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Spectroscopic study of Y210C λ-repressor: implications for cooperative interaction

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A non-cooperative mutant of λ-repressor, Y210C, has been purified and characterized. The mutant protein does not show any evidence of cooperative interaction as judged by difference near-UV circular dichroism spectra of DNA. The mutant protein also shows much weaker self-assembly as revealed by fluorescence anisotropy measurement. The far-UV circular dichroism spectrum of the protein shows a modest but significant reduction in the 220 nm range, suggesting a structural change. The Lehrer plot of acrylamide quenching of Y210C repressor at a predominantly dimeric concentration (0.5 μM) is almost identical with that of the wild-type protein at the same concentration. Transmission of operator-induced conformational change is also preserved in the mutant protein. Like that of the wild-type protein, cysteines of the mutant protein are unreactive to sulfhydryl reagents under native conditions. Most importantly, C210 is unreactive to sulfhydryl reagents under native conditions. This fact, coupled with the structural change observed in the far-UV CD spectra, suggests that C210 is located at the interior of the protein and exerts its effect indirectly on cooperative contact probably through destabilization of a reverse turn, of which it is an important part.

Keywords: circular dichroism spectroscopy/cooperative interaction/Y210C λ-repressor

Introduction

The isolation and characterization of mutant proteins have played a very significant role in understanding structure–function relationships of many proteins (Ruppel and Spudich, 1995; Dean *et al.*, 1996; Pressley, 1996). These successes have led to the development of site-directed and scanning mutagenesis procedures, which are now capable of generating mutants in every amino acid residue of a protein within a relatively short period of time (Trower, 1996). Such massive screening has given rise to spectacular successes in mapping functional regions and pathways of information transfer of complex proteins (Niu *et al.*, 1994; Tang *et al.*, 1996). Random and site-directed mutagenesis have also been used to delineate structure–function relationships of proteins involved in regulation of transcription. However, there is an inherent assumption in all such mutant studies that the functional properties of the mutant are directly attributable to the mutated residue. Complete biophysical and biochemical characterization of a mutant protein is, however, necessary to elucidate the residue–function relationship (Burz and Ackers, 1994, 1996; Burz *et al.*, 1994; Chang *et al.*, 1994; Chen *et al.*, 1994; Chang and Matthews, 1995).

The operator–repressor system of bacteriophage λ has been developed as a model system for regulation and switching of gene expression (Ptashne, 1992). The right operator of bacteriophage λ consists of three contiguous operator sites, O_R1, O_R2 and O_R3. Two proteins, λ-repressor and cro, control the activities of two critical promoters, P_R and P_{RM}, by binding to these operator sites (Ptashne, 1992). The λ repressor binds to these operator sites, with alternate pairwise cooperativity (Johnson *et al.*, 1979), and such cooperativity is essential for functioning of the switch. Cooperative binding occurs when λ repressor binds to the adjacent operator sites and establishes a protein–protein contact (Hochschild and Ptashne, 1986). This protein–protein contact occurs through the C-terminal domain of the lambda repressor, whereas the DNA binding occurs through the N-terminal domain.

Cooperative binding of proteins to multi-partite operators has now been described in many gene regulatory systems and is an essential component of them (Adhya, 1989). It is generally believed that such cooperative binding takes place through protein–protein contact of two DNA-bound protein molecules, as has been described for the λ repressor–operator system. Recent work indicates that the system is probably fairly complex, involving DNA-induced allosteric changes that determine the nature of the protein–protein contact (Bandyopadhyay *et al.*, 1996). In addition, adaptation of the λ-repressor to maintain cooperative binding to various pairs of natural operator sites separated by variable numbers of base pairs suggests the existence of a complex molecular machinery.

One of the most important motivations for studying non-cooperative mutants is to identify protein–protein interfaces involved in cooperative interaction. The large size of the protein–DNA complexes and the difficulty in crystallizing them have made it difficult to study the structure of this type of complex by NMR and X-ray crystallography. Hence the study of non-cooperative mutants remains the only viable option for the elucidation of the nature of protein–protein interfaces in this type of complex. Many such non-cooperative mutants of λ-repressor have now been isolated (Beckett *et al.*, 1993; Benson *et al.*, 1994; Whipple *et al.*, 1994), but only a few have been biochemically and biophysically characterized (Burz and Ackers, 1994, 1996; Burz *et al.*, 1994). These mutants form a fairly extensive set and are distributed over at least three regions of the protein structure (when judged from the homologous UmD′ structure), but further structural characterization is needed before one can identify the residues that are the direct participants in the cooperative dimer–dimer interaction. We have undertaken the biophysical and biochemical characterization of these mutant proteins in order to identify residues that are directly involved in cooperative interaction and shed light on the structure of the cooperatively bound λ-repressor–operator complex. In this paper we show that the mutant Y210C affects cooperative binding indirectly

and the mechanism of such interference may be through destabilization of a reverse turn.

