0.66	1.17	1.12
Purified IgG	(107–120) 1:40 + (143–157) 1:10	0.72	1.35	1.07
	(107–120) 1:40 + (181–195) 1:40	0.73	1.32	1.11
	(143–157) 1:10 + (181–195) 1:40	0.75	1.45	1.03
Fab	(107–120) no dil + (143–157) no dil	0.37	0.69	1.07
	(107–120) no dil + (181–195) no dil	0.56	0.97	1.15
	(143–157) no dil + (181–195) no dil	0.59	1.06	1.11

[a] Antibody dilutions correspond to the lowest concentrations at which saturation of antigens was achieved. no dil, No dilution.
[b] Sum of optical densities obtained for each serum.
[c] Defined as $2 \times (A_{1+2})/A_1 + A_2$, where A_1 and A_2 are the optical densities of each antibody alone and A_{1+2} is the optical density obtained with the two antibodies in the same reaction.

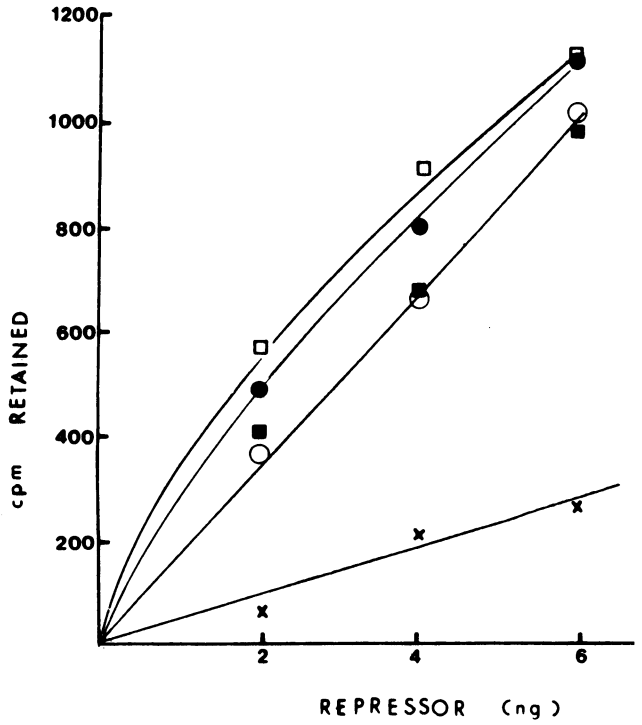

FIG. 6. Effect of antirepressor and antipeptide IgGs on the operator-binding activity of repressor. Operator-binding assays were performed according to the method of Johnson et al. (20). Repressor was diluted in Tris hydrochloride (pH 7.4), 100 mM NaCl, 2 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, BSA (100 μg/ml), and 1 mM dithiothreitol to a concentration of 1 μg/ml. Portions of 2, 4, and 6 μl were added to an excess of purified IgGs in IP buffer (reaction volume, 13 μl). After a 1-h incubation in ice, 50 μl of lambda [^{32}P]DNA (500 cpm/ng; 0.1 μg/ml) in binding buffer was added; incubation was continued for another 15 to 20 min in ice, and the preparation was filtered through nitrocellulose membranes. After being washed, the filters were counted in a scintillation counter. Radioactivity retained on the filters is plotted against the amount of repressor present in each reaction. Background counts of mixtures with each of the antibodies but lacking repressor have been subtracted. Symbols: ×, antirepressor IgG; ■, anti-peptide (107-120) IgG; ○, anti-peptide (143-157) IgG; ●, anti-peptide (181-195) IgG; □, preimmune serum.

autodigestion, which may dissociate the antibodies from the immunocomplex or render them inactive.

Autodigestion assays carried out in the presence of a large (10-fold) excess of synthetic peptides showed no anomalies compared with the control (data not shown). A second type of control, incubation of repressor with the synthetic peptides at physiological pH (7.4 to 7.6), did not cause autodigestion.

