View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Publications of the IAS Fellows

Protein Engineering vol.12 no.3 pp.225–233, 1999

Amino acid changes in the repressor of bacteriophage lambda due to temperature-sensitive mutations in its *c*I gene and the structure of a highly temperature-sensitive mutant repressor

Nandan Kumar Jana[1], Siddhartha Roy[2], Bhabatarak Bhattacharyya[1] and Nitai Chandra Mandal[1,3]

[1]Department of Biochemistry and [2]Department of Biophysics, Bose Institute, Acaharya J.C. Bose Birth Centenary Building, P-1/12, CIT Scheme VII M, Calcutta 700 054, India

[3]To whom correspondence should be addressed.
E-mail: ncmandal@boseinst.ernet.in

The mutant *c*I*ts* genes from seven different λ*c*I*ts* phages carrying *ts*U50, *ts*U9, *ts*U46, *ts*1, *ts*U51, *ts*I-22 and *ts*2 mutations were cloned in plasmid. The positions of these mutations and the resulting changes of amino acids in the repressor were determined by DNA sequencing. The first four mutations mapping in the N-terminal domain show the following changes: I21S, G53S, A62T and V73A, respectively. Of the three remaining mutations mapping in the C-terminal domain, *c*I*ts*I-22 and *c*I*ts*2 show N207T and K224E substitutions respectively, while the mutant *c*I*ts*U51 gene carries F141I and P153L substitutions. Among these *ts* repressors, CI*ts*2 having the charge-reversal change K224E was overexpressed from *tac* promoter in a plasmid and purified, and its structure and function were studied. Operator-binding studies suggest that the *ts*2 repressor is somewhat defective in monomer–dimer equilibrium and/or cooperativity even at permissive temperatures and loses its operator-binding ability very rapidly above 25°C. Comparative studies of fluorescence and CD spectra, sulfhydryl group reactivity and elution behaviour in size-exclusion HPLC of both wild-type and *ts*2-mutant repressors at permissive and non-permissive temperatures suggest that the C-terminal domain of the *ts*2 repressor carrying a K224E substitution has a structure that does not favor tetramer formation at non-permissive temperatures.

Keywords: bacteriophage λ/lambda repressor/structure of λ repressor/temperature-sensitive mutant repressors of λ

Introduction

The structural analysis of functionally defective mutant proteins forms a very important part of strategies aimed at understanding the structure–function relationships of proteins at the level of residues and atoms. Temperature-sensitive (*ts*) mutants form an interesting subset, because at permissive temperatures, the function of the *ts* protein is preserved, implying a conservation of the functional form of its structure; only at non-permissive temperatures is the functionality of such protein lost, implying an alteration of that structure due to denaturation. Many of the *ts* mutations lead to changes in amino acids that may not be directly involved in the functional regions of the proteins, but are needed for maintaining the integrity of their structure. Hence a study of the structure of *ts*-mutant proteins at permissive and non-permissive temperatures offers certain unique avenues of exploration of protein structure–function relationships.

The repressor of bacteriophage λ, the product of its *c*I gene, negatively regulates the expression of the genes from the two early promoters, P_L and P_R, by specific interaction with two operators, O_L and O_R, respectively (Ptashne *et al.*, 1980; Ptashne, 1992). The functional form of the repressor is a dimer of two identical subunits. The repressor binds to three operator sites, O_R1, O_R2 and O_R3, that constitute the operator O_R, in order of decreasing affinity with alternate pair-wise cooperativity. The selectivity and cooperativity are essential for proper functioning of the switch. The cooperativity is a consequence of protein–protein interaction between two repressor dimers bound to two operator sites.

The λ repressor monomer has 236 amino acid residues and two functional domains: the N-terminal domain spanning from 1 to 92 and the C-terminal domain from 132 to 236 residues. The N-terminal domain is responsible for specific operator binding and contact with RNA polymerase for activation of P_{RM} promoter, while the C-terminal domain is responsible for dimer formation and higher order protein–protein contacts. Recent studies suggest that a communication occurs between the two domains of λ repressor through conformational changes, and the nature of the protein–protein interaction is significantly modulated following binding of the repressor with the operator sites (Saha *et al.*, 1992; Bandyopadhyay *et al.*, 1995). The amino acid sequence of the repressor has been determined by both DNA sequencing of the *c*I gene (Sanger *et al.*, 1982) and protein sequencing of the purified repressor (Sauer and Anderegg, 1978). The intact λ repressor has not been crystallized, but the crystal structures of the N-terminal domain (Pabo and Lewis, 1982) and its complex with the operator DNA (Jordan and Pabo, 1988) have been solved. Normal affinity of the wild-type repressor for operators could be altered by mutations. Thus, both low- and high-affinity mutations in the *c*I gene in phage (Kaiser, 1957; Sussman and Jacob, 1962; Lieb, 1966; Nag *et al.*, 1984) and the latter type of mutations in plasmid-borne *c*I gene (Nelson and Sauer, 1985) have been isolated. Recently, several *c*I mutants having mutations in the C-terminal domain have also been isolated which are defective in monomer–dimer equilibrium and cooperative binding; some of these non-cooperative mutants have been studied in detail using the tools of structural biology (Burz and Ackers, 1994; Burz *et al.*, 1994). This has led to the identification of several residues that are important in various protein–protein interactions.

There are several *ts* mutations in the *c*I gene of λ which are distributed in the two functional domains (Lieb, 1966). However, no *ts* mutation mapping in the connecting hinge region (93–131) has been reported. It has been reported that all of these *ts*-mutant repressors lose DNA-binding activity at 42°C in cell-free extracts (Mandal and Lieb, 1976). *In vivo*, certain *ts* mutations in the N-terminal domain complement certain others in the C-terminal domain in *trans* (Lieb, 1966, 1976). However, when any two of these mutations are present in *cis* in the same *c*I gene, the resulting repressor is non-

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

© Oxford University Press

functional even at 30°C (Nag *et al.*, 1982). The existence of many of these *c*I*ts* mutants of λ with such interesting properties led us to initiate the characterization and detailed structural studies of the *ts*-mutant repressors. In this work, we determined the changes of amino acids induced by seven different *ts* mutations and their exact positions in the repressor protein by sequencing DNA of the relevant mutant *c*I genes. As the lysogen of λ*c*I*ts*2 mutant was shown to be the most temperature sensitive and most susceptible to UV induction among the known λ*c*I*ts* mutants (Lieb, 1966), and in this study we found that the mutant CI*ts*2-protein carries a charge-reversal K224E substitution, a detailed structural investigation on this mutant repressor was also carried out.

Materials and methods

Materials

Acrylamide, DTNB, β-mercaptoethanol, IPTG, PMSF, bovine serum albumin (BSA), DTT, calf thymus DNA, glycerol, DNase, RNase, QAE-Sephadex and polyethyleneimine were purchased from Sigma Chemical (St Louis, MO). Restriction enzymes were purchased from GENEI (Bangalore, India). Bactotryptone, bactoagar and yeast extract were obtained from Difco Laboratories (Detroit, MI) and hydroxyapatite from Bio-Rad (USA). The DNA sequencing kit Sequenase version 2.0 was bought from Amersham. Oligo primers for DNA sequencing were synthesized in an automated DNA synthesizer (Applied Biosystems, Model 380A). [^{32}P-α]dATP and [^{3}H]thymidine were obtained from Bhaba Atomic Research Center (Mumbai, India). Millipore filters (HAWP, HA 0.45 μm, 25 mm) were purchased from Millipore India (Bangalore, India). All other chemicals and reagents were of analytical grade and were purchased from local suppliers.