Materials and methods

Materials

Plasmid pLRB152 containing Y210C mutant λ-repressor was a gift from Drs S.Adhya and P.Youderian. Bis-ANS was purchased from Molecular Probes (Eugene, OR). Crude O_R1–O_R2 oligonucleotide (5′ CCTATCACCGCCAGAG GTAAAA-TAGTCAAC ACGCACGGTGTTACCA 3′ and its complementary sequence) was purchased from IMMCO Genetics (New Delhi, India). QAE-Sephadex A-50, all restriction enzymes and T4 DNA ligase were supplied by Pharmacia (Uppsala, Sweden). Ampicilin, DTNB, IPTG and PMSF were obtained from Sigma Chemical (St Louis, MO). Three-times recrystallized acrylamide used for quenching studies was purchased from Spectrochem (Bombay, India). Bacto-tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI). All other reagents were of analytical grade.

Purification of oligonucleotides

O_R1 oligonucleotide (5′ GTACCTCTGG CGGTGATAG 3′ and its complementary oligonucleotide) was synthesized on a ABI 381A DNA synthesizer using reagents purchased from Applied Biosystems. All the oligonucleotides were synthesized with 5′-terminal trityl on and purified by reversed-phase high-performance liquid chromatography (HPLC) (μBondapack C_{18} column). The mixture was annealed in 0.1 M potassium phosphate buffer, pH 8, by heating at 80°C and then cooling slowly to room temperature.

Cloning and transformation

Plasmid pLRB152 contains the Y210C under control of *lacUV5* promoter. To increase the expression further, we subcloned the Y210C under the *tac* promoter. This was performed by exchanging a *Hin*dIII fragment from pLRB152 containing the C-terminal part of the *cI* gene to the corresponding fragment of plasmid pEA305 which contains the wild-type *cI* gene under *tac* promoter (Amann *et al.*, 1983). The identity of the mutant was verified by DNA sequencing.

Isolation and purification of Y210C λ-repressor

The repressor was purified according to Saha *et al.* (1992) with modifications as noted below. The cell lysate supernatant was mixed with 50 ml of SB [10 mM Tris–HCl, pH 8, containing 1 mM calcium chloride, 0.1 mM EDTA, pH 8, 0.1 mM 2-mercaptoethanol, 5% (v/v) glycerol] containing 100 mM KCl and 50 ml of pre-swollen QAE-Sephadex A-50 in SB containing 100 mM KCl and the mixture was stirred for 1 h at 4°C. It was then poured into a column and allowed to settle. The column was washed with 50 ml of SB containing 100 mM KCl. The protein was then eluted with a linear gradient of 100 ml of SB containing 100 mM KCl and 100 ml of SB containing 600 mM KCl. Fractions of 5 ml were collected. Y210C was eluted in tubes 13–20.

A hydroxyapatite column was run as described previously (Saha *et al.*, 1992). The appropriate fractions were pooled and dialyzed overnight in SB containing 600 mM KCl, then 80% ammonium sulfate precipitation was carried out at 4°C. The precipitate was suspended in 1 ml of 0.1 M KP, pH 8, and dialyzed against the same buffer. In contrast to the native repressor, Y210C repressor dialyzed under these conditions and at very high protein concentrations partially precipitates without loss of any biological activity. The Y210C repressor, however, is soluble in 0.1 M potassium phosphate, pH 8.0, at lower concentrations, where all the experiments were conducted. The precipitate was dissolved in 0.1 M potassium phosphate, pH 8, containing 0.3 M KCl, 1 mM EDTA and 2-mercaptoethanol for storage. SDS–PAGE at this stage indicated >95% purity. The stored protein was dialyzed against 0.1 M potassium phosphate buffer, pH 8.0, at lower concentrations before use. The repressor concentration is always expressed in terms of monomer unless stated otherwise.

Circular dichroism

Circular dichroism (CD) spectra were measured in a JASCO J-600 spectropolarimeter using a 0.1 cm pathlength cuvette for far-UV measurements and 1.0 cm for near-UV measurements. The measurements in both cases were performed in 0.1 M potassium phosphate buffer, pH 8.0, at ambient temperature (25 ± 1°C). For far-UV measurements, the protein concentration was 1.2 μM. The mutant and the wild-type protein concentrations were first determined by measuring the absorbance at 280 nm and the concentrations were appropriately adjusted by diluting with buffer. The near-UV CD spectra were measured as described by Bandyopadhyay *et al.* (1996).