Effect of antibodies on the operator-binding activity of repressor. The N terminus of the repressor molecule functions in the recognition and binding to the operator sequences (37). We wished to determine whether the antibodies complexed to the carboxyl end of the molecule could cause a conformational change of the N-terminal region of the repressor. To this end, we performed filter binding assays, using repressor previously exposed to antibody (Fig. 6). The antipeptide IgGs did not affect significantly the operator-binding capacity of the repressor, in contrast to the inhibition observed with the antirepressor IgG.

DISCUSSION

In the absence of crystallographic data on the structure of lambda repressor or LexA protein involved in induction, we pursued an immunological approach. Interpretation of the relative reactivities of a specific antibody toward different antigens assumes that the highest affinity occurs when the IgG fits perfectly the complementary residues that constitute the epitope. In the case of different antigens bearing epitopes with the same amino acid sequence, the highest reactivity is assumed to define those molecules that have the cognitive sequence on the surface either (i) as part of a stable tertiary structure, (ii) because the molecules are folded but flexible in that region (thus, the epitope is exposed in the majority of the population at any given time), or (iii) because the molecules are disordered, as in the case of small peptides. Sachs et al. (35) have demonstrated that it is possible to obtain conformationally specific antibodies directed against either native or disordered antigen, the latter by using fragments of the protein. They were able to use these antipeptide antibodies to measure conformational equilibria of staphylococcal nuclease (14).

The three antipeptide IgGs that we have characterized in this study show the highest reactivity toward the immunogenic peptide and no cross-reaction with the other two oligopeptides, which demonstrates their specificity for the primary structure of that region of the repressor. The fact that they reacted better with the denatured than with the native forms of repressor (Fig. 2) could be interpreted to mean that those regions of the repressor are normally folded, becoming more exposed after treatment.

In the competition experiments, it was possible to distinguish between thermally denatured and urea-treated repressors. By using the protease thermolysin to analyze the products of denaturation, it was possible to demonstrate that the repressor had attained a different set of resistant domains with each treatment. According to the reactivity observed, heating of repressor to 75°C followed by cooling seemed to produce a larger number of disordered molecules than did urea treatment.

Each IgG displayed a distinctive pattern of reactivities, which probably reflects the flexibility of the molecular structure containing the epitope. It is obvious that there are many parameters, such as temperature and ionic strength, that are capable of influencing local structure, including the way the antigen is attached to the plastic wells of a microdilution plate (data not shown). With all of these factors taken into consideration, it is still possible to gain some insight, although tentative, with this type of analysis. For example, the (107-120) sequence of repressor, which contains the labile Ala-Gly bond and forms part of the 40-amino-acid region called the connector or hinge, has been considered to be flexible, with no discernible tertiary structure (29). The analysis with anti-peptide (107-120) IgG suggests that it is folded when the native protein is in solution under approximately physiological conditions, becoming more exposed after certain denaturing treatments. The fact that a large proportion of radiolabeled repressor (up to 92% with a large excess of antibody) can be immunoprecipitated seems to indicate that this region is flexible under the conditions used. In contrast, anti-peptide (143-157) IgG could precipitate at most 35% of the soluble radiolabeled repressor and reacted very weakly with repressor in immunoblots (Fig. 4). Moreover, native repressor failed to inhibit all activity present in the antipeptide IgG in the competition experiments (Fig. 2). Denatured repressor competed better

than did native repressor but also did not reach completion. We can deduce from these observations that the (143-157) sequence is probably less accessible to IgG in the native state and does not become totally exposed even after being subjected to the denaturing procedures used in this study. According to its probable secondary structure, this region has a high turn index and high hydrophobicity, which implies internal structure. In this respect, the results just described confirm the supposition of Slilaty and Little (41). Using site-directed mutagenesis on LexA, they have shown that Ser-119 and Lys-159 are essential for the cleavage reaction, which indicates that the sequence Gly-Met-Ser-Met may be the catalytic site that acts like a serine protease. Their attempts to support this thesis with the specific inhibitor diisopropyl fluorophosphate were unsuccessful; one of the possible reasons that they mention for the negative result is that the active serine is in a pocket not easily accessible to the reagent. The (143-157) sequence contains the equivalent presumed catalytic site in repressor (Glu-Asn-Ser-Met), and it seems likely, from probing with the antibody, that it is not flexible or fully exposed to external reagents.