Bacteria, bacteriophages and plasmids

Escherichia coli RR1 [*supE44 hsdS20 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl1*] and DH5 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) were obtained from S.Adhya (National Institutes of Health, Bethesda, MD). *E.coli* 594 (*F*$^-$ *galK*$^-$ *galT*$^-$ *lac*$^-$ *str*^{r} *su*$^-$) and Y-mel (*suIII*$^+$) and bacteriophage λ strains, λ*c*I$^-$, λ*c*I*ts*U50, λ*c*I*ts*U9, λ*c*I*ts*U46, λ*c*I*ts*1, λ*c*I*ts*U51, λ*c*I*ts*I-22 and λ*c*I*ts*2 from M.Lieb. The plasmid pEA305 (Amann *et al.*, 1983) was a gift from M.Ptashne. pNM1 (Das and Mandal, 1986) and pBR322 were used from laboratory stocks. Other plasmids were constructed during this work.

Bacterial growth

E.coli was routinely grown in tryptone broth (TB) (1% bactotryptone and 0.5% NaCl) or Luria–Bertani broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.1) with shaking at the desired temperature. The growth was monitored by measuring the optical density (OD) at 590 nm. [^{3}H]λ DNA was prepared as described by Mandal *et al.* (1974).

Recombinant DNA methods

Transformation of plasmid DNA in *E.coli* was done by the $CaCl_2$ method as described by Sambrook *et al.* (1989). The strategies of cloning of the mutant *c*I*ts* genes of λ are described in Figure 1.

Molecular cloning of cIts-mutant genes of λ

The mutant *c*I*ts* genes from seven different λ*c*I*ts* mutant phages, λ*c*I*ts*U50, λ*c*I*ts*U9, λ*c*I*ts*U46, λ*c*I*ts*1, λ*c*I*ts*U51, λ*c*I*ts*I-22 and λ*c*I*ts*2 were cloned in pBR322 (see Figure 1 for details) to obtain the plasmids pMJU50, pMJU9, pMJU46, pMJ1, pMJU51, pMJI-22 and pMJ2, respectively.

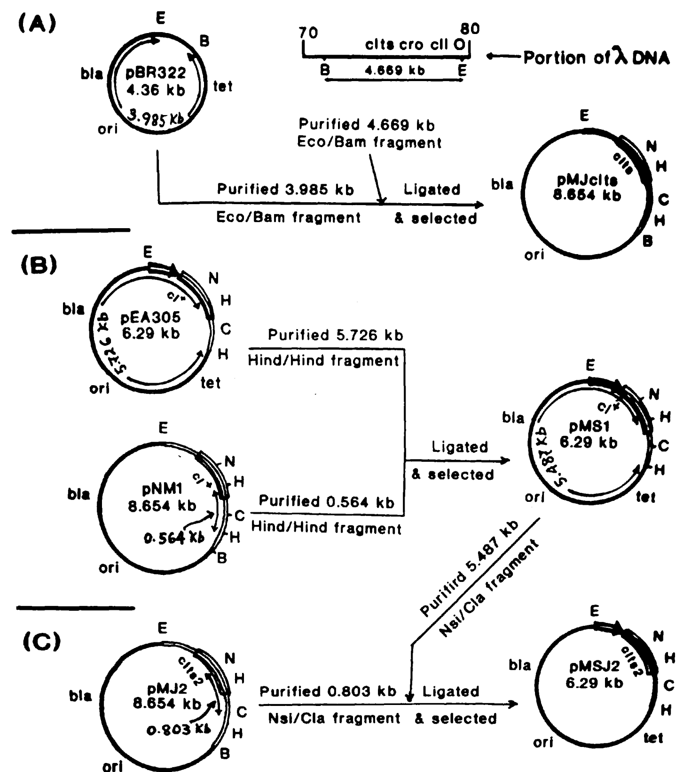

Fig. 1. Cloning of mutant *c*I*ts* genes of λ. (**A**) The mutant *c*I*ts* genes from λ*c*I*ts*U50, λ*c*I*ts*U9, λ*c*I*ts*U46, λ*c*I*ts*1, λ*c*I*ts*U51, λ*c*I*ts*I-22 and λ*c*I*ts*2 phages were cloned in pBR322 as shown. The constructed plasmid pMJcIts represents the general name for pMJ50, pMJ9, pMJ46, pMJ1, pMJ51, pMJI-22 and pMJ2 for the clones of *c*I*ts* genes, respectively, from the above *c*I*ts* mutant phages. These plasmids were selected in *E.coli* DH5 by their immunity to superinfection by λ*c*I$^-$ at 32°C and sensitivity to λ*imm*434*c*I$^-$ at 32°C and λ*c*I$^-$ at 42°C. Finally, each clone was confirmed by restriction analysis. (**B**) The plasmid pEA305 carries the wild-type *c*I gene of λ tagged to the *tac* promoter and expresses the repressor at very high level (around 30% of the total cellular protein) when induced by IPTG in a *lacI*^{q} host (Amann *et al.*, 1983). This plasmid does not contain the *Cla*I site between the C terminus of *c*I gene and the downstream *Hin*dIII site, whereas the plasmid pNM1 (Das and Mandal, 1986) carrying also the wild-type *c*I gene tagged to its P_{RM} promoter has the *Cla*I site at the analogous position. A cassette plasmid pMS1 carrying the *c*I$^+$ gene tagged to the *tac* promoter and containing the *Cla*I site between C terminus and the downstream *Hin*dIII site was constructed by replacing the 0.564 kb *Hin*dIII/*Hin*dIII fragment of pEA305 by the same restriction fragment from pNM1. pMS1 was selected by testing its immunity to λ*c*I$^-$ superinfection at both 32 and 42°C and detecting the presence of the *Cla*I site, and by restriction analysis. (**C**) The plasmid pMSJ2 carrying the mutant *c*I*ts*2 gene tagged to the *tac* promoter was constructed by exchanging the 0.803 kb *Nsi*I/*Cla*I fragment of pMS1 with the same fragment from pMJ2. The selection of pMSJ2 was done exactly as described in (A). Symbols: in the portion of λ DNA in (A), the numbers (70 and 80) above the map represent the % coordinates on λ DNA. The letters above the map indicate gene symbols and those below restriction enzyme symbols. Restriction enzyme symbols in plasmids: C, *Cla*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I. For further details, see Materials and methods.