Fluorescence methods

All fluorescence spectra were measured with a Hitachi F 3010 spectrofluorimeter having a spectral addition and subtraction facility. The excitation and emission bandpasses were 5 nm, unless mentioned otherwise. Fluorescence quenching studies were carried out as described by Bandyopadhyay *et al.* (1995). Operator-induced conformational change was measured by titrating 1 μM Y210C repressor with a 19-mer O_R1 containing duplex oligonucleotide in 0.1 M phosphate buffer, pH 8.0. The excitation wavelength was 295 nm and the emission was measured at 340 nm. For titration of the bis-ANS–repressor complex, 1 μM Y210C λ-repressor was mixed with 0.5 μM bis-ANS in 0.1 M phosphate buffer, pH 8.0, and titrated with the 19-mer O_R1 containing duplex oligonucleotide. The excitation wavelength was 385 nm and the emission wavelength was 500 nm. Tetramer–dimer dissociation of Y210C λ-repressor was determined using dansyl chloride-labeled protein as described by Banik *et al.* (1993).

DTNB titration of cysteines

The repressor was dialyzed extensively in 0.1 M phosphate buffer, pH 8.0. The protein solution was placed in the sample cuvette and the dialyzate buffer was placed in the reference cuvette of a double-beam spectrophotometer. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) solution was added to each cuvette to a final concentration of 0.1 mM and the reaction was followed for 1 h. Similar protocols were observed for urea-denatured repressor, except that urea was added to a final concentration as desired. Protease cleavage was as described in Banik *et al.* (1992). Protein solution (9 μM) was incubated with subtilisin, trypsin, chymotrypsin and proteinase K at a final concentration of 1.3 μg/ml each. After 88 h, sulfhydryl titers were measured as described by Banik *et al.* (1992).

Results

The mutant studied here, Y210C, was obtained by random mutagenesis by Youderian and co-workers (Benson *et al.*, 1994) based on a screen that selects for mutants that are defective in cooperative binding without affecting single operator binding. To verify that the mutant protein does indeed

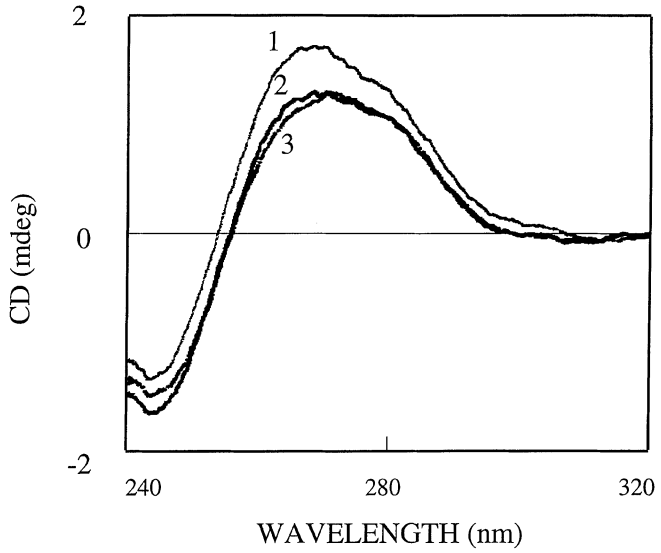

Fig. 1. Difference CD spectra of O_R1–O_R2 oligonucleotide in the free state and various protein complexes. (1) O_R1–O_R2 oligonucleotide complex with wild-type λ repressor minus the same concentration of λ-repressor. (2) O_R1–O_R2 oligonucleotide complex with Y210C λ-repressor minus the same concentration of Y210C λ-repressor. (3) O_R1–O_R2 oligonucleotide minus buffer. The spectra were taken at ambient temperature (25 ± 1°C). The solution conditions were 0.1 M phosphate buffer, pH 8.0. Ten spectra were signal averaged to improve the signal-to-noise ratio. The protein concentrations were 0.5 μM and the oligonucleotide concentrations were 0.125 μM.

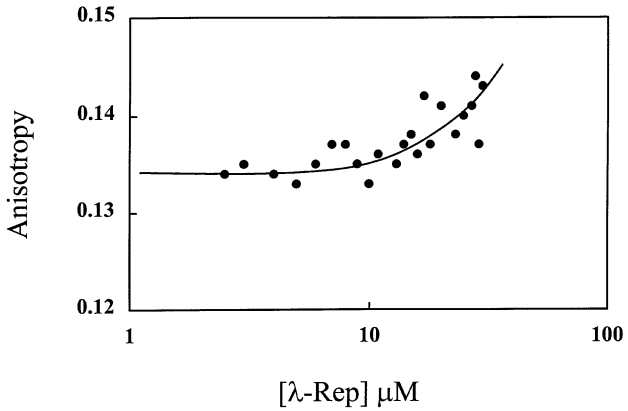

Fig. 2. Fluorescence anisotropy versus protein concentration for dansylated Y210C λ-repressor. The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0, at 25°C. The excitation wavelength was 340 nm and the emission wavelength was 520 nm. The bandpasses were 10 nm each. Four spectra were signal averaged to improve the signal-to-noise ratio.

