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Multiphasic denaturation of the λ repressor by urea and its implications for the repressor structure

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Urea denaturation of the λ repressor has been studied by fluorescence and circular dichroic spectroscopies. Three phases of denaturation could be detected which we have assigned to part of the C-terminal domain, N-terminal domain and subunit dissociation coupled with further denaturation of the rest of the C-terminal domain at increasing urea concentrations. Acrylamide quenching suggests that at least one of the three tryptophan residues of the λ repressor is in a different environment and its emission maximum is considerably blue-shifted. The transition in low urea concentration (mid-point approximately 2 M) affects the environment of this tryptophan residue, which is located in the C-terminal domain. Removal of the hinge and the N-terminal domain shifts this transition towards even lower urea concentrations, indicating the presence of interaction between hinge on N-terminal and C-terminal domains in the intact repressor.

Proteins that bind specifically to double-stranded DNA sequences are well known. Repressors are one such class of DNA-binding proteins that bind specifically to operators. Apart from physically occupying these operator sites and causing inhibition of transcription, they cause other effects such as activation of transcription from adjacent promoters (Bushman and Ptashne, 1986), inhibition or activation at sites far away (Dunn et al., 1984) etc. Recently, a number of groups have shown that these functions may involve protein–protein contact and, in the latter case, looping out of the intervening DNA sequences (Hochschild and Ptashne, 1986). But detailed molecular characterisation of such interactions has not been established.

The λ repressor is a dimeric protein of identical subunits. Each monomer folds into an N-terminal domain (amino acid residues 1–92), which is responsible for DNA binding (Jordan and Pabo, 1988) and contact with RNA polymerase for activation function (Guerente et al., 1982) and the C-terminal domain (amino acid residues 132–236), which is responsible for subunit interaction and protein–protein contacts required for co-operative binding (Pabo et al., 1979). The intervening protease-sensitive region (amino acid residues 93–131), called the connector or hinge region, has not yet been studied in any detail and very little is known about its structure or its role in λ repressor functions.

The λ repressor and its interaction with operators have been studied intensively. In addition to specific recognition of operator sequences, the λ repressor can activate transcription from promoters adjacent to the operator (Hawley and McClure, 1983) and can bind co-operatively to three contiguous operator sequences O_R1, O_R2 and O_R3 or O_L1, O_L2 and O_L3 (Senear et al., 1986). It is thus an important model system for studying many aspects of DNA–protein interaction. Such complex and multiple functions carried out by a single protein may have their basis in complex structural organization within the protein and in intramolecular interactions.

Equilibrium denaturation of proteins is a useful tool to study protein structure and folding. Urea is one of the most commonly used denaturants. Distinct transitions at different urea concentrations may indicate the existence of distinct structural elements while transitions at lower urea concentrations imply less stability. In this paper, urea denaturation has been used to explore different structural organizations of the λ repressor and their implications for function.

MATERIALS AND METHODS

Materials

The compounds eosin 5-isothiocyanate, dansyl chloride and fluorescein 5-isothiocyanate adsorbed on 10% celite were purchased from Molecular Probes Inc. (Eugene, OR, USA). Ultrapure urea was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). 5,5′-Dithio-bis(2-nitrobenzoic acid) (Nbs_2), SP-Sephadex C-25-120, Koshland reagent III and Sephadex G-50 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and acrylamide (four times recrystallised) was purchased from Spectrochem (India). All other reagents were of analytical grade quality.