DNA sequencing

The nucleotide sequence of the complete λ DNA molecule is known (Sanger *et al.*, 1982). Hence four suitable 20-mer primers were designed such that the whole of the *c*I gene in a plasmid could be sequenced using those primers. The above mutant *c*I genes in plasmid were sequenced by the double-stranded DNA-sequencing method of Sanger *et al.* (1977) using synthetic oligo primers and the plasmid DNAs carrying the mutant *c*I*ts* genes as templates and the Sequenase kit

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

(version 2.0). Each mutation was sequenced at least three times from three independent sequencing reactions using the respective plasmid clones isolated from different colonies.

Subcloning of cIts2-mutant gene in an expression vector

The wild-type *c*I gene of λ is expressed under the control of *tac* promoter in a plasmid pEA305 (Amann *et al*., 1983) to a very high level. We first prepared a cassette plasmid pMS1 which could be used for subcloning most of the above *c*I*ts* mutations for high expression under the control of *tac* promoter (see Figure 1). The *c*I*ts*2 mutation was subcloned in pMS1 to give pMSJ2. The presence of *ts*2 mutation in the *c*I gene tagged to the *tac* promoter in pMSJ2 was confirmed by restriction analysis and DNA sequencing: the high level of expression of the repressor was confirmed by SDS–PAGE analysis of the extract of IPTG-induced cells carrying pMSJ2 (data not shown).

Isolation of repressors

Wild-type repressor was isolated from *E.coli* RR1 carrying pEA305 plasmid and the *ts*2 repressor from *E.coli* RR1 bearing the plasmid pMSJ2 (see Figure 1). The cells containing the above plasmids were separately grown to an OD_{590} of 0.6, IPTG was added to a final concentration of 1 mM and the plasmids were grown further for 2 h at the same temperature. The cells were then harvested and washed and the pelleted cells were stored at –20°C. The wild-type repressor was purified following exactly the procedure described by Saha *et al*. (1992). The *ts*2-mutant repressor was purified basically by the above procedure with the following modifications. After centrifugation of the sonicated extract at 12 000 *g*, the supernatant was subjected to ammonium sulfate fractionation. The precipitate obtained at 60–80% saturation was dissolved in sonication buffer [10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 2 mM $CaCl_2$, 0.1 mM β-mercaptoethanol and 5% (v/v) glycerol] containing 25 mM KCl. This was then subjected to QAE-Sephadex column (instead of CM-Sephadex column) chromatography followed by hydroxyapatite column chromatography. The purified repressors were stored in the presence of 50% glycerol at –20°C. The repressor was assayed by the DNA filter-binding method of Riggs *et al*. (1968) and the protein concentration was determined from the relation $A^{1\%}{}_{280} = 11.3$.

Determination of sulfhydryl reactivity

The sulfhydryl reactivity was determined by the procedure described by Banik *et al*. (1992). This was done by titrating protein with DTNB under various conditions when 1 mol of 2-nitro-5-mercaptobenzoate was liberated per mole of exposed sulfhydryl (SH) group in the protein. This liberated mercaptobenzoate was quantitated from its absorption at 412 nm using the relation $\varepsilon = 1.36 \times 10^4$ l/mol. cm at pH 8.0 (Ellman, 1959).

Fluorescence methods

For all spectroscopic studies, the repressors were dialyzed against 0.1 M potassium phosphate buffer, pH 8.0. All fluorescence spectra were measured in a Hitachi F 3000 spectrofluorimeter with a computer for spectral addition and subtraction. For tryptophan fluorescence, the excitation and emission wavelengths were kept at 295 and 340 nm, respectively. All fluorescence values were corrected for volume changes, inner filter effects and blank values according to Bandyopadhyay *et al*. (1995). The excitation and emission bandpass were 5 nm unless mentioned otherwise. Acrylamide quenching of tryptophan fluorescence was studied as described by Bandyopadhyay *et al*. (1995).

Circular dichroism

Far-UV-circular dichroism (CD) spectra were measured in a Jasco J600 spectropolarimeter using a 10 mm pathlength cuvette at the required temperature controlled at ±1°C. The scan speed was 50 nm/min and 10 scans were signal-averaged to increase the signal-to-noise ratio.

Size-exclusion HPLC

Size-exclusion HPLC was performed with a Waters HPLC system using a Protein Pak TM 300 SW column (7.5 × 300 mm) having a fractionation range of molecular weight 10 000–400 000 Da native globular proteins. The column was pre-equilibrated and eluted with 0.1 M phosphate buffer, pH 8.0, and a 0.4 ml/min flow rate was used throughout. Carbonic anhydrase, bovine serum albumin, yeast alcohol dehydrogenase, β-amylase and apoferritin were used as molecular weight markers.

Results

Codon changes in the cI gene and amino acid changes in the repressor of λ due to seven different cIts mutations

Seven *c*I*ts* mutations, *c*I*ts*U50, *c*I*ts*U9, *c*I*ts*U46, *c*I*ts*1, *c*I*ts*U51, *c*I*ts*I-22 and *c*I*ts*2 were sequenced using the plasmids pMJU50, pMJU9, pMJU46, pMJ1, pMJU51, pMJI-22 and pMJ2, respectively, as described in Materials and methods. The results are summarized in Table I. Of the four *ts* mutations in the DNA-binding domain, U50 and U46 have changes at the 21st (Ile to Ser) and 62nd (Ala to Thr) positions in the α-helices 1 and 4, respectively; U9 has replaced Gly by Ser at the 53rd position just outside the helix 3 on the C-terminal side, while *ts*1 has a change at the 73rd position (Val to Ala) in between the helices 4 and 5. Of the three *ts* mutations in the oligomerizing domain, *c*I*ts*U51 carries two changes, one at the 141st (Phe to Ile) and the other at the 153rd (Pro to Leu) position. The *c*I*ts*I-22 mutation has replaced Asn by Thr at position 207. In *ts*2 repressor, a charge reversal has occurred at position 224 (Lys to Glu), which is located very close to the C terminus.

Purification of CIts2 repressor

The *ts*2 mutant repressor is very heat labile and highly sensitive to UV induction in a lysogen (Lieb, 1966). Its DNA-binding activity in cell-free extract prepared from a λ*c*I*ts*2 lysogen could not be demonstrated even at 20–30°C (Mandal and Lieb, 1976). Association of all these interesting properties with the CI*ts*2 repressor led us to concentrate further on the structure and function studies of this mutant gene-regulatory protein.

The CI*ts*2 repressor was purified by the procedure of Saha *et al*. (1992) with a slight modification as described in Materials and methods. It was observed that this mutant protein did not bind to CM-Sephadex to which the wild-type repressor could bind. On the other hand, this mutant repressor bound very tightly to QAE-Sephadex, to which the wild-type repressor bound very weakly. The purified *ts*2 repressor obtained from the hydroxyapatite column gave a single band corresponding to a monomeric molecular weight of 26 kDa on SDS–PAGE gel (data not shown).