lack cooperative binding, we studied the nature of the binding of pure Y210C mutant to an oligonucleotide encompassing two operators, O_R1 and O_R2, separated by same base pairs as in the wild-type sequence. In a previous paper we showed that the CD spectrum of the oligonucleotide changes significantly when the repressor binds cooperatively, indicating distortion of the DNA (Bandyopadhyay *et al.*, 1996). Such a spectral change, and hence DNA distortion, do not take place when the two operators are on the opposite face of the DNA helix. Hence this characteristic CD spectral change of the oligonucleotide may be taken as a signature of cooperative binding and we attempted to determine if the mutant protein induces a similar change in the CD spectrum of the O_R1–O_R2 oligonucleotide. Figure 1 shows the CD spectra of the O_R1–O_R2 oligonucleotide alone and in the presence of wild-type λ-repressor and Y210C λ-repressor. As was observed in a previous study, the wild-type λ-repressor binding causes the CD spectral intensity in the region of 260 nm to increase significantly. An equivalent amount of Y210C, however, was unable to elicit any change in the CD spectra. At these protein and oligonucleotide concentrations, it is expected that the repressor would be bound to the operators. Since no significant change in the CD spectrum is observed, it may be concluded that the two Y210C dimers bound to operator sites are not interacting as in a cooperative complex.

Another aspect of the cooperative dimer–dimer interaction is the oligomerization of λ-repressor in solution. Free λ-repressor undergoes a concentration-dependent oligomerization from dimer to tetramer to octamer and even higher order structures (Brack and Pirrotta, 1975; Banik *et al.*, 1993; Laue *et al.*, 1993; Senear *et al.*, 1993; Bandyopadhyay *et al.*, 1995). It was generally assumed that the dimer–dimer contacts involved in free repressor tetramerization and cooperative binding are the same. Recent studies, however, suggest that they are different in some respects (Burz *et al.*, 1994; Bandyopadhyay *et al.*, 1996; Burz and Ackers, 1996). At least one mutant, S228N, is characterized that is defective in tetramer formation but not in cooperativity. No mutant is known that is defective in cooperativity but forms tetramer and higher order oligomers. Since Y210C is defective in cooperativity, we tested whether Y210C can self-assemble to higher order structures. We used a fluorescence anisotropy assay as described by Banik *et al.* (1993), using dansyl chloride-labeled repressor. Figure 2 shows the plot of fluorescence anisotropy versus protein concentration of dansyl-labeled Y210C λ-repressor. The initial anisotropy values at low protein concentrations, i.e. ~1 μM, are very similar to those for the wild-type dimer, which is the predominant species at that concentration. This indicates that like the wild-type repressor, the Y210C mutant is dimeric at ~1 μM concentration. An increase in protein concentration, however, did not change the anisotropy significantly. This is in contrast to the wild-type repressor, which undergoes assembly to higher order structures in this concentration range (Banik *et al.*, 1993). This indicates that Y210C is defective in both cooperative binding and tetramer and higher order assembly. Only above 20 μM protein concentration is some increase in anisotropy seen, which suggests that there may be dimer–tetramer association at high protein concentrations. Thus Y210C mutation seems to affect that part of the interface which is common to both the self-assembly and the cooperative contact.

In previous studies, we have shown that upon binding of the operator to the N-terminal domain, a conformational change occurs in the C-terminal domain (Saha *et al.*, 1992). This conformational change affects the way in which the two λ-repressor molecules interact with one another. Hence this DNA-induced conformational change is a crucial element in the proper manifestation of binding cooperativity. Theoretically some non-cooperative mutant may be defective in transmission of such allosteric change. Similar types of mutants are known in the cyclic AMP receptor protein (CRP) (Ryu *et al.*, 1993; Adhya *et al.*, 1995). To test whether Y210C is capable of transmitting such DNA-induced conformational changes, we monitored the tryptophan fluorescence and bis-ANS fluorescence of the bis-ANS–repressor complex as a function of added single operator O_R1. In a previous study we showed that bis-ANS binds exclusively to the C-terminal domain of

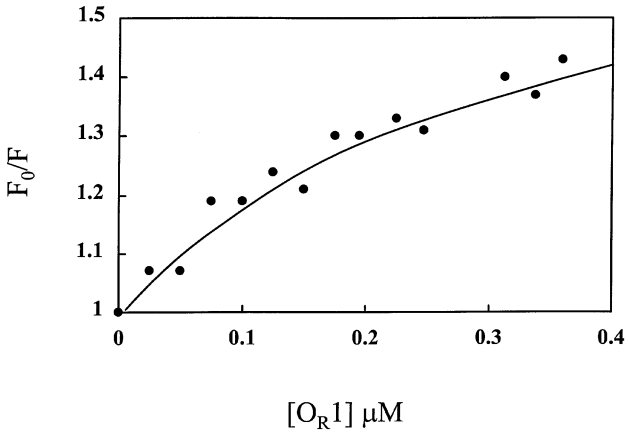

Fig. 3. Tryptophan fluorescence quenching of Y210C λ-repressor upon titration with O_R1 oligonucleotide. The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0, at 25°C. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The bandpasses were 5 nm each. The protein concentration was 1 μM.

