pH-dependent autocleavage of λ repressor occurs in the operator-bound form: characterization of λ repressor autocleavage

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The first-order rate constants for the RecA-independent, spontaneous, pH-dependent autocleavage of the λ cI repressor was measured in the present study at pH 10.6 at 27, 37 and 42 °C respectively. Autocleavage of the repressor occurs also at pH 9 and 8, although at progressively slower rates. We demonstrate that the spontaneous autocleavage occurs also in the operator-bound state, at a rate either higher than or equal to the rate in solution, depending on the pH value. Owing to the near equality of the rate constant in both operator-free and operator-bound repressors, it can be inferred that the cleavage site has a similar structure and dynamics with respect to the catalytic site in both forms at neutral pH. Covalent modification using PMSF, brought about by a large molar excess of the reagent, inhibits autocleavage of the λ repressor. The difficulty in obtaining this covalent modification is rationalized using our recent λ repressor models. Bimolecular type II *trans*-cleavage was observed previously for mutant LexA repressors lacking a crucial catalytic serine or lysine residue [Kim and Little (1993) *Cell* (Cambridge, Mass.) 73, 1165–1173], but it

could still be cleaved by an 85–202 ‘enzyme’ fragment possessing an improved or hypercleavable character lacking its own cleavage site. Such a type II *trans*-cleavage was not observed with the covalently modified intact λ repressor used as substrate and the purified wild-type λ repressor 112–236 fragment used as the ‘enzyme’. All these results show that for the wild-type λ repressor, the catalytic site is close to the cleavage site in both operator-free and -bound states. In the lytic pathway, the repressor is mainly cleaved via RecA-mediated cleavage, which occurs much faster than the spontaneous autocleavage; the possible biological significance of this slow, spontaneous, but constant, autocleavage is related to the lysogenic state, when RecA-mediated cleavage is absent.

Key words: λ repressor autocleavage, covalent modification by inhibitor, gene regulation, operator-binding and repressor function, self-proteolysis, *trans*-cleavage.

INTRODUCTION

At the heart of the gene regulatory system of the double-stranded DNA bacteriophage λ is its 236 residue cI repressor [1,2]. The switching between the lytic and lysogenic states of the λ phages uses cI as a regulatory protein binding to six different operator sites [1,2]. Although the N domain of cI makes all the contacts with the operator sites and can mediate both positive and negative control of transcription, its C domain modulates and regulates the entire activity, since the intact repressor binds to operator DNA approx. 1000-fold more strongly when compared with the isolated N domain [3]. After exposure to a moderate dose of UV light or other DNA-damaging conditions, cleavage of cI repressor at its Ala¹¹¹-Gly¹¹² occurs in the presence of activated RecA protein *in vivo* or *in vitro*; this results in loss of operator binding and, hence, loss of repressor function [4,5] and turns on transcription of the *cro* gene. Thus an important function of the cI repressor is its ability to undergo autodigestion at a specific peptide bond, Ala¹¹¹-Gly¹¹². The intramolecular cleavages in both LexA and λ repressors were further characterized and their pH dependence was studied [6], but the temperature dependence of the λ repressor's autodigestion has not been studied. We have measured the first-order rate constant for the λ repressor's autodigestion at different temperatures and, thereby, calculated the activation energy (E_a) and pre-exponential Arrhenius factor.

The involvement of Ser¹⁴⁹ and Lys¹⁹² as catalytic groups in the λ repressor's self-proteolysis was implicated by comparison with studies on LexA, and a possible mechanism was suggested [7]. λ Repressor mutants that either resist the cleavage (*ind*[−])

[8] or are better substrates for the RecA-mediated cleavage [9] were studied previously; the results supported a model in which repressor monomers are the only substrate in the RecA-mediated cleavage [9]. The fact that the λ repressor is dimeric when bound to operator DNA [1] implies that RecA-mediated cleavage does not affect the operator-bound form of the repressor. Papain is known to cleave the λ repressor in solution [10], but we have shown that papain does not cleave the repressor when bound to operator DNA [11]. Similarly, characterization of the autodigestion of the repressor when bound to operator DNA can reveal whether it also requires a monomeric form similar to RecA-mediated cleavage or can occur in the dimeric, operator-bound state. Our results show that the operator-bound dimeric repressor autocleaves itself.