Operator-binding activity of ts2 repressor at permissive and non-permissive temperatures

Figure 2 shows the binding of *ts*2 repressor to whole λ DNA containing wild-type O_L (O_{L1}, O_{L2} and O_{L3}) and O_R (O_{R1}, O_{R2}

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

Table I. Nucleotide changes due to different *c*I*ts* mutations and the resulting amino acid changes in the repressor of λ

Mutations	Mapping domain	Codon position	Codon sequence		Amino acid changes
			Wild	Mutant	
*c*I*ts*U50	N-terminal	21	ATT	AGT	Ile → Ser (I → S)
*c*I*ts*U9	N-terminal	53	GGC	AGC	Gly → Ser (G → S)
*c*I*ts*U46	N-terminal	62	GCC	ACC	Ala → Thr (A → T)
*c*I*ts*1	N-terminal	73	GTT	GCT	Val → Ala (V → A)
*c*I*ts*U51	C-terminal	141	TTC	ATC	Phe → Ile (F → I)
	C-terminal	153	CCA	CTA	Pro → Leu (P → L)
*c*I*ts*I-22	C-terminal	207	AAC	ACC	Asn → Thr (N → T)
*c*I*ts*2	C-terminal	224	AAA	GAA	Lys → Glu (K → E)

The mutant *c*I*ts* genes from λ*c*I*ts* phages were cloned in pBR322 and the nucleotide sequences of the *ts*-mutant codons were determined by DNA sequencing as described in Materials and methods. Changes of the amino acids by the *ts* mutations were translated from the mutant codon sequences.

and O_{R3}) at 20°C. As only the dimer form of λ repressor binds to operator DNA, the nature of the sigmoidicity in the curve showing the binding of repressor to the operator DNA as a function of the protein concentrations may be taken as a qualitative measure of monomer–monomer association of the protein to form dimers and their subsequent cooperative interaction. It is clear from the data in Figure 2A that the wild-type repressor shows sigmoidicity in the 0–2 nM range of protein concentrations, while the *ts*2-mutant repressor shows much less prominent sigmoidicity in the 2–6 nM concentration range. This possibly suggests a much weaker association of the *ts*2 monomer to form dimer, especially at low concentrations of the protein, and/or a defect in the cooperative interaction of the dimers. Even at higher concentrations of protein (16–20 nM) where the plateau in binding reached, the mutant protein showed about 57% binding compared with the wild-type repressor under identical conditions. This indicates that even at higher concentrations of the protein, either the mutant dimer formation is not as efficient as that of the wild-type repressor and/or the binding of mutant repressor may not be stabilized well owing to weaker cooperative (tetrameric) interaction of the adjacently bound repressor dimers. The fact that a λ*c*I*ts*2 lysogen is relatively more susceptible to UV induction than a wild-type λ lysogen supports the above views.

Figure 2B shows the DNA-binding ability of the mutant and wild-type repressors as a function of temperature. Maximum binding of the *ts*2 repressor occurred at ~20°C and then fell off gradually with further increase in temperature such that at 42°C only 8% of the maximum binding activity was retained. On the other hand, the wild-type repressor showed its maximum binding at ~30°C, and nearly 50% of this maximum binding was retained at 42°C. Hence maximum binding of *ts*2 repressor occurred ~10°C lower than that of the wild-type protein, but beyond the respective temperatures of their maximum binding both the repressors showed a gradual loss of binding with increase in temperature. The wild-type λ lysogen is stable at 42°C, whereas the λ*c*I*ts*2 lysogen is stable at 30°C but is induced at 35°C (Lieb, 1966). This suggests that the wild-type and *ts*2 repressors could still remain bound to the operators at 42 and 30°C, respectively, in their lysogens. Under *in vitro* conditions, the temperatures of maximum binding are lowered for both the repressors by ~10°C compared with the respective maximum temperatures of the stability of their lysogens. This may be explained as follows: in lysogens also the monomer–monomer association may be weakened with increase in temperature, as seen in *in vitro* interaction of the purified repressor to operators (Figure 2A), but in the former condition, the effective concentration of dimer may not change significantly owing to more synthesis of repressor monomers from P_{RM} promoter unless at certain higher temperatures the monomeric protein undergoes denaturation, thereby preventing dimer formation. *In vivo*, there may be another factor such as chaperon-assisted maintenance of native conformation (Bukau and Horwich, 1998) of the repressor proteins to a certain extent at higher temperatures beyond the temperatures of their *in vitro* maximum binding.

Structure of the C-terminal domain of ts2 repressor is different from that of the wild-type repressor

Clearly, the *ts*2-mutant repressor has a weak DNA-binding activity compared with the wild-type, even at permissive temperatures. Thus, a study of the structure of this mutant protein may shed much light on the state of the C-terminal domain. Figure 3 shows the CD spectra of the wild-type and *ts*2 repressors at 25°C. The spectrum of the mutant repressor is clearly different from that of the wild-type, with a significant loss of intensity at ~220 nm and an enhancement at ~206–208 nm. If we assume that the secondary structure of the N-terminal domain is unchanged (the *ts*2 mutation is located at the C-terminal end), it is likely that the secondary structure of the C-terminal domain is significantly changed since the CD spectrum of the whole repressor is dominated by the spectrum of the helix-dominated N-terminal domain. This suggests that even at permissive temperatures, the mutant protein may be partially denatured.

The λ repressor has three SH groups, all of which are situated in the C-terminal domain at 180, 215 and 219 codon positions (Sauer and Anderegg, 1978), and the reactivities of these SH groups provide another measure of the conformational state of the C-terminal domain of this protein. In the wild-type protein, these SH groups are totally unreactive, even at 6 M urea (Whipple *et al.*, 1994). This suggests a very stable core of the C-terminal domain in the wild-type protein. We measured the sulfhydryl reactivities of the *ts*2 protein under a variety of conditions, and the results are shown in Table II. The wild-type protein showed negligible reactivity at 25 and 42°C in both the presence and absence of 6 M urea, while the *ts*2 repressor showed a significant reactivity at 25°C even in the absence of urea, which was further enhanced at 42°C and in 6 M urea. The data can be interpreted as partial exposure of one sulfhydryl residue at permissive temperatures and its much enhanced exposure at non-permissive temperatures. It is