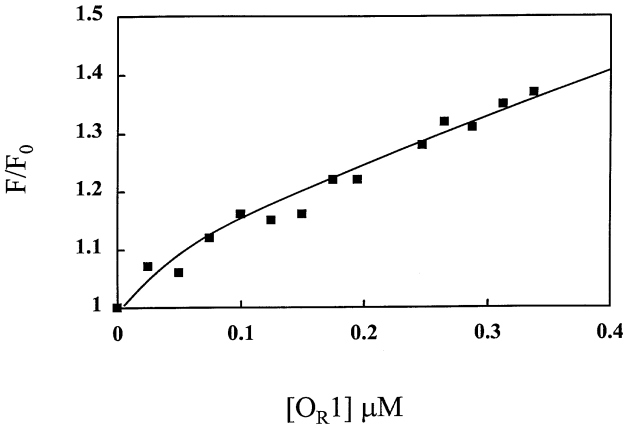

Fig. 4. Enhancement of bis-ANS fluorescence upon titration of the bis-ANS–Y210C λ-repressor with O_R1 containing oligonucleotide. The solution conditions were 0.1 M potassium phosphate, pH 8.0, at 25°C. The excitation and emission wavelengths were 385 and 500 nm, respectively. The bandpasses were 10 nm each. The protein and bis-ANS concentrations were 1 and 0.5 μM, respectively.

the repressor and hence acts as a C-terminal domain probe (Saha *et al.*, 1992). The tryptophans are all situated at or near the C-terminal domain. Figures 3 and 4 show the effect of operator titration on Y210C repressor as monitored by tryptophan fluorescence and on the Y210C repressor–bis-ANS complex as monitored by bis-ANS fluorescence. In both cases, the fluorescence changes are very similar to that of the wild-type repressor (Saha *et al.*, 1992), indicating that the DNA-induced conformational change is unaffected by the mutation.

The reactivity of cysteine residues in proteins towards DTNB can be used to determine the degree of solvent exposure of cysteine residues. In mutants that introduce a cysteine residue, the reactivity of this cysteine residue may be taken as a measure of solvent exposure of the substituted residue, provided the mutation does not trigger a massive conformational change. Y210C introduces a cysteine in the protein sequence in addition to the three cysteines normally present in the protein. In a previous study, we showed that the three cysteines in the wild-type protein are unreactive to sulfhydryl reagents such as DTNB under native conditions and this lack

Table I. Sulfhydryl reactivity of λ-repressor under different conditions[a]

Solution conditions	No. of sulfhydryl groups reacted per monomer
Native Y210C[b]	0.1
Y210C in 2 M urea[c]	0.13
Y210C in 6 M urea[c]	0.43
Y210C after exhaustive protease digestion[d]	4.1
Wild-type repressor after exhaustive protease digestion[e]	2.98

[a]DTNB titrations were carried out in 0.1 M phosphate buffer, pH 7.5, containing additives as described. Final concentration of DTNB, 100 μM.
[b]Protein concentration, 6.8 μM.
[c]Protein concentration, 6.5 μM.
[d]Protein concentration, 6.7 μM. The protein was digested with subtilisin, trypsin, chymotrypsin and proteinase K at concentrations of 1.3 μg/ml for 88 h.
[e]Protein concentration, 6.9 μM.

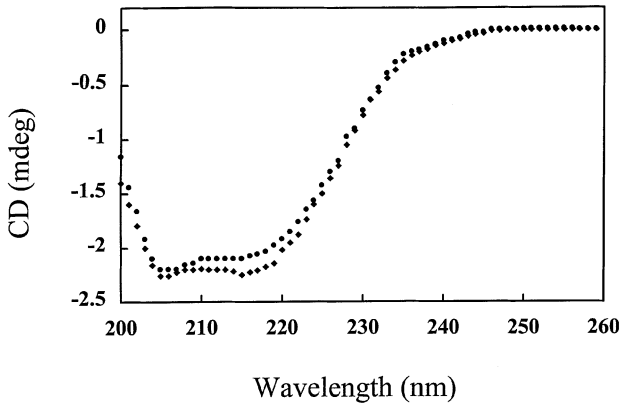

Fig. 5. Far-UV circular dichroism spectra of (◆) wild-type and (●) Y210C λ-repressor. The spectra were obtained in a 0.1 cm pathlength cuvette in 0.1 M potassium phosphate buffer, pH 8.0, at ambient temperature (25 ± 1°C). The protein concentrations were 1.2 μM. Ten spectra were signal averaged to improve the signal-to-noise ratio.

of reactivity is observed even in 6 M urea. Table I shows the sulfhydryl reactivity of the cysteines of Y210C repressor under normal buffer conditions and increasing concentrations of urea and with prolonged incubation. Under native conditions none of the four sulfhydryl groups reacts and even upon incubation in 6 M urea less than one cysteine residue shows reactivity towards DTNB. However, upon exhaustive protease digestion, complete sulfhydryl reactivity is observed. This indicates that the three cysteines of the wild-type sequence in addition to C210 are buried within the protein structure and hence are inaccessible to sulfhydryl reagents.