The difficulty of covalently modifying the catalytic Ser¹⁴⁹ of LexA was reported previously; it needs a 220-fold molar excess of the DIFP (di-isopropyl fluorophosphate) reagent [12]. Similar studies have not been reported for the λ repressor. We investigated the incubation conditions needed for covalently modifying Ser¹⁴⁹ of a λ repressor using PMSF, another serine protease inhibitor, by monitoring the ability of the covalently modified repressor to autodigest later. Intermolecular cleavage or *trans*-cleavage has been studied for LexA and λ repressors [13]. We investigate whether the purified fragment 112–236 of the λ repressor can cleave a covalently modified intact λ repressor that has lost its ability to autodigest through such a bimolecular *trans*-cleavage.

Thus, through various approaches, the RecA-independent autocleavage of the λ repressor has been characterized in the present study. Our measurements do not indicate any significant difference in the structure and dynamics of the cleavage-site

Abbreviations used: DIFP, di-isopropyl fluorophosphate; PMS, phenylmethylsulphonyl; SB, standard buffer; wt, wild-type.

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region of the repressor between the free and bound states. It is shown that PMSF covalently modifies the catalytic Ser¹⁴⁹ only when used in large molar excess, indicating that the cleavage-site region covers it well. We have discussed the possible biological significance of this RecA-independent slow autocleavage by relating it to the lysogenic state.

MATERIALS AND METHODS

Materials

Sephadex G-50 and CM-Sephadex C-25 column materials, CAPS [3-(cyclohexylamino)-1-propanesulphonic acid] and PMSF were purchased from Sigma (St. Louis, MO, U.S.A.). Spectra/Por dialysis membrane (molecular mass cut-off, 3500 Da) was obtained from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Tris was purchased from Spectrochem (Bombay, India) and glycine was from Sisco Research Laboratories (Mumbai, India). Other reagents of guaranteed-reagent grade were mainly purchased from Merck (Mumbai, India). Both strands of the 17-mer O_L1 operator DNA [1] were obtained from Bangalore Genei (Bangalore, India).

Buffers

SB (standard buffer) containing 10 mM Tris/HCl (pH 8.0), 2 mM CaCl₂, 0.1 mM EDTA, 5 % (v/v) glycerol and 0.1 mM 2-mercaptoethanol was used. TGC buffer was prepared by dissolving equimolar amounts of Tris, glycine and CAPS [6] and by adding sodium hydroxide solution to bring the pH value to 10.6 or HCl to bring the pH value to 9.0 or 8.0.

Purification of λ repressor

The intact wt (wild-type) repressor and its SN228 (Ser → Asn²²⁸) mutant were purified as described by Johnson et al. [14], dialysed against SB also containing 200 mM KCl and stored at -20 °C.

Autocleavage reaction in the absence of operator DNA

Protein solutions were diluted to a concentration of $\leq 10 \mu\text{M}$ in 0.2 M TGC buffer [3] stock and the pH value was checked after all the additions using an ION83 Ionmeter (Radiometer, Copenhagen, Denmark). The mixtures were incubated at 27, 37 or 42 °C at pH 10.6. The aliquots were withdrawn after different time periods, and the reaction was monitored by boiling samples with SDS/PAGE gel loading dye, electrophoresis on an SDS/18 % polyacrylamide gel and staining the gel with silver nitrate. The rate of cleavage of the wt and SN228 repressors were compared at 27, 37 and 42 °C at 5 μM protein concentration by scanning the silver-stained polyacrylamide gel with a Bio-Rad GS700 imaging densitometer system using the Molecular Analyst software package. Percentage of cleavage was calculated by measuring the amount of intact repressor remaining in comparison with the total amount of protein present in the lane (i.e. intensity of the intact band divided by the sum of the intensities of the three bands, i.e. intact and the two fragments). Proteins of different lengths and sequences do not stain similarly; however, in our method, it is assumed that the two fragment bands, necessarily produced in equimolar amounts, together stain in the same manner as the intact repressor, i.e. cleavage at 111–112 does not significantly alter the total stain. The natural logarithm of the fraction of intact repressor remaining was plotted against the incubation time. For additional experiments at pH 9.0 or 8.0, 0.2 M TGC buffer containing 0.1 M NaCl was prepared separately and adjusted to these values by adding HCl. Incubation temperatures of 37 °C (pH 9.0) or 42 °C

(pH 8.0) were used and a similar procedure was followed for the determination of first-order rate constants.