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

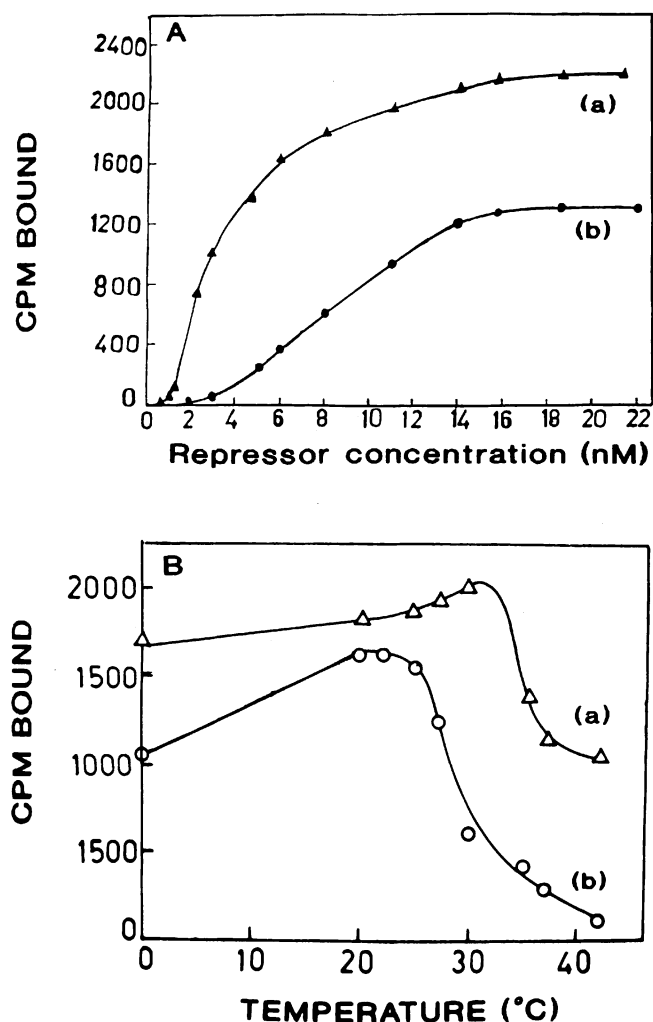

Fig. 2. Binding of mutant *ts*2 and wild-type repressors to λ DNA. (**A**) Operator binding of λ repressor as a function of protein concentration. Increasing amounts of repressors were used in a total volume of 0.25 ml of binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2 mM $CaCl_2$, 50 mM KCl, 5% DMSO, 100 µg/ml BSA, 100 µg/ml calf thymus DNA) containing 0.8 µg of [^{3}H]λ DNA. The mixtures were incubated at 20°C for 10 min to reach equilibrium. Then 0.1 ml aliquots were filtered through Millipore filters (HAWP, HA-0.45 µm) and washed with 0.5 ml of wash buffer (binding buffer minus BSA and calf thymus DNA) at 20°C. CPM bound represents the radioactivity (corrected for blank) retained on filter from a 0.1 ml aliquot of reaction mixture. Each aliquot filtered contained a total input of 3030 c.p.m. of [^{3}H]λ DNA. (**B**) Effect of temperature on the binding of repressors to double-operator (containing both O_L and O_R) λ DNA. The experiment was carried out exactly as described in (A) except that each repressor was used at 11.5×10^{-9} M (fixed) in the presence of 1 µg of [^{3}H]λ DNA in a total volume of 0.25 ml at different temperatures and washing was done with the wash buffer at the respective temperatures used for binding reaction. Input counts on the filter were 5200 c.p.m., of which the c.p.m. bound are shown. Curves (for both A and B): (a) wild-type repressor and (b) *ts*2 repressor.

not known which of the three C residues in the C-terminal domain are partially exposed. As C219 and C215 are very close to K224E mutation, it may be that one of these two or both of them are partially exposed. Whichever may be exposed, the above sulfhydryl reactivity data support the notion that the overall structure of the C-terminal domain of *ts*2 repressor is perturbed at permissive temperatures and becomes more so at non-permissive temperatures.

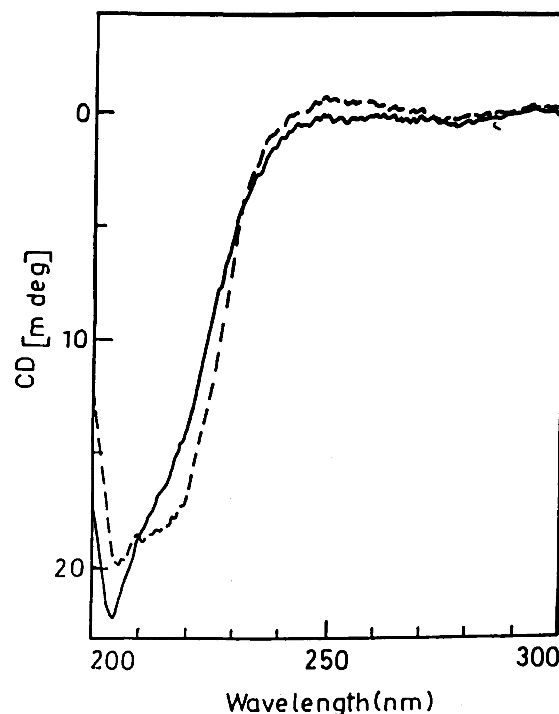

Fig. 3. CD spectra of *ts*2 mutant and wild-type repressors. The spectra were measured in 0.1 M potassium phosphate buffer, pH 8.0, at 25°C. A protein concentration of 1 µM was used for both the repressors. Curves: solid line, *ts*2 repressor ; broken line, wild-type repressor. For further details, see Materials and methods.

Table II. Reaction of sulfhydryl groups of wild-type and *ts*2-mutant repressors of λ with DTNB under different conditions

	Repressor type	Conditions	Equivalence of SH group reacted
(A)	Wild-type	25°C	0.004
	*ts*2 mutant	25°C	0.247
(B)	Wild-type	42°C	0.050
	*ts*2 mutant	42°C	0.973
(C)	Wild-type	6 M urea treated at 25°C	0.070
	*ts*2 mutant	6 M urea treated at 25°C	0.934

Repressor at 10 µM concentration in 0.1 M potassium phosphate buffer, pH 8.0, was incubated for 1 h with 100 µM DTNB at 25°C in (A) and 42°C in (B). The repressor samples in (C) were incubated for 18 h with 6 M urea containing 1 mM EDTA at 25°C (C) and then dialyzed against 6 M urea for 18 h at 25°C followed by DTNB (110 µM) reaction at 25°C as above. For further details, see Materials and methods.

Tryptophan residues which are buried in the wild-type protein are completely exposed in the ts2-mutant protein even at permissive temperatures

Tryptophan fluorescence of a protein is often used as a sensitive probe for studying its structure. The λ repressor has three Trp residues, two of which are situated within the C-terminal domain and the third in the hinge region bordering the C-terminal domain. Acrylamide quenching of these Trp residues has been used to resolve conformational and structural aspects of the C-terminal domain of λ repressor (Bandyopadhyay *et al*., 1995). It has been shown that at 25°C in the presence of low concentrations of acrylamide, the fluorescence of wild-type repressor at 0.5 µM concentrations is quenchable to the

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

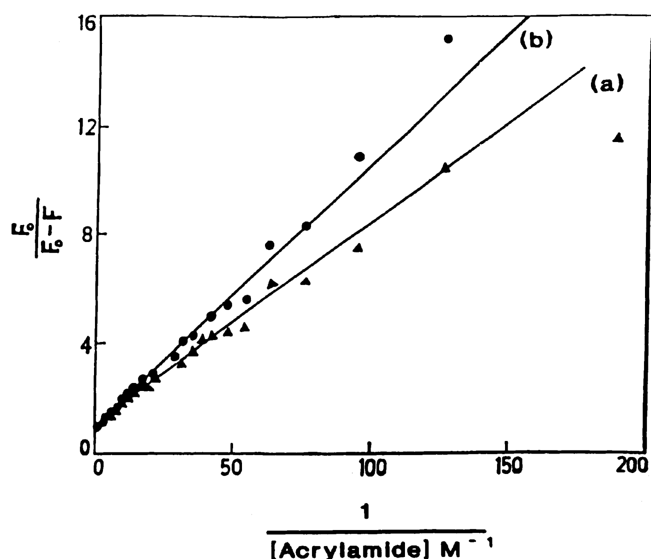

Fig. 4. Lehrer plot of acrylamide quenching of tryptophan fluorescence of *ts*2-mutant repressor at 25°C. The spectra were measured in 0.1 M potassium phosphate buffer, pH 8.0, in the presence of various concentrations of acrylamide. The excitation and emission wavelengths were 295 and 345 nm, respectively. The data corresponding to each point are an average of four independent determinations. The repressor concentrations used were (a) 0.5 and (b) 10 µM.