Circular dichroism spectroscopy is a sensitive monitor of the secondary structure of proteins. It may be used to determine structural changes in mutant proteins when compared with wild-type proteins. Figure 5 shows the far-UV CD spectra of 1.2 μM wild-type and Y210C λ-repressor. The general nature of the two spectra is very similar. The spectral intensities are almost identical up to 225 nm, but there is a small but significant reduction in the Y210C spectrum in the 210–225 nm range. The reduction is most pronounced at ~215 nm, but still is less than 5%.

Tryptophan fluorescence quenching by collisional quenchers is a sensitive method for assessing the accessibility of tryptophan residues. Since accessibility is a function of the general

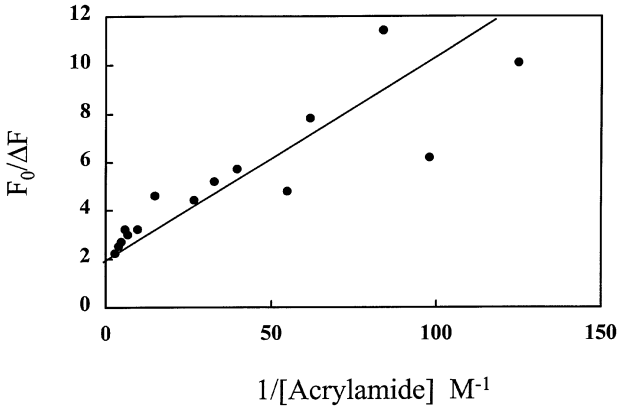

Fig. 6. Lehrer plot of acrylamide quenching of tryptophan fluorescence Y210C λ-repressor. The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0, at 25°C. The excitation wavelength was 295 nm and the emission was monitored at 340 nm. The bandpasses were 5 nm each.

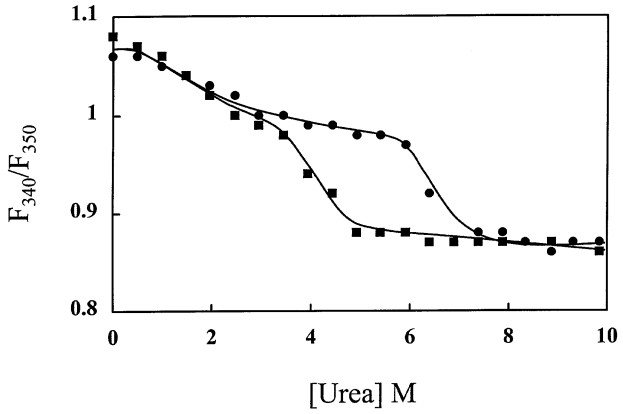

Fig. 7. Urea denaturation profile of (●) the wild-type and (■) Y210C λ-repressor. Wild type or Y210C λ-repressor (1 μM) was incubated in a given concentration of urea overnight at room temperature. The fluorescence intensity was then measured at 340 and 350 nm, with excitation at 295 nm. The bandpasses were 5 nm each. The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0, at 25°C.

Table II. Quenching parameters at different Y210C concentrations obtained from the Lehrer plot

[Repressor] (μM)	Intercept	K_{sv} (M^{-1})
0.5	2	23.75
1.0	2	19.4
2.5	2	11.4
10	2	7.0

conformational properties of the protein, dynamic and static, it is also a sensitive measure of the conformational integrity of the protein. Previously we have shown that the fluorescence contributions of three tryptophan residues (W129, 142 and 230) may be partially resolved by acrylamide quenching and this is a sensitive monitor of several processes involving the C-terminal domain. Since all the tryptophan residues are situated at or near the C-terminal domain as is the Y210C mutation, the acrylamide quenching pattern may be a sensitive probe of the C-terminal domain structure in the mutant protein. Figure 6 shows the Lehrer plot of 0.5 μM Y210C repressor. The initial part of the Lehrer plot cuts the ordinate at 2, indicating that about 50% of the initial fluorescence is quenchable by low concentrations of acrylamide. The K_{sv} of the initial quenchable part is 23.75. Both the K_{sv} and the intercept values are similar to those for the wild-type repressor at the same concentration, indicating general preservation of the structural and conformational integrity of the C-terminal domain of the Y210C repressor. Increasing the protein concentration does not significantly alter the ordinate intercept value and the K_{sv} value is only affected marginally (Table II). This is in contrast to the wild-type repressor, which undergoes a significant change in the ordinate intercept value upon transition from dimer to tetramer in this protein concentration range. Hence the quenching data and fluorescence anisotropy data both suggest a lack of oligomerization in this concentration range. Taken together, the fluorescence quenching, CD and sulfhydryl reactivity data suggest that the integrity of the C-terminal domain is largely preserved with only modest changes in secondary structure, probably around the mutation site.