Staining intensity of bands

The linearity of staining intensity with the amount of protein loaded was checked by loading various percentages of the maximum amount used in different lanes of an SDS/polyacrylamide gel. It was found that the staining intensity [calculated using the equation 'Adj Volume' = absorbance $\times$ area (in mm²) using the Molecular Analyst software package] was linear with the amount of protein loaded in the range 0.05–7.0. Below 0.05 units, the signal-to-noise ratio is low, whereas for readings above 10 units, there may be some non-linearity. The bands measured were in the range 0.05–7.0 for calculating the first-order rate constants.

Purification of the autocleaved fragment

The preparative autocleavage reaction for the purification of autocleaved fragments was set at pH 10.7 at 37 °C. Approx. 3–4 mg of the intact protein was used for the reaction. The mixture was incubated for 63 h at 37 °C and then dialysed against SB containing 50 mM KCl at 4 °C. After a thorough dialysis with two changes, the mixture was loaded on to an SP (sulphopropyl)-Sephadex C-25 column, equilibrated in SB containing 50 mM KCl. The N-terminal fragment 1–111 remained bound to the column and it was later eluted with a high-salt buffer. The 112–236 fragment and the trace amount of intact repressor remaining eluted in the flow-through fractions, as monitored by the subsequent SDS/PAGE analysis. To remove the trace amount of the intact repressor from the 112–236 fragment, gel-filtration chromatography was then performed using a Sephadex G-50 gel-filtration column (73 ml bed vol.) with a flow rate of about 6 drops/min (approx. 0.2 ml/min). Fractions containing pure 112–236 fragment were pooled, concentrated, dialysed against 0.1 M potassium phosphate buffer (pH 8.0) and stored at -20 °C.

Determination of the melting temperature for 17 bp O_L1 duplex

Since a high pH value may, in principle, destabilize the DNA double helix of O_L1 and thereby interfere with repressor binding, an experiment was performed in which the melting temperature of O_L1 was determined in 0.2 M TGC buffer (pH 10.6) containing 0.1 M NaCl; the melting temperature was found to be approx. 75 °C for a 2.25 μM solution of the duplex. Similarly, the melting temperature of O_L1 was found to be > 90 °C in 0.2 M Tris/HCl (pH 8.8), containing 0.1 M NaCl. Since the concentration of O_L1 used in our autocleavage reactions was 7.88 μM at temperatures much below the melting temperatures just mentioned, the operator DNA was in double helical conformation during our autocleavage experiments.

Autocleavage of λ repressor in the presence of operator DNA

Equimolar amounts of the two strands of O_L1 operator were mixed together, heated to approx. 95 °C in an Eppendorf tube by submerging in a water bath for approx. 2 min, allowed to cool to room temperature (25–27 °C) and dialysed against 1 mM Tris and 10 mM NaCl. Ultimately, a stock O_L1 solution was obtained by concentrating this solution 7-fold to a duplex concentration of approx. 0.79 mM. A 2-fold molar excess of the DNA compared with the dimeric concentration (or an equal concentration of both repressor monomer and DNA) was used to ensure that all the protein remained in the operator-bound state during the autocleavage reaction. In autocleavage reactions the same TGC buffer

was used, which contained 0.1 M NaCl to keep the DNA in the duplex state. The procedure followed was similar to the operator-free experiment described above, except that at pH 10.6, the incubations were performed at 23, 30 and 37 °C. For pH 9.0 and 8.0, incubations were performed at 37 and 42 °C, since the reaction would be too slow at lower temperatures. First-order rate constants were obtained from semi-logarithmic plots as described above.