Isolation of the λ repressor

The λ repressor was isolated from an *Escherichia coli* strain (RR1 Δ15 *lacZ*) bearing a plasmid pEA305 which contains the wild-type *cI* gene under the control of the *tac* promoter (Amann et al., 1983). The *tac* promoter is a tryptophan–*lac* fusion promoter, which is repressed by *lac* repressor and

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Abbreviation. Nbs_2, 5,5′-dithio-bis(2-nitrobenzoic acid).

inducible with isopropyl β-D-thiogalactoside. When fully induced, the λ repressor constitutes about 30% of total cellular protein. The cells containing the above plasmid were grown to $A_{590} = 0.6$, then isopropyl β-D-thiogalactoside at a final concentration of 1 mM was added and grown for a further 2 h. Repressor was purified according to method I of Johnson et al. (1980) involving poly(ethyleneimine) precipitation followed by chromatography on CM-Sephadex A-50 and hydroxyapatite columns. The λ repressor was assayed by the DNA filter-binding procedure of Riggs et al. (1970). SDS gel electrophoresis in a 13.5% polyacrylamide gel (Laemmli, 1970) showed a single band corresponding to a molecular mass of 26 kDa. For all studies, the repressor was dialysed against 0.1 M potassium phosphate pH 8.0 and the repressor concentration was determined with the value $A^{1\%}_{280} = 11.3$. The repressor was stored in aliquots at −70 °C.

Spectroscopic studies

Steady-state fluorescence was measured on an Hitachi F3000 spectrofluorimeter equipped with a computer for spectra addition and subtraction facilities. The bandpass of both excitation and emission monochromators was 5 nm unless stated otherwise. The fluorescence of buffers containing the corresponding urea concentrations was always subtracted from any measured fluorescence. All fluorescence measurements were performed at 25 °C and the experiments were carried out in 1-cm path-length cuvettes.

Polarisation experiments were performed using an Hitachi polarisation accessory. The fluorescence intensity components (I_{vv}, I_{vh}, I_{hv}, I_{hh}), in which the subscripts refer to the horizontal (h) or vertical (v) positioning of the excitation and emission polarizers, respectively, were used to calculate the steady-state fluorescence polarization (P):

$$P = \frac{(I_{vv} - G I_{vh})}{(I_{vv} + G I_{vh})} \quad (1)$$

where G is the grating factor that corrects for wavelength-dependent distortions of the polarizing system.

$$G = I_{hv}/I_{hh}. \quad (2)$$

Absorbance measurements were performed on a Shimadzu UV-160 spectrophotometer after proper baseline correction. Circular dichroic measurements were made on a Jasco 500C spectropolarimeter using a 1-cm path-length quartz cell. In urea denaturation experiments, fluorescence and circular dichroic measurements were performed as follows. A series of freshly prepared solutions of ultrapure urea in 0.1 M potassium phosphate pH 8.0 of appropriate concentrations was prepared and the repressor was added. Equal volumes of buffer were added to the same volume of urea solution and used as a blank.

Isolation and characterization of λ repressor fragments

N- and C-terminal domain fragments of the λ repressor (1−92, 92−236 and 132−236) were isolated after papain digestion according to Pabo et al. (1979) with slight modification. The kinetics of papain digestion was calibrated by quenching the papain digestion at different times with Koshland reagent III and running it on SDS/PAGE. The pattern obtained was very similar to that of Pabo et al. (1979). The N-terminal domain was purified on SP-Sephadex C-25. After an initial wash through, the N-terminal domain was eluted with a gradient of 1−100 mM KCl. Appropriate fractions were pooled, concentrated by lyophilization and dialysed in a 2-kDa cutoff dialysis tubing against 0.1 M potassium phosphate pH 8.0. The purified material showed a single band on SDS/PAGE corresponding to a molecular mass of approximately 10 kDa and a gave fluorescence emission maximum at 303 nm, which is characteristic of proteins not containing tryptophan (there are no tryptophans in the N-terminal domain). For purification of the C-terminal fragment (93−236), the λ repressor was digested with papain for the appropriate time and the mixture was then put on a SP-Sephadex C-25 column and the flow-through collected. The flow-through was then further purified over a Sephadex G-75 column equilibrated with 0.1 M phosphate pH 8.0. Domain 132−236 was purified according to the same protocol, only a longer digestion time was used. The identity of the fragments was established by comparing the mobility of the fragments on SDS/PAGE with that of a papain digest which contains all three C-terminal fragments. The protein concentration of the fragments was determined by calculated absorption coefficients based on the known tyrosine and tryptophan content of the fragments.