Clearly the mutation affects the structure of the C-terminal domain modestly without any global unfolding. Many mutations, however, alter the global stability of the native state of proteins. One of the ways to estimate the stability of the native state is to measure the denaturation profile of the protein as a function of urea or guanidine hydrochloride. Previously, we have shown that λ-repressor denatures in several distinct phases, and at least two of these phases involve the C-terminal domain and can be monitored by measuring tryptophan fluorescence (Banik *et al.*, 1992). Figure 7 shows the F_{340}/F_{350} ratio for wild-type and Y210C λ-repressor. The wild-type repressor shows two distinct transitions centered around 2 and 6.5 M urea, as was observed before (Banik *et al.*, 1992). The high urea transition in the wild-type repressor is shifted dramatically to lower urea concentrations in the Y210C repressor, indicating significant destabilization of the native structure. In the UmuD′ structure, the amino acid residues equivalent to the tryptophan residues are at a considerable distance away from Y210 equivalent residue, suggesting that the effect of the mutation may not be confined to the local area. Hence we conclude that although the general integrity of the C-terminal domain structure is preserved, there are conformational changes and change of interactions which are not confined to the mutation area only.

Discussion

Negative regulation of transcription is one of the earliest types of regulation known. Recent studies suggest that many negative regulator binding sites are multi-partite in nature. The repressors bind to these multi-partite sites with cooperativity which involves protein–protein interaction. This protein–protein interaction is thought to be the crucial element that determines the stability of the cooperatively bound tetramer and allows the functioning of negative regulation. Like other negative regulators, λ-repressor binding sites are also multi-partite. Interaction between two operator site-bound repressor dimers is crucial in maintaining the integrity of the negative regulation. A large collection of C-terminal domain mutants have recently been described that are defective in cooperative interaction but not in DNA binding. A number of independent isolations have yielded mutations in residue 210 (Benson *et al.*, 1994; Whipple *et al.*, 1994). The Y210 residue is part of an

'NPXY' sequence which has been shown to form a reverse turn in solution (Bansel and Gierasch, 1991).

The C-terminal domain of λ-repressor is homologous with the UmuD′ protein. These two proteins (residues 25–139 of UmuD′ and 112–236 of λ-repressor) have ~31% sequence identity and a high degree of conservative substitutions. In addition, both the proteins undergo recA-mediated self-cleavage reactions, suggesting that they adopt similar fold. In the UmuD′ crystal structure, the analogous 'NSAY' sequence forms a tight turn between two anti-parallel β-strands [β5–β6 in the nomenclature of Peat *et al.* (1996)]. This region interacts with a 3_{10} helix (between β1–β2 in the same nomenclature) to enclose one of the core segments. In addition, λ-repressor contains a five amino acid insert in the 3_{10} helix region when compared with UmuD′. Secondary structure prediction of the last 40 residues of λ-repressor suggests a high propensity for sheet and turn formation. This makes it likely that the NPQY sequence in λ-repressor also forms a turn.

Although we have not studied the dimerization or DNA binding ability of this mutant, Y210N and Y210H have been studied by Whipple *et al.* (1994) and Burz and Ackers (1994). Both the mutants have normal DNA binding and dimerization, suggesting that Y210C is likely to have normal dimer formation capability and DNA binding ability. Recent NMR studies of the UmuD′ protein in solution suggest that the monomer–monomer interface may be different from the interface inferred from the crystallographic data (Ferentz *et al.*, 1997). The mutant data seem to agree with the latter interface. In this new suggested interface, Y210 is far from the dimer interface, suggesting normal dimer formation in this mutant. The mutant repressor also transmits the operator-induced conformational change, suggesting that it is not blocked in such a function. Fluorescence quenching, CD studies and reactivity of other sulfhydryl groups suggest that general integrity of the C-terminal domain is roughly intact.

The C210 residue in this mutant is completely unreactive under native conditions and in 6 M urea, suggesting that residue 210 is buried in the protein structure. This conclusion is supported by the fact that in the UmuD′ structure, the equivalent residue Y114 is surrounded by several residues such as D59, S60 and N111. Several non-hydrogen atoms from these residues are within a 3–4 Å distance of the side-chain non-hydrogen atoms of Y114 (Figure 8a and b). As described above, the λ-repressor contains an additional five amino acid insert in the 3_{10} helix that interacts with the 'NSAY' reverse turn in UmuD′, suggesting that elements of this turn may be buried further in the λ-repressor structure. It is unlikely that such a buried residue is a direct participant in the dimer–dimer interaction.