Effect of PMSF on the autocleavage of λ repressor

To examine if the autocleavage reaction could be inhibited by the serine protease inhibitors, the reaction was performed in the presence of different concentrations of PMSF in DMSO at pH 10.7 or 8.0. The volume of PMSF solution added was adjusted in such a way that the final concentration of DMSO was > 10 % (v/v). The reaction mixture was incubated at 37 °C, aliquots were withdrawn at different time intervals and the reaction was monitored by SDS/PAGE (18 % gel). The gel was then stained with silver nitrate and analysed using the Molecular Analyst software package.

Type II *trans*-cleavage of λ cI repressor

To examine if the λ repressor undergoes type II *trans*-cleavage [13] or not, the repressor (5 μ M) was first treated with PMSF (100 mM) for 1 h and then dialysed overnight at 4 °C against buffer to remove any unbound PMSF. The molar absorption coefficient ϵ_{280} of the 112–236 fragment was calculated to be 18 350 M⁻¹ · cm⁻¹ (1.337 litres · g⁻¹ · cm⁻¹) based on the tryptophan and tyrosine content [15]. A purified 112–236 fragment (purified as described above) was then added to a final concentration of 1 μ M; the pH value was increased by adding TGC buffer and the reaction mixture was incubated at 37 °C for 20, 28 or 44 h. In control experiments, an equal volume of the buffer was added instead of the 112–236 fragment and treated similarly. At the end of the incubation period, the aliquots were taken out, boiled with the gel loading dye and subjected to SDS/PAGE (18 % gel).

RESULTS

Kinetics of autocleavage in the absence of operator DNA

The rate of autocleavage of the wild-type cI repressor at different temperatures is shown in Figure 1 as a plot of the natural logarithm of the fraction of intact repressor remaining versus the incubation time at pH 10.6. The rate of cleavage increases appreciably with increase in the incubation temperature between 27 and 42 °C. Nearly identical results were obtained for the SN228 mutant, which is known to be defective in dimerization [16]. In contrast, for RecA-mediated cleavage, the mutants defective in dimerization are cleaved faster [9]. The rate constants for both repressors were 1.35×10^{-6} , 4.36×10^{-6} and 7.35×10^{-6} s⁻¹ at 27, 37 and 42 °C respectively (Table 1), calculated from the slopes of straight lines passing through the origin and the centroid of the data points. The errors in these first-order rate constants were estimated to be less than ± 4 % of the values mentioned above. The enthalpy of activation for the autocleavage reaction was calculated to be 21.6 kcal/mol (1 cal = 4.184 J). The activation energy ($E_a = \Delta H + RT$) was calculated to be 22.2 kcal/mol at 300 K. For this value of E_a , the Arrhenius exponential factor is approx. 7×10^{-17} ; hence, the pre-exponential Arrhenius factor A is approx. 1.9×10^{10} s⁻¹.

Kinetics of autocleavage in the presence of operator DNA

The rates of autocleavage of the wild-type and the SN228 mutant cI repressors (as both of them show nearly identical rate of

Figure 1 Effect of temperature on the rate of autocleavage of λ repressor in the absence of operator DNA

The natural logarithm of the fraction of intact repressor remaining is plotted against the incubation time at 27 °C (◆), 37 °C (○) and 42 °C (□) at pH 10.6 for the wt fragment. The first-order rate constants are calculated from a straight line passing through the centroid of the data points within a group and the origin.

Table 1 First-order rate constants for repressor autocleavage under various conditions

The errors in the rate constants given below are estimated to be within 6 % of the values. Generally, rate constants (in s⁻¹) were calculated from five data points having 10–70 % cleavage.

pH 8.0	pH 9.0	pH 10.6
Reaction in the presence of operator DNA		
0.30×10^{-6} (37 °C)	1.19×10^{-6} (37 °C)	2.29×10^{-6} (23 °C)
0.43×10^{-6} (42 °C)	1.84×10^{-6} (42 °C)	5.48×10^{-6} (30 °C)
		12.56×10^{-6} (37 °C)
Reaction without operator DNA		
0.49×10^{-6} (42 °C)	0.69×10^{-6} (37 °C)	1.35×10^{-6} (27 °C)
		4.36×10^{-6} (37 °C)
		7.35×10^{-6} (42 °C)
Control experiment with GalO _E operator DNA		
		3.76×10^{-6} (37 °C)