Chemical modification

The repressor was fluorescein-labeled by treatment with a 35-fold molar excess of fluorescein 5-isothiocyanate adsorbed on celite which was suspended in 0.1 M potassium phosphate pH 8.0 and added to the repressor (1 mg/ml). The reaction was performed at 4 °C for 3 h with occasional vortexing. Excess reagent was removed by exhaustive dialysis against 0.1 M potassium phosphate pH 8.0. Finally, the labeled repressor concentration was determined by the Bio-Rad protein assay (Bradford, 1976) using purified λ repressor as standard. Molar concentrations of repressor were always calculated in terms of monomer subunit, unless otherwise stated.

The repressor was eosin-labeled by the same protocol. The only exception was that the labeled protein was loaded directly onto a column (0.5 cm × 30 cm) of Sephadex G-50 for separation of unreacted reagent and eluted with 0.1 M potassium phosphate pH 8.0; 1-ml fractions were collected. The labeled repressor concentration was determined as stated before. Repressor was dansyl-labeled by treatment with a 10-fold molar excess of dansyl chloride. An *N,N'*-dimethyl formamide solution of dansyl chloride was added to a 1 mg/ml solution of λ repressor in 0.24 M potassium phosphate pH 8.0 so that the final dimethyl formamide concentration was 10% in the reaction mixture; the repressor retained full DNA-binding activity at or below this concentration (data not shown). The reaction was performed at 25 °C for 15 min and finally quenched with 1 M Tris/Cl pH 7.8 to a final concentration of 5 mM Tris, followed by loading onto a column (0.5 × 15 cm) of Sephadex G-50. The remainder of the procedure was as described for labeling with eosin 5-isothiocyanate. The incorporation ratios of fluorescein, eosin- and dansyl-labeled repressor were calculated to be 1.2, 1.0 and 1.7 using molar absorption coefficients of 72 mM^{-1} cm^{-1} at 495 nm (Garel, 1976), 83 mM^{-1} cm^{-1} at 520 nm (Haugland, 1985) and 4500 M^{-1} cm^{-1} at 340 nm (Matthews and Hsieh, 1985) for fluorescein, eosin and dansyl respectively.

Nbs_2 titration of the λ repressor was carried out in different conditions as described in Table 1. The number of sulfhydryl groups reacted was calculated by measuring absorbance at 412 nm using the molar absorption coefficient of the thionitrobenzoate anion of 13.6 mM^{-1} cm^{-1}.

Energy transfer

Fluorescein- and eosin-labeled λ repressor in 0.1 M potassium phosphate pH 8.0 were mixed with different concentrations of urea to final concentrations of 1 μM each. Excitation was at 450 nm and emission at 520 and 560 nm. Energy transfer at each urea concentration was measured in three different sets. First, by measuring the emission of 1 μM fluorescein-labeled repressor (F_{fl}); second, by measuring the emission of 1 μM eosin-labeled repressor (F_{eo}); third, by measuring the emission spectra of a mixture of 1 μM each of fluorescein- and eosin-labeled repressor (F_{fl+eo}). The background-subtracted emission spectra of fluorescein- and eosin-labeled repressor were added into the computer of the fluorimeter. In all cases, emission at 520 nm and 560 nm was noted and the ratio of F_{560}/F_{520} was calculated. This ratio for computer-added and physically mixed fluorescein- and eosin-labeled repressor was taken as a measure of energy transfer.

$$\text{Energy transfer efficiency} = \frac{(F^{560}_{fl+eo}/F^{520}_{fl+eo})}{(F^{560}_{fl} + F^{560}_{eo})/(F^{520}_{fl} + F^{520}_{eo})}.$$

If the subunits are fully dissociated, then there should be no difference in computer-added and physically mixed spectra and the ratio should be 1.