The anti-peptide (181-195) IgG had higher reactivity for denatured than for native repressor in competition ELISA (Fig. 2), and up to 89% of the native molecules could be immunoprecipitated. This finding indicates that there is some folding and flexibility in this region.

We presume that the oligopeptides (and, as a consequence, antipeptide IgG specificity) did not attain structures comparable to those of the homologous sequences in the intact molecule for the following reasons. (i) The antirepressor IgGs from two rabbits failed to react with the synthetic peptides in ELISA, competition ELISA, or immunoblots (Fig. 2 and unpublished results). (ii) The peptides did not compete with repressor for sites on the RecA protein in the cleavage assay. Another small fragment, tryptic peptide (99-119), also gave negative results in a similar cleavage reaction, whereas papain fragments of the COOH terminus were able to compete for RecA sites (38). (iii) Antipeptide IgGs failed to cross-react with LexA protein in ELISA and immunoblots (results not shown). All of these facts are consistent with the idea that the oligopeptides are too small or require interaction with other sequences in the molecule to generate a three-dimensional structure. The lack of cross-reactivity with LexA could also be attributed to those amino acid residues which are not shared with repressor. Even a single amino acid change in the epitope is capable of diminishing (1) or eliminating (28) the binding of antipeptide antibodies if the amino acid involved happens to be one of the residues that make contact with the IgG.

The fact that all three antipeptide IgGs, which recognize separate sequences in the linear structure, inhibit the same activity (RecA-mediated cleavage) supports the notion that these regions may form a more compact tertiary structure. There is scant evidence for the existence of this hypothetical three-dimensional structure of the C terminus of repressor: (i) upon thermal denaturation, only one cooperative melting transition of the C terminus is observed (29), which suggests folding of these two regions; (ii) in the case of P22 repressor, the hinge and the C domain remain associated after proteolysis (11); (iii) three mutations in the hinge region of repressor (17) and a small deletion in LexA (25) render these proteins more sensitive to proteases, which indicates folding of the wild type; (iv) mutations in repressor that confer resistance to RecA-mediated cleavage but do not affect autodigestion lie in the hinge and the (181-195) regions (16), which implies that those are the areas of interaction with RecA and that therefore they must have some proximity; and (iv) Ser-119 and Lys-156 in LexA, which correspond to Ser-149 and Lys-192 in lambda repressor, are necessary for hydrolysis of the Ala-111–Gly-112 bond and therefore must attain close contact, as shown in the model of autodigestion (41). The results that we obtained with the ELISA additivity test, indicating that the three different antibodies compete for binding to repressor even when Fab preparations are used (Table 2), strongly suggest that the conserved regions we studied are present on the same side of the molecule and may be adjacent in the tertiary structure. The large size of the immunoglobulin molecule prevents us from drawing any firm conclusion in this respect; however, in some cases size does not seem to be a significant factor, since antibodies to synthetic peptides of $pp60^{src}$ recognize epitopes which are eight residues apart and only one of them inhibits enzymatic function (15).

The operator-binding activity of repressor was not significantly affected after treatment with antipeptide IgGs but was strongly inhibited by antirepressor IgGs (Fig. 5). We surmise that the antipeptide antibodies did not disrupt repressor dimer formation, since this is the active species that binds to operator sequences (37). It is interesting to note that a large adduct on the hinge or C domain of repressor did not interfere with its function or cause steric hindrance for DNA.

The findings of this study suggest that with more quantitative experiments using site-directed antibodies, it may be possible to estimate the local conformational equilibria of a specific sequence in the molecule. We expect that this information will be complemented by studies of those monoclonal antibodies that are conformational, thus allowing identification of the primary sequences that form the surface of the repressor and are adjacent in the tertiary structure.

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