autocleavage) at different temperatures in the presence of O_L1 operator are shown in Figure 2 as semi-logarithmic plots for pH 10.6. The rate of cleavage increases appreciably with increase in the incubation temperature between 23 and 37 °C. The rate constants were 2.29×10^{-6} , 5.48×10^{-6} and 12.56×10^{-6} s⁻¹ at 23, 30 and 37 °C respectively. The enthalpy of activation for the autocleavage reaction was calculated to be 22.2 kcal/mol. E_a was calculated to be 22.8 kcal/mol at 300 K. However, owing to errors inherent in the first-order rate constants, the error in E_a could be approx. ± 2 kcal/mol. For this value of E_a , the Arrhenius exponential factor is approx. 8.4×10^{-17} . Hence, the pre-exponential Arrhenius factor A is approx. 2.7×10^{10} s⁻¹, almost the same as that obtained for the operator-free state, although there could be a large error in estimating this due to the error in the E_a value. At 37 °C, the first-order rate constant in the operator-bound state is, thus, 2.9 times the rate constant in the operator-free state. It is seen that, in the presence of the GalO_E operator DNA, there was no enhancement of the rate at pH 10.6 (Table 1), in contrast with O_L1.

Figure 2 Effect of temperature on the rate of autocleavage of λ repressor in the presence of operator DNA

The natural logarithm of the fraction of intact repressor remaining is plotted against the incubation time at 23 °C (○), 30 °C (●) and 37 °C (△) at pH 10.6 for the wt fragment. The first-order rate constants are calculated from a straight line passing through the centroid of data points within a group and the origin.

Kinetics of autocleavage at pH 9.0 and 8.0

The various first-order rate constants at pH 8.0 and 9.0 are presented in Table 1 (first and second columns respectively). From these, it is found that both in the presence and absence of operator DNA, the rate constants progressively increased with pH values. For a given pH value, the rate constant progressively increased with incubation temperature. The rate constants were generally calculated from values corresponding to five different incubation periods having 90–30% intact repressor remaining (10–70% cleavage). For lower pH values, longer incubation periods became necessary to observe significant amounts of cleavage. At 42 °C and pH 8.0, the rate constant was determined to be $0.49 \times 10^{-6} \text{ s}^{-1}$ in the absence of operator DNA and $0.43 \times 10^{-6} \text{ s}^{-1}$ in the presence of operator DNA (Table 1). Thus, at pH 8.0, the rate of autodigestion is almost equal for both operator-free and -bound repressors.

Effect of PMSF on the autocleavage of λ CI repressor

It was not straightforward to modify the catalytic serine group of the repressor using PMSF, since reaction conditions were unknown. An equimolar amount or even 100-fold excess of PMSF did not inhibit the autocleavage reaction of the repressor, whereas a very high concentration (approx. 10000-fold excess) of PMSF in DMSO did. From the gel shown in Figure 3, incubations with 50, 100 and 200 mM PMSF [17] resulted in 46, 13.5 and 6.6% of the normal autocleavage reaction during a subsequent 62 h period. The inhibition of the autocleavage reaction was probably due to non-specific reaction of PMSF with the serine and threonine residues of the protein, leading to an alteration of the structure. However, the observation that the PMSF-treated repressor underwent autocleavage on prolonged incubation indicates that the effect might be more specific and the activity returns after a loss of the PMS (phenylmethylsulphonyl) moiety. This reversal was also noticed for chymotrypsin [17].

Type II *trans*-cleavage of λ repressor

Kim and Little [13] investigated the conditions under which an intermolecular cleavage reaction occurs where one molecule of LexA acts as an enzyme to cleave other molecules [13]. In their type I cleavage reaction, they mixed a truncated 1–98 residue LexA substrate containing an LP89-QW92 hypercleavable muta-

Figure 3 Inhibition of λ repressor autodigestion by PMSF

Mixtures with no (lane 1), 50 mM (lane 2), 100 mM (lane 3) and 200 mM (lane 4) PMSF were examined after 62 h. Lanes 5–8 show the same mixtures respectively after 83 h. The upper bands, all of which are saturated, represent the intact repressor, whereas the lower bands represent the cleaved fragments, their intensities diminishing with higher concentrations of PMSF. Comparison of the product intensities after 83 h, particularly lanes 3 and 4 with lanes 7 and 8, reveals that more cleavage appeared after 83 h; there is a hydrolysis of the covalent adduct formed by the PMS moiety, as noted for chymotrypsin previously [17].