Acrylamide quenching

The classical Stern-Volmer equation for quenching describes the decrease in fluorescence intensity as a function of quencher concentration, [Q].

$$F_o/F = 1 + K_{sv}[Q] = 1 + k_q \tau_o [Q] \quad (3)$$

where F_o is the initial fluorescence intensity, F is the intensity in the presence of added quencher, K_{sv} is the Stern-Volmer constant, τ_o is the fluorescence lifetime in the absence of quencher, and k_q is the bimolecular rate constant for quenching. When there are fluorophores of different degrees of quenchability in the system, it is convenient to use a modification of the Stern-Volmer equation as suggested by Lehrer (1971):

$$F_o/(F_o - F) = 1/(f_a k_q \tau_o [Q]) + 1/f_a \quad (4)$$

where f_a is the contribution to the total fluorescence of the emission which is quenched. A plot of $F_o/(F_o - F)$ vs $1/[Q]$ gives information on f_a and the effective quenching constant is determined from the slope. In quenching experiments, the neutral quencher acrylamide was used, since it does not denature proteins even when present in molar concentrations (Eftink and Ghiron, 1981).

RESULTS

Denaturation of the λ repressor

The λ repressor contains three tryptophan residues at positions 129, 142 and 230; the latter two are in the C-terminal domain while the first is in the connector (i.e. the hinge) region. Fig. 1 shows the effect of urea concentration on the tryptophan emission spectrum of the λ repressor. As the urea concentration increases from 0 to 10 M, there is a progressive shift of the emission maximum towards the red. Four representative fluorescence emission spectra are shown in Fig. 1. In the absence of any urea, the repressor has an emission maximum at 340 nm. This spectrum was compared with those obtained in 3 M, 6 M and 10 M urea. In 3 M and 6 M urea

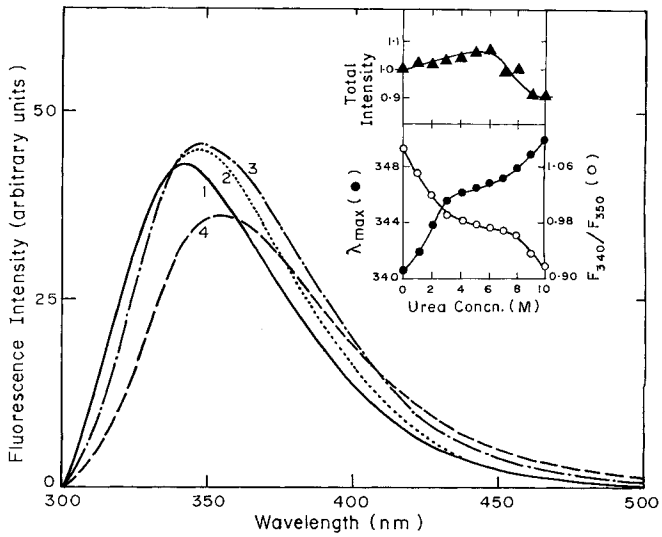

Fig. 1. Effect of urea concentration on the tryptophan fluorescence of the λ repressor. Fluorescence spectra were measured after mixing with urea at 25 °C at a protein concentration of 0.75 μM in 0.1 M potassium phosphate pH 8.0. The excitation wavelength was 295 nm. Emission spectra in the presence of (1) 0, (2) 3 M, (3) 6 M, (4) 10 M urea. The inset shows the total emission intensity, F_{340}/F_{350} ratio and wavelength maximum as a function of urea concentration. The total emission intensity reported here is normalized with respect to the spectrum with no urea.

(curves 2 and 3), the emission spectra of the repressor are almost identical and the maximum is shifted to 346 nm. In 10 M urea, the emission maximum is shifted to 350 nm, but the intensity at 340 nm falls to about 80% of that of the repressor in the absence of any urea. The inset of Fig. 1 shows the total intensity, F_{340}/F_{350} ratio and wavelength maxima as a function of urea concentration. The total emission intensity increases up to 6 M urea concentration and then it declines indicating two distinct transitions. The F_{340}/F_{350} ratio, as well as the wavelength maximum shift, also indicates at least two distinct transitions, one between 0–3 M urea and another between 6–10 M urea.