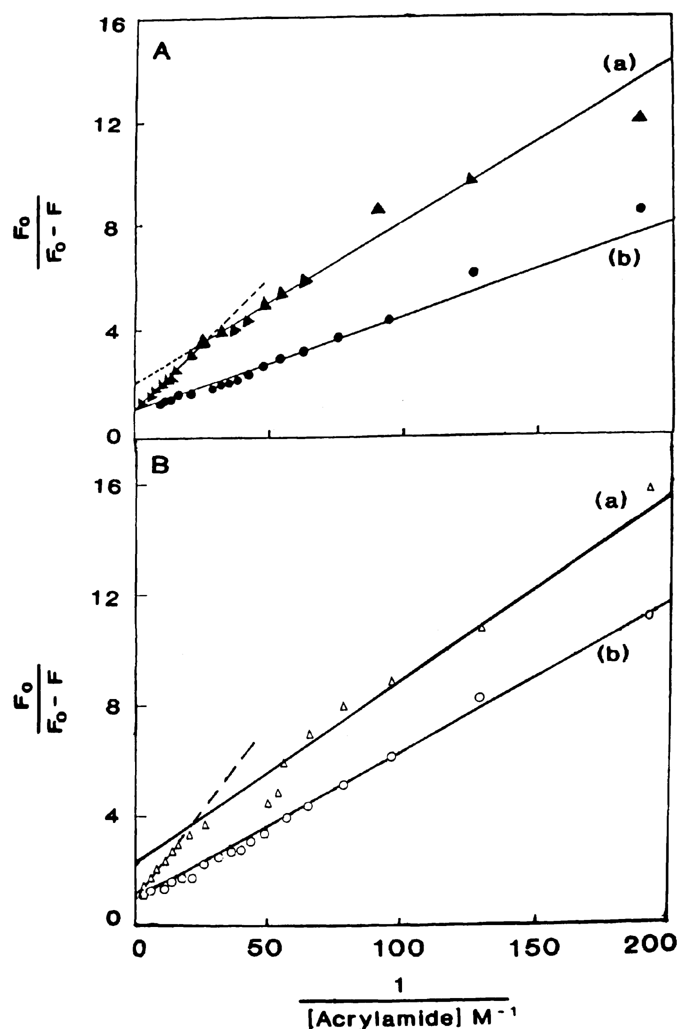

Fig. 5. Lehrer plot of acrylamide quenching of tryptophan fluorescence of *ts*2 and wild-type repressors at 42°C. The experiment was carried out exactly as described in Fig. 4 except that the temperature was 42°C instead of 25°C. Protein concentrations were (**A**) 0.5 µM and (**B**) 10 µM. Curves: (a) wild-type repressor and (b) *ts*2 repressor.

extent of 40% (K_{sv} = 45 M^{-1}) and that at 10 µM to only about 25% (K_{sv} = 38 M^{-1}) (Bandyopadhyay *et al.*, 1995). The Lehrer plot of acrylamide quenching of the *ts*2-mutant protein at 25°C using 0.5 and 10 µM concentrations is shown in Figure 4. The plots are similar at these two concentrations of the protein with only a modest difference in the initial part of the quenching. At 10 µM concentration of *ts*2 repressor, the plot is approximately linear and cuts the *y*-axis at ~1 with a K_{sv} of 10 M^{-1} (Figure 4). This suggest a much enhanced exposure of the two buried Trp residues at 25°C [K_{sv} of the buried tryptophans in the wild-type protein is ~3 M^{-1} or lower (Bandyopadhyay *et al.*, 1995)]. At 42°C, the Lehrer plot for *ts*2 repressor at 0.5 µM cuts the *y*-axis at ~1 with a K_{sv} of 29 M^{-1} (Figure 5A and B). This is in contrast to that of the wild-type protein, which shows a biphasic behavior and cuts the *y*-axis at ~2. The two phases have K_{sv} of 29 and 9 M^{-1}, respectively (summarized in Table III). This again suggests that the Trp residues which are buried in the wild-type protein are completely exposed in the *ts*2-mutant protein at non-permissive temperatures.

Tryptophan environments in ts2 repressor do not change with increasing concentration of the protein

Lambda repressor is known to tetramerize in solution (Brack and Pirrotta, 1975; Banik *et al.*, 1993). In a previous study, it has been shown that the tetramerization of repressor is accompanied by a dramatic effect on the Trp230 residue resulting in an overall shift of emission maxima of 2 nm in buffers containing 0.5 M acrylamide (Bandyopadhyay *et al.*, 1995). At comparable protein concentrations at 25°C, the tryptophan fluorescence of *ts*2 repressor was found to be red shifted (data not shown). Figure 6 shows the emission maximum of tryptophan fluorescence of *ts*2 and wild-type repressors in 0.5 M acrylamide as a function of protein concentration at 25°C. As noted previously, the wild-type repressor showed a blue shift of ~2 nm when the protein concentration was changed from 0.2 to 10 µM. The *ts*2 repressor, however, showed a blue shift of only a few tenths of a nanometer in the same protein concentration range. Clearly, the change of W230 environment, which is characteristic of the wild-type protein association, is absent in the *ts*2 repressor. In order to determine whether this lack of environment change in the latter repressor was due to the lack of tetramer formation, we performed size-exclusion HPLC at 25°C. The *ts*2 protein eluted as a dimer at all loading concentrations in the range 1–50 µM, whereas under identical conditions, the wild-type repressor showed a significant shift towards higher molecular weight species (data not shown). This suggests that the *ts*2 protein is defective in tetramer formation. Hence it appears that the lack of emission maxima shift in the mutant protein is probably due to the lack of tetramer formation in this concentration range. Taken together with the operator-binding data in Figure 2A, this indicates that at permissive temperatures, the cooperativity of *ts*2 repressor may be weaker than that of the wild-type.

Discussion

The study of mutant proteins impaired in one or more functions has always been a powerful approach to studying their struc-

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

Table III. Summarized data of tryptophan fluorescence quenching of wild-type and *ts*2-mutant repressors by Lehrer plot

Temperature (°C)	Protein concentration (μM)	K_{sv1}		K_{sv2}	
		Wild-type	*ts*2	Wild-type	*ts*2
25	0.5	45.00	13.33	<6.00	–
	10.0	38.00	10.44	<6.00	–
42	0.5	28.62	29.10	9.30	–
	10.0	35.20	22.20	7.70	–

These data were taken from Figures 4 and 5. All Stern–Volmer constants are in M^{-1}.