Figure 4 Type II *trans*-cleavage

A silver-stained gel showing type II *trans*-cleavage is not possible with the λ repressor. Lanes 1–4 represent reactions after 16 h, whereas lanes 5–8 represent reactions after 45 h. Lanes 1 and 5 are controls for lanes 2 and 6 respectively and represent reactions in the presence of 0.34 mol of enzyme/mol of substrate; similarly, lanes 3 and 7 are controls for lanes 4 and 8 respectively and represent reactions in the presence of 1.7 mol of enzyme/mol of substrate. In lanes 2, 4, 6 and 8, the band intensity of 112–236 produced by cleavage was calculated by subtracting the amount of enzyme 112–236 added in each lane. The upper bands represent the covalently modified intact repressor, whereas lower bands represent the 112–236 fragment; the 1–111 fragment stains relatively weakly in silver and is not apparent in this gel.

tion with the C-terminal 85–202 fragment containing another hypercleavable mutation, EA152, as enzyme. In their type II *trans*-cleavage, non-cleavable LexA containing either an SA119 mutation at the catalytic serine residue or a KA156 mutation at the catalytic lysine residue was exposed to the 85–202 fragment of hypercleavable EA152 as enzyme. Our choice of substrate was covalently modified λ repressor molecules with the attached PMS group. After the covalent modification, excess PMSF was removed by dialysis and the substrate was exposed to separately purified wild-type 112–236 fragment as enzyme; thus, ours is a variant of the type II reaction. Intramolecular cleavage was prevented by the covalent modification using PMSF. For the 16 h incubation, 98% of the band intensity in a lane (no enzyme or with 0.34 mol of enzyme/mol of substrate) corresponded to the intact repressor; for the 45 h incubation, a band intensity of 90% (no enzyme) and 91% (with enzyme) corresponded to the intact repressor (Figure 4). Thus the purified 112–236 fragment apparently did not act as an enzyme in a type II *trans*-cleavage reaction.

Figure 5 Ribbon diagram of the C domain of λ repressor

Residues 103–236 from our PDB entry 1j5g are displayed. The region 103–115 is closest to the viewer in this ribbon diagram, covering the catalytic serine and lysine residues responsible for autocleavage of the repressor. The α carbon and a few other non-hydrogen atoms of the side chains of Ala¹¹¹, Ser¹⁴⁹, Lys¹⁹² and Tyr²¹⁰ are displayed as ball-and-stick models. In an alternative model 1lwq for the repressor tetramer, two out of four subunits possess a similar disposition of the cleavage-site region compared with the rest of the C domains [18], but two other catalytic sites are involved in interdomain interactions and have their cleavage-site regions further away. Both 1j5g and 1lwq are homology-based models.

Interaction of cleavage site with catalytic site

The experimental results obtained in the present study may be rationalized in terms of our three-dimensional models for λ repressor bound to two adjacent operator sites [18]. In Figure 5, a ribbon diagram of residues 103–236 of a single repressor molecule shows Ala¹¹¹–Gly¹¹² as a part of a cleavage-site region close to the catalytic Ser¹⁴⁹ and Lys¹⁹² side chains. This accounts for the large value of the pre-exponential Arrhenius factor. Furthermore, both the Tyr²¹⁰ side chain and residues 105–115 serve as lids for the catalytic groups. These lids prevent the PMSF molecules from reacting with the catalytic serine residue.