Fig. 2 (a) shows the effect of urea concentration on the emission maximum and the F_{340}/F_{350} ratio of the repressor at several protein concentrations. The repressor at three different concentrations (0.7, 1.4 and 2.8 μM) shows the same profile. A sigmoidal curve is obtained for both the fluorescence ratio and the emission maximum. This transition could be due to the denaturation of the C-terminal domain or the hinge region. It is unlikely to be due to denaturation of the N-terminal domain since this does not contain any tryptophan residues and global N-terminal unfolding occurs at higher urea concentrations (see later). The emission maximum shifted from 340 nm to 346 nm between 0–6 M urea, which indicates a gradual shift of one or more tryptophan residues to a more polar and solvent-exposed environment. The existence of an isoemissive point at 337 nm implies a two-state equilibrium. In addition, our recent results have shown that this transition is fully reversible (R. Saha and S. Roy., unpublished observation). Thus a reversible two-state equilibrium for this transition was assumed and an equilibrium constant was calculated at several different urea concentrations (Bowie and Sauer, 1989). From the equilibrium constant, the free energy change for the transition was calculated at each urea concentration. Fig. 2 (b) shows a plot of unfolding free energy (ΔG_u)

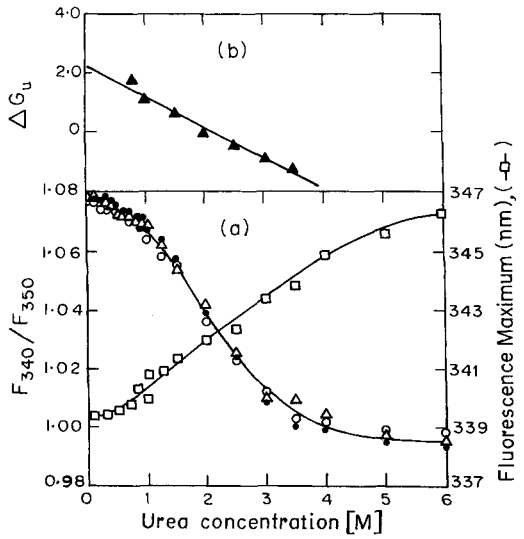

Fig. 2. Dependence of the tryptophan fluorescence and the emission maximum of the repressor on urea concentration. (a) The repressor concentration was (●) 0.7 μM (○) 1.4 μM or (△) 2.8 μM in 0.1 M potassium phosphate pH 8.0 at 25 °C. The excitation wavelength was 295 nm and emission at 340 nm and 350 nm was noted. (□) 2.8 μM repressor was used for the emission maximum shifting experiment. (b) The free energy of unfolding versus the urea concentration. (Number on the axis are kcal/mol and need to be multiplied by 4.18 to obtain values in kJ/mol).

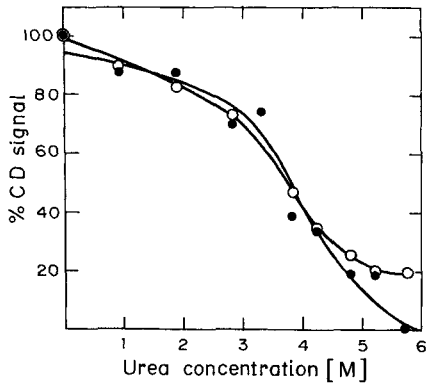

Fig. 3. Dependence of the percentage of CD signal of (○) 4 μM repressor and (●) 8 μM purified N-terminal domain of repressor on urea concentration.

versus urea concentration, which was extrapolated to zero urea concentration to yield the free energy of transition in the absence of urea. A value of 8.74 kJ/mol indicated a modest free energy change that accompanies this transition.