How this mutation affects the dimer–dimer interaction is unclear, but several lines of evidence suggest it may be through the destabilization of the reverse turn and subsequent conformational change elsewhere in the protein. (1) It was pointed out by Whipple *et al.* (1994) that this residue is part of a sequence (NPXY) which forms a reverse turn in solution. This sequence was first observed in native low-density lipoprotein receptor and was thought to be an important element of the internalization signal. A tyrosine to cysteine mutation in this sequence completely abolishes internalization. Bansal and Gierasch (1991) have shown that a similar mutation abolishes the propensity of a peptide containing this sequence to form a reverse turn, thus underlining the role of tyrosine in maintaining the conformational integrity of the reverse turn. (2)

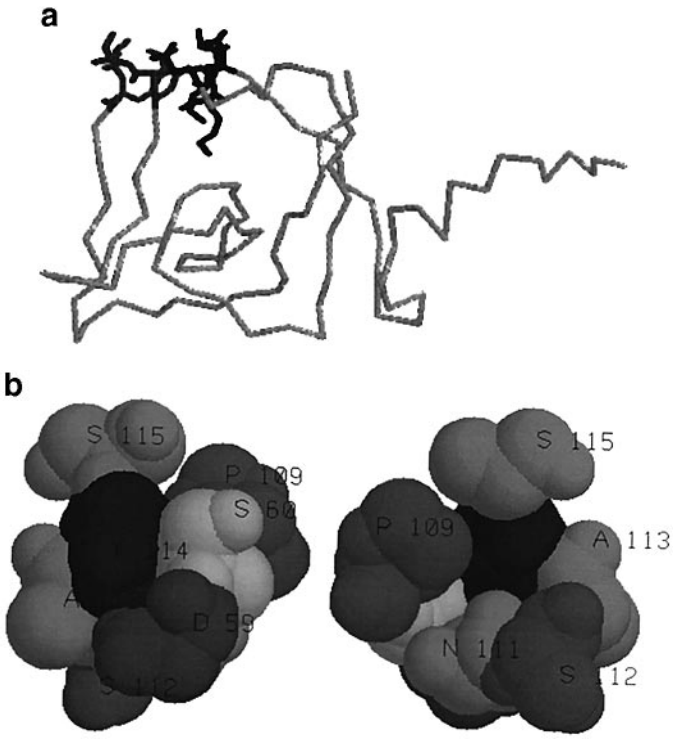

Fig. 8. (**a**) Backbone trace of the UmuD′ protein. The turn residues NSAY (111–114) are highlighted in light gray and residues 58–60 in darker shades of gray. (**b**) The space-filling model of the residues in close proximity to Y114 (darkest gray) viewed from two different orientations. The figures were constructed using *Rasmol* and UmuD′ coordinates obtained from the Protein Data Bank.

Recently, we have sequenced two previously isolated temperature-sensitive mutations in the *cI* gene. One is a double mutant FI141/PT153 and another is a single mutant NT207 (N.Jana, S.Roy and N.C.Mandal, unpublished observations). P153 falls into the extra five amino acid insert in the 3_{10} helix as mentioned above and N207 belongs to the 'NPXY' reverse turn. Clearly, at lower temperatures these mutations largely preserve the dimer–dimer cooperative interaction, whereas at higher temperatures they destabilize the structure enough to disrupt some essential function. This suggests that integrity of the structural region containing the reverse turn may be important in cooperative interaction. (3) The urea denaturation profile of Y210C mutant is significantly different and part of the C-terminal domain is denatured at significantly lower urea concentrations, suggesting destabilization of the native state with respect to the denatured state. Monitoring of the denaturation by tryptophan fluorescence makes it likely that this destabilization effect is not confined to the local area, as the tryptophan residues are distant from the reverse turn. This suggests that Y210C mutant has a general destabilizing effect on part of the C-terminal domain. (4) N207 and Y210 are in close contact in the UmuD′ structure, It has been speculated that they may form hydrogen bonds of unusual nature to stabilize the turn (Bansal and Gierasch, 1991). Mutation in both residues leads to loss of cooperative interaction, either conditionally or constitutively. Hence the most straightforward explanation of the mechanism of loss of cooperative contact may be through destabilization of the reverse turn and consequent changes in conformation of other regions important for direct interaction.

As stated by Whipple *et al.* (1994), the C-terminal domain of λ-repressor has three regions which have specificity determinants for cooperative interactions: the 147, 196–202 and 210–212 regions, in addition to the disputed tail region 228–234. It now appears that S228N is not a cooperative mutant as was previously thought (Burz and Ackers, 1994; Burz *et al.*, 1994; Bandyopadhyay *et al.*, 1996). Hence it appears that the role of this region in cooperative interaction is questionable. In the 210 region, Y210 appeared to be the most promising candidate for cooperative interaction. This work, however, indicates that the effects of 210 mutations are indirect and the specificity of Y at this position may be for a structural requirement of a reverse turn. We speculate that the 196–202 region and the G147 region may be the predominant interaction points between the two dimers in the cooperative complex.

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