DISCUSSION

Our observed rate constant of $4.36 \times 10^{-6} \text{ s}^{-1}$ at pH 10.6 for the (operator-free) autocleavage reaction is approx. 14 times lower compared with the previously reported rate constant of $6.1 \times 10^{-5} \text{ s}^{-1}$ at 37 °C at 1 nM protein concentration [6]. However, our extrapolated rate constant of $12 \times 10^{-6} \text{ s}^{-1}$ at 46 °C is nearly twice the rate constant of $7.3 \times 10^{-6} \text{ s}^{-1}$ reported by another group [8]. Although the previous study [6] used a nanomolar concentration of the repressor, the first-order rate constant at pH 10.6 was judged to be independent of repressor concentration; hence, the trichloroacetic acid precipitation method used to evaluate the amount of cleavage seems to be the source of the disparity by overestimating the amount of cleavage. We used a procedure similar to the one used in a previous study [8] to measure the cleavage for various incubation periods, except that we used the TGC buffer instead of Tris and did not use BSA in the reaction mixture. The autocleavage rates in the SN228 repressor that has a defective dimerization [16] were found to be identical with those in the wild-type repressor, indicating that defective dimerization does not affect the autocleavage rate. The pre-exponential Arrhenius factor is found to be approx. $1.9 \times 10^{10} \text{ s}^{-1}$, indicating the number of collisions between the cleavage and the catalytic groups for the operator-free reaction, which in turn requires the presence of the catalytic groups quite close to the substrate Ala–Gly.

The present study has shown for the first time that autocleavage of the repressor continues in the operator-bound state. At pH 10.6, the collisional frequency is apparently increased slightly in the operator-bound state, and there is also a small change in the E_a value. Furthermore, near neutral pH, the autocleavage rates of the repressor in the operator-free and -bound states seem to be nearly equal. Thus the structure and dynamics of this cleavage site relative to the catalytic site are probably similar in both states near neutral pH.

Our interest in the autocleavage reaction evolved out of our model-building efforts for the operator-bound forms of LexA [19] and its homologous λ repressor [18] [PDB (<http://www.rcsb.org/pdb>) accession numbers for comparative models of the λ repressor are in RCSB001477 (1gfx) processed on 22 June 2000, RCSB001635 (1j5g) processed on 3 May 2002 and RCSB016355 (1lwq) processed on 30 May 2002; our related model of LexA repressor in RCSB000408 (1qaa) processed on 2 February 1999 and an improved model of it in RCSB017215 (1mvd) processed in September 2002], since we reasoned that the substrate Ala–Gly must be near the catalytic groups. We predicted a comparative model of the λ repressor C domain in the PDB entry 1gfx, which turned out to have only 1.24 Å r.m.s. deviation (1 Å = 0.1 nm) in the main chain atoms from the crystal structure proposed in the entry 1f39 released later for residues 139–236 [20]. We also predicted the proximity of the Ala–Gly to the catalytic groups for our LexA dimer model [19] and all our repressor models (1gfx, 1j5g, 1lwq), since we believed that the autocleavage reaction required this. The present study verifies this experimentally. Although the number of collisions per second is numerous, only a fraction of them result in autocleavage, since the E_a barrier is high. The slowness of the reaction was explained for LexA by molecular modelling [19], since the geometry was found to be not suitable for a nucleophilic attack from the catalytic serine O γ to the alanine carbonyl carbon atom. The structure of the cleavage site [19] needs modification in the light of the recently proposed crystal structure of various LexA mutant dimers [21] showing two conformations for the cleavage site, termed ‘cleavable’ and ‘non-cleavable’, and this has been incorporated in our later λ repressor models 1j5g and 1lwq. Additionally, the LexA crystal structure [21] shows that the glycine residue has an unusual conformation in its main chain ($\Phi = +163^\circ$, $\Psi = -142^\circ$), which is generally not favoured for other residue types. However, the fact that the GA112 mutant of the λ repressor cleaves at all [8] indicates that such an unusual conformation, although helpful for cleavage, may not be required. It is difficult to reconcile the non-cleavable form of the cleavage-site region [21] with the experimentally measured Arrhenius factor in the present study and in a previous study [6], since in that form the cleavage site is approx. 20 Å away from the catalytic site. The non-cleavable form, which was proposed to be 10000 times higher in concentration compared with the cleavable form [12,21], shows up almost as frequently as the latter in the various mutant crystal structures [21]. It is to be noted that neither of the mutants used is for a wild-type structure (which is cleavable), but the ‘cleavable form’, in our opinion, is the closest approximation. However, in the λ repressor, something close to a non-cleavable form is possible in half of the monomers comprising a tetramer, since half of the subunits seems to use the catalytic serine residue for dimer–dimer contacts [20]; we have incorporated this idea in our PDB entry 1lwq.