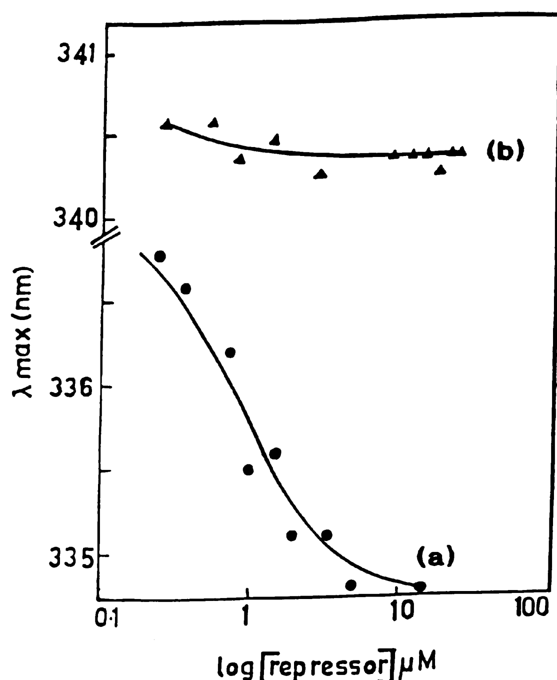

Fig. 6. Emission maxima of *ts*2 and wild-type repressors as a function of protein concentration at 25°C. The experiment was carried out exactly as described in Fig. 4 except that the repressors were used at different concentrations in the presence of 0.5 M (fixed) acrylamide. The excitation and emission bandpasses were 5 and 1.5 nm, respectively. Curves: (a) wild-type repressor and (b) *ts*2 repressor.

ture–function relationships. For this purpose, the most powerful approach is to combine the functional studies of mutant proteins with studies of their structure. With the development of expression systems capable of producing large quantities of proteins and also advances in the techniques of structural biology, it has now become possible to explore systematically the role of each individual amino acid in a particular function. In the λ repressor system, this approach was initiated by Burz and co-workers (Burz and Ackers, 1994; Burz *et al*., 1994) with respect to cooperative interaction of the C-terminal domain.

Several *ts* mutations in the *c*I gene of λ originally isolated in phage show a nearly even distribution in the N- and C-terminal domains of the repressor (Lieb, 1976). In this work, the exact positions and the amino acid changes due to seven such *c*I*ts* mutations were determined by DNA sequencing (Table I). It appears that all seven *c*I*ts* mutations show base changes at either the first (5′) or second (middle) position but not at the third (3′) position of the codon; this is possibly due to the third base flexibility in the codon dictionary. In this group of *c*I*ts* mutants, no mutation was found in the hinge region between residues 92 and 132. The amino acid changes caused by these mutations appear to be random with respect to their different properties such as the polarity, charge and size of the side-chain. The mutations *ts*U50, *ts*U46, *ts*U9 and *ts*1 in the DNA-binding domain are possibly not in the residues that are involved directly in DNA binding but have a profound effect on the conformation of the DNA-binding domain (Lim and Sauer, 1991) [(the *c*I857*ts* mutation is known to replace Ala by Thr at 66th position in helix 4 of the DNA-binding domain (Sanger *et al*., 1982)]. It is likely that the positions at which such changes of amino acids have occurred are very important for the maintenance of the functional conformation of the protein. It is interesting to know that the *ts*U51 mutation in the C-terminal domain has two changes, F141I and P153L (Table I). It may be noted that λ*c*I*ts*U51 also shows the *ind*⁻ phenotype (Lieb, 1966), but which of the above two mutations confers the *ind*⁻ property is not known. Beckett *et al*. (1993) isolated a non-cooperative mutation at position 147 (G147D) which is located in between the two U51 mutations. Although the crystal structure of the C-terminal domain of λ repressor is not known, the structural importance of the residues may be guessed from the crystal structure of the homologous protein UmuD′. The residue F141 is semi-conserved (F or Y) among the four homologous proteins, UmuD′, MucA, LexA and CI (Peat *et al*., 1996). If the structure of UmuD′ is used as a rough guide of CI C-terminal domain structure, then F141 appears to be removed from the clusters of non-cooperative mutant residues in CI. P153L, on the other hand, falls in a five amino acid insert that is unique to CI of λ among the above four homologous proteins. This insert is adjacent to the residues the equivalent of which in UmuD′ forms the only 3_{10} helix in the structure. The short 3_{10} helix in the UmuD′ structure interacts with the region containing NASY reverse turn. The equivalent residues of non-cooperative mutations, Y210H, G147D and M212R, are located close to these structural elements. It may be that the above two U51 mutations destabilize this important region in a temperature-dependent manner and the conformation of the hinge region of this mutant protein is altered in such a way that the protein is not accessible to RecA, thereby imposing the *ind*⁻ property.

The N207T change by *ts*I-22 mutation appears to be very close to a predicted reverse turn around which some non-cooperative mutations have been isolated (Whipple *et al*., 1994). It has been argued that this region is involved in direct protein–protein cooperative contact. Hence, the *ts*I-22 mutation may destabilize the region around position 207 at non-permissive temperatures. Recently, it has been shown that the non-cooperative mutant Y210C may exert its effect through indirect means (Deb *et al*., 1998). This also suggests that N207 may not be directly involved in protein–protein interaction, and its role may be in the formation of the putative reverse turn.

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

The importance of protein–protein contact in negative regulation in prokaryote is now well appreciated. The exact role of such contact is, however, not clear. It certainly augments the strength of the protein–DNA interaction. However, a different role such as the transmission of the effect of conformational change may also be involved. Structural and functional studies of non-cooperative mutants may shed light on these aspects. The C-terminal domain of the *ts*2 repressor was found to have significant perturbation in the structure and possibly a weaker cooperativity even at permissive temperatures. This weakened cooperativity, however, is still sufficient to allow the establishment and maintenance of lysogeny to take place at permissive conditions. At higher temperatures, the structure appears to be severely disrupted, leading, perhaps, to a complete loss of cooperativity and a loss of the ability to lysogenize as well as to maintain lysogeny (Lieb, 1966). Even with the availability of the crystal structure, the nature of the monomer–monomer interface of UmuD′ remains controversial. The conclusions drawn about the nature of the monomer–monomer interface from the NMR study of Ferentz *et al*. (1997) are not compatible with the interface seen in the crystal structure. The charge reversal by *ts*2 (K224E) mutation is dramatic. The residue K224 is located near the residue V222. Equivalent residues in UmuD′ are part of a β sheet and the side chains of these residues would be immediately adjacent. In the crystal structure of UmuD′, F128, which is equivalent to V222 in λ CI, is part of the monomer–monomer interface. In the proposed NMR structure, however, the C-terminal tail region forms part of the monomer–monomer interface and the residue corresponding to K224 is adjacent to this important interaction point. Therefore, it is possible that in either case K224 is close to the monomer–monomer interface. Hence a charge reversal at this point exerts a strong destabilizing effect on protein–protein interactions, causing impaired function particularly at higher temperatures. The observed effect of this mutation on the accessibility of several Trp and sulfhydryl residues clearly suggests that the destabilization is not confined to local dimensions.