The fluorescence data only give an incomplete picture of the denaturation, as they reflect changes in local environments of the tryptophan residues, which in turn reflect the conformational changes of the tryptophan-containing regions. CD, however, predominantly monitors changes in secondary structure. The repressor exhibits a negative CD signal at 223 nm. The strength of this signal which monitors the secondary structure slowly diminishes in intensity as the urea concentration increases from 0 to 6 M urea. Fig. 3 shows a plot of the CD signals of both the intact λ repressor and its purified N-terminal domain at 223 nm as a function of increasing urea concentration. In both the cases a similar denaturation profile is obtained. The mid-point of transition is around 3.5 M urea.

Table 1. Reaction of sulfhydryl groups of the λ repressor with Nbs_2. The λ repressor concentration was 250 μg/ml. To obtain protease-cleaved repressor, it was incubated in 0.1 M potassium phosphate with a mixture of proteases: trypsin, chymotrypsin, subtilisin, proteinase K (E/S = 1:200, by mass at 25 °C for 18 h followed by addition of 100 μM Nbs_2; control experiments were done with an identical protocol except the repressor and the value was subtracted from the protein value. Native repressor was incubated with 100 μM Nbs_2 at 25 °C for 1.5; a control without repressor was subtracted from the actual measurement with protein. The urea-treated repressor was either incubated with 6 M urea containing 1 mM EDTA at 25 °C for 1.5 h and dialysed against 6 M urea for 18 h at 25 °C, or incubated with 9 M urea containing 1 mM EDTA at 25 °C for 1 h; 100 μM Nbs_2 was then added. A_{412} was noted to calculate reacted −SH groups; values for the control experiment with 100 μM Nbs_2 were subtracted.

Conditions	−SH group reacted
	mol/mol
Protease-cleaved repressor	2.70
Native repressor	0.05
Repressor treated with:	
6 M urea	0.07
9 M urea	0.97

These two similar transitions reflect the loss of secondary structures, probably the α-helices, upon urea treatment; the helix-containing N-terminal domain denatures at 3.5 M urea.

The reactivity of cysteine residues of the λ repressor, which are all situated in the C-terminal domain at positions 180, 215, and 218, was used as a probe for C-terminal domain denaturation. Cysteine residues of the repressor did not react with Nbs_2 under normal conditions or even in 6 M urea, as shown in Table 1. Exhaustive protease digestion under non-reducing conditions, however, caused near-complete exposure of the cysteine groups as measured by Nbs_2 reaction. This observation indicated that the low reactivity of cysteine residues in the intact λ repressor is due to inaccessibility of the reagent and not due to disulfide bond formation. Thus the two distinct phases of λ repressor denaturation that occur below 6 M urea do not involve global C-terminal denaturation.

Subunit dissociation and energy transfer

Fluorescence polarisation of a fluorophore rigidly attached to a macromolecule can reflect the rotational correlation time of the macromolecule. It is expected that subunit dissociation would lead to decreased correlation time and, hence, a reduced polarisation value, if the ratio of fluorescence life-time/correlation time is not very small. Dansyl chloride was chosen as the fluorophore because of its relatively long life time (> 10 ns). It was also observed that incorporation of 1.7 mol dansyl chloride/λ repressor did not lead to loss of any DNA-binding activity (data not shown). Fig. 4 (a) shows the change of polarisation of dansyl-labeled λ repressor versus increasing urea concentrations at two different protein concentrations. The transition midpoints are around 7 M and 7.7 M at 0.375 μM and 0.75 μM protein concentrations, respectively.