A 220-fold molar excess of DIFP, a serine protease inhibitor, is required to inhibit the autodigestion of the LexA repressor [12]. [We have used PMSF instead of DIFP, since the manufacturer (Aldrich) of the latter toxic chemical does not deliver it by post.] Higher concentrations of PMSF inhibit the autodigestion of the λ repressor more (Figure 4). We had to use a 10000-fold

molar excess of PMSF to observe a significant inhibition of the autocleavage reaction. This underscores the difficulty of the reaction, suggesting that the catalytic Ser¹⁴⁹ side chain [7] is buried in the λ repressor C domain. Alternatively, Ser¹⁴⁹ may be buried as a part of the dimer–dimer interface as observed in the crystal structure [21], but it still leaves half of the catalytic serine residues exposed, unless they too are covered by the cleavage-site region. As the repressor treated with the high concentration of the PMSF ultimately cleaved itself, the effect of PMSF was not due to any major structural changes resulting from non-specific modifications; PMSF-treated enzymes were found to lose the inhibitor group when incubated at high temperature and/or high pH [17]. So the cleavage of the covalently modified repressor after longer periods (≥ 48 h) of incubation was quite probably due to loss of the inhibiting PMS moiety after incubation at 37 °C and pH 10.7.

The λ repressor enzyme (112–236) was almost as efficient as the LexA enzyme in terms of *trans*-cleavage of the LexA substrate and is capable of recognizing the LexA Ind⁺ mutation [13]. A truncated λ cI (containing the N-terminal domain of cI repressor, the cleavage site and additional 19 amino acid residues) is not a good substrate for either the λ or the LexA enzymes [13]. We have performed similar experiments by using a purified 112–236 fragment of the λ cI repressor as the enzyme and the PMSF-treated λ cI repressor as the substrate, but there was no *trans*-cleavage product. Thus the λ repressor is not a good substrate for the *trans*-cleavage reaction. This observation is in agreement with the crystal structures [20,21], since the serine-containing catalytic portion is similar in both λ and LexA repressors; however, the cleavage-site region may possess slightly different structures in the two repressors. A proteolytic fragment AB, containing the Ala-Gly in the homologous P22 repressor, remained bound to BC unless mild denaturing conditions were used [22], suggesting that, in the phage repressors, the Ala-Gly region interacts strongly with the rest of the C domain. This is corroborated by the larger value of the pre-exponential factor in λ of $1.9 \times 10^{10} \text{ s}^{-1}$ compared with 10^9 s^{-1} for LexA [6].

There are normally approx. 1200 RecA molecules/cell in *Escherichia coli*, and their number increases up to 50-fold after induction [23]. Assuming a volume of 10^{-15} litre for the cell, the concentration of RecA could be as high as 100 μM after induction, i.e. a thousand times higher than 0.1 μM , the physiological concentration of the λ repressor [20]. In the *in vitro* assays of Gimble and Sauer [9], where 4.5 μM of the repressor and 2.7 μM RecA were chosen, approx. 50% of the repressor molecules were cleaved within 4 h at 37 °C in the presence of single-stranded DNA and ATP. Since the repressor concentration *in vivo* is only 0.1 μM and there is a large excess of RecA under conditions of induction, the RecA-mediated cleavage in solution is estimated to take only approx. 5 min for 50% cleavage. The assay represents cleavage in the absence of operator DNA, and according to their model, RecA-mediated cleavage prefers monomeric repressors [9]. Hence, RecA-mediated cleavage cannot directly affect the repressor dimers bound to operator sites: it waits for those dimers to fall off the DNA first. Approx. 40 min after induction and subsequent growth, a plate clearing assay could detect lysis [24]. Whereas autodigestion, a much slower process, does not seem to contribute substantially to the lytic pathway, a physiological significance of the autocleavage rate is connected to the lysogenic state. To keep the repressor concentration constant, the transcription of the repressor through

P_{RM} has to be at least equal to the rate of self-proteolysis of the repressor.

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