Study of several non-cooperative mutant repressors (Burz and Ackers, 1994; Burz *et al*., 1994) suggests that many of them have significantly enlarged hydrated volumes, indicating disruption of their native structure. Unfortunately, not many of them have been studied from a structural point of view. Study of the *ts*2 repressor clearly underlines the possibility that many of the mutants may have indirect functional effects and, hence, cannot be used to identify the regions of protein–protein contact. Such *ts* mutants in many cases, however, clearly provide information about structural connectivities to important functional regions. Many non-cooperative and *ts* mutants are now known in the C-terminal domain of λ repressor. Lack of a crystal or NMR structure makes it difficult to relate it to specific structural roles. The recent availability of a homologous UmuD′ structure (Peat *et al*., 1996), however, may allow one to speculate about the structural regions involved in the dimer–dimer interaction. Equivalent residues of one group of non-cooperative mutation fall within a general area of the UmuD′ structure. These mutations are G147D, E188K, K192N and M212R. The Cα atom of the equivalent residue of G147 in UmuD′ is within 6 Å of NH of the equivalent residue of N192 in UmuD′. Similarly, the equivalent residue of M212 of λ CI, an isoleucine in UmuD′, is within 8 Å of the equivalent residue of K192 in UmuD′. For another group of mutations in CI of λ, the equivalent residues in UmuD′ form the 3_{10} helix and NASY reverse turn, which are close together in space. These are P153L (*ts*51), N207T (*ts*I-22) and Y210C or Y210H. The existence of *ts* mutants in this region strongly suggests that these mutations exert their influence through structural alteration at non-permissive temperatures and are not the interaction points as such. Our recent studies with Y210C also suggest that it is an indirect mutant. Equivalent residues of a third group of mutations, R196G, D197G, D197A, S198N, S198R, G199D, G199V and F202S, fall on an exposed loop in the UmuD′ structure. Equivalent residues of a fourth group of mutations falls in the C-terminal tail side, which include S228N, T234K and, possibly, K224E (*ts*2). Both S228N and T234K have been shown to be defective in dimer–monomer transition and are not likely to exert their effect on cooperativity, if any, by direct contact. K224E is unlikely to be a direct-contact mutation and probably exerts its effect through destabilization of the protein structure as revealed by spectroscopic studies (Figures 2–6). The general destabilization seen in the *ts*2-repressor structure strongly argues in favor of this. Hence it appears likely that the regions around G147 and the 196–199 loop may be the primary contact points for dimer–dimer interactions.

Acknowledgements

We thank Drs M.Ptashne, M.Lieb and S.Adhya for microbial strains and Dr P.Chakrabarti for critically reading the manuscript. This work was supported by an Ad Hoc Research Fellowship to N.K.Jana and Research Grant No. 37/849/94-ERM-II from the Council of Scientific and Industrial Research, Government of India, New Delhi.

References

Amann,E., Brosius,J. and Ptashne,M. (1983) *Gene*, **25**, 167–178.

Bandyopadhyay,S., Banik,U., Mandal,N.C., Bhattacharyya,B. and Roy,S. (1995) *Biochemistry*, **34**, 5090–5097.

Banik,U., Saha,R., Mandal,N.C., Bhattacharyya,B. and Roy,S. (1992) *Eur. J. Biochem*., **206**, 15–21.

Banik,U., Saha,R., Mandal,N.C., Bhattacharyya,B. and Roy,S. (1993) *J. Biol. Chem*., **268**, 3938–3943.

Beckett,D., Burz,D.S., Ackers,G.K. and Sauer,R.T. (1993) *Biochemistry*, **32**, 9073–9079.

Brack,C. and Pirrotta,V. (1975) *J. Mol. Biol*., **96**, 139–152.

Bukau,B. and Horwich,A.L. (1998) *Cell*, **92**, 351–366.

Burz,D.S. and Ackers,G. (1994) *Biochemistry*, **33**, 3406–3416.

Burz,D.S., Beckett,D., Benson,N. and Ackers,G. (1994) *Biochemistry*, **33**, 8399–8405.

Das,T. and Mandal,N.C. (1986) *Mol. Gen. Genet*., **204**, 540–542.

Deb,S., Bandyopadhyay,S. and Roy,S. (1998) *Protein Engng*, **11**, 481–487.

Ellman,G.L. (1959) *Arch. Biochem. Biophys*., **82**, 70–77.

Ferentz,A.E., Opperman,T., Walker,G.C. and Wagner,G. (1997) *Nature Struct. Biol*., **4**, 979–983.

Jordan,S.R. and Pabo,C.O. (1988) *Science*, **242**, 893–899.

Kaiser,A.D. (1957) *Virology*, **3**, 42–61.

Lieb,M. (1966) *J. Mol. Biol*., **16**, 149–163.

Lieb,M. (1976) *Mol. Gen. Genet*., **146**, 291–298.

Lim,W.A. and Sauer,R.T. (1991) *J. Mol. Biol*., **219**, 359–376.

Mandal,N.C. and Lieb,M. (1976) *Mol. Gen. Genet*., **146**, 299–302.

Mandal,N.C., Crochet,M. and Lieb,M. (1974) *Virology*, **57**, 85–92.

Nag,D.K., Chattopadhyay,D.J. and Mandal,N.C. (1982) *Virology*, **118**, 448–450.

Nag,D.K., Chattopadhyay,D.J. and Mandal,N.C. (1984) *Mol. Gen. Genet*., **194**, 373–376.

Nelson,H.C.M. and Sauer,R.T. (1985) *Cell*, **42**, 549–558.

Pabo,C.O. and Lewis,M. (1982) *Nature*, **298**, 443–447.

Peat,T.S., Frank,E.G., McDonald,J.P., Levine,A.S., Woodgate,R. and Hendrockson,W.A. (1996) *Nature*, **230**, 727–730.

Ptashne,M. (1992) *A Genetic Switch: Gene Control and Phage Lambda*. Cell Press, Boston.

Ptashne,M., Jeffrey,A., Johnson,A.D., Maurer,R., Meyer,B.J., Pablo,C.O., Roberts,T.M. and Sauer,R.T. (1980) *Cell*, **19**, 1–19.

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010

Riggs,A.D., Bourgeois,S., Newby,R.F. and Cohn,M. (1968) *J. Mol. Biol*., **34**, 365–368.
Saha,R., Banik,U., Bandyopadhyay,S., Mandal,N.C., Bhattacharyya,B. and Roy,S. (1992) *J. Biol. Chem*., **267**, 5862–5867.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Sanger,F., Coulson,A.R., Hong,G.F., Hill,D.F. and Peterson,G.B. (1982) *J. Mol. Biol*., **162**, 729–773.
Sauer,R.T. and Anderegg,R. (1978) *Biochemistry*, **17**, 1092–1100.
Sussman,R. and Jacob,F. (1962) *C. R. Acad. Sci*., **254**, 1517–1519.
Whipple,F.W., Kuldell,N.H., Cheatham,L.A. and Hochschild,A. (1994) *Genes Dev*., **8**, 1212–1223.

Received June 20, 1998; revised November 10, 1998; accepted December 7, 1998

Downloaded from peds.oxfordjournals.org by guest on December 3, 2010