Singlet−singlet energy transfer has been used to measure distances between suitably matched donor and acceptor pairs, in the range of 2−8 nm. In a hybrid protein dimer containing donors in one subunit and acceptor in another, energy transfer

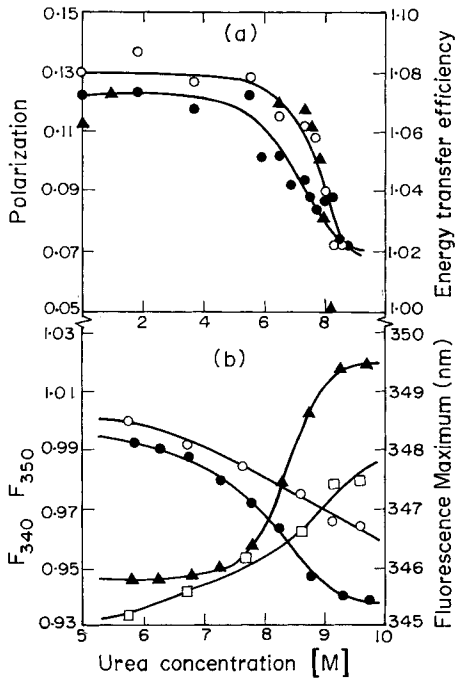

Fig. 4. (a) Polarisation and energy transfer efficiency and (b) F_{340}/F_{350} ratio and emission maximum of the λ repressor as a function of urea concentration. (a) Dansylated repressor at final concentrations of (○) 0.75 μM and (●) 0.375 μM was mixed with different concentrations of urea. Excitation was at 340 nm and emission at 520 nm. Polarisation was calculated as described under Materials and Methods. (▲) Fluorescence energy transfer efficiency between fluorescein-eosin hybrid repressor at different urea concentrations. (b) F_{340}/F_{350} ratio of (●) 0.75 μM and (○) 4 μM λ repressor at various urea concentrations. Emission maxima of (▲) 0.75 μM and (□) 4 μM λ repressor at different urea concentrations. The excitation wavelength was at 295 nm. All other solution conditions were the same as in Fig. 1.

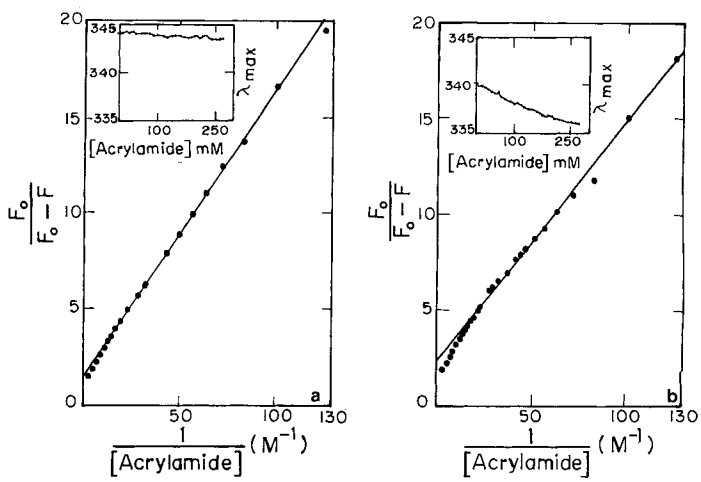

Fig. 5. Lehrer plot for quenching of tryptophan fluorescence by acrylamide. (a) 3 M urea; 0.1 M potassium phosphate pH 8.0. (b) Repressor at 4 μM was titrated with increasing concentrations of acrylamide. The excitation wavelength was at 295 nm and emission was measured at 340 nm. The insets of both (a) and (b) show the change in emission maximum with increasing acrylamide concentration.

would vanish upon dissociation, as the average distance between the subunits will be too large (Shore and Chakravarti, 1976). We have used fluorescein/eosin as a donor/acceptor pair to measure energy transfer. Fluorescein labeled λ repressor was mixed with an equimolar amount of eosin labeled λ repressor. Under these conditions, 50% of dimers should exist as hybrids. If any energy transfer takes place, the energy transfer ratio (as defined in Materials and Methods) would be greater than 1. It should revert to 1 upon complete dissociation. Thus the efficiency of energy transfer was monitored as a function of urea concentration. As the urea concentration increases, the energy transfer efficiency remains constant till about 6 M urea, then slowly decreases and approaches a value of 1. This decrease in energy transfer efficiency reflects the subunit dissociation of the repressor; the midpoint of the transition profile is around 7.7 M urea at a concentration of 0.75 μM repressor.

Previously, we have shown that, upon going from 6 M to 10 M urea, there is a further red shift and quenching of tryptophan fluorescence. In order to discover if they correlate with subunit dissociation, we have followed tryptophan fluorescence and emission maxima in the urea concentration range 6–10 M. As shown in Fig. 4 (b), the tryptophan emission maximum shifts from 346 nm to 350 nm, accompanied by fluorescence quenching. This change in tryptophan environment when monitored at 0.75 μM repressor concentration approximately followed the subunit dissociation curve. To determine if this transition is concentration-dependent, the same experiment was conducted at 4 μM protein concentration. As can be seen from the figure, the transition at this higher concentration clearly shifts towards higher urea concentrations.

Acrylamide quenching and environment of tryptophan residues

Acrylamide quenching of tryptophan residues provides information on their accessibility in proteins. The urea denaturation studies reported above indicated changes in tryptophan environments which may be accompanied by concomitant changes of accessibilities. Acrylamide quenching at different urea concentrations can thus provide a more detailed picture of such transitions. Fig. 5 (a) shows the Lehrer plot for quenching of tryptophan fluorescence by acrylamide in 0.1 M potassium phosphate pH 8.0. The initial linear portion when extrapolated to infinite acrylamide concentration yields a value of 2 for $F_o/(F_o - F)$. This indicates approximately 50% of the initial fluorescence can be quenched very efficiently by acrylamide. At higher acrylamide concentrations, deviations from linearity occur as $F_o/(F_o - F)$ approaches a lower value. We interpret this as meaning that at least one and possibly two out of the three tryptophan residues are highly exposed and can be quenched readily by low concentrations of acrylamide. As shown in the inset of Fig. 5 (a), consistent with the above explanation, the quenching of tryptophan fluorescence of the λ repressor results in a gradual blue shift of emission maxima from 340.4 nm to 335 nm at 0.275 M acrylamide. Fig. 6 (a) shows the emission spectra of the λ repressor in the absence of any acrylamide (curve 1) and at 0.02 M acrylamide (curve 2) concentration. Since at this (0.02 M) concentration of acrylamide, only the most exposed tryptophans are quenched to a significant degree, the difference spectra (curve 1 − curve 2 = curve 4) reflect the emission of the most exposed tryptophan residues (Lehrer, 1971). Curve 4 has an emission maximum at 346.8 nm, indicating a high degree of solvent exposure of the quenchable tryptophan residues.

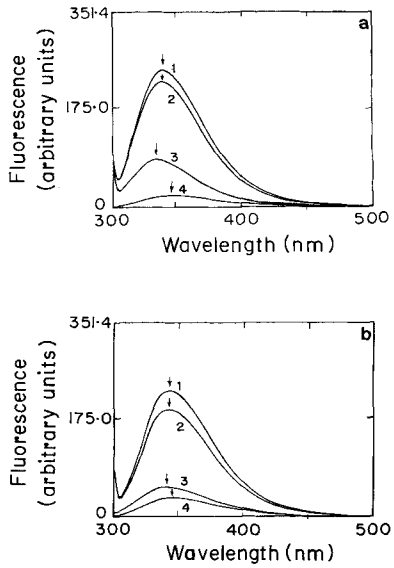

Fig. 6. Effect of acrylamide quenching on the tryptophan emission spectra of the λ repressor in 0.1 M potassium phosphate pH 8.0. (a) In the absence of urea; (b) in 3 M urea. The excitation wavelength was 295 nm. The emission spectra are as follows: (1) λ repressor; (2) λ repressor in 0.02 M acrylamide in (a) and 0.027 M acrylamide in (b); (3) λ repressor in 0.5 M acrylamide; (4) difference spectra of (1) and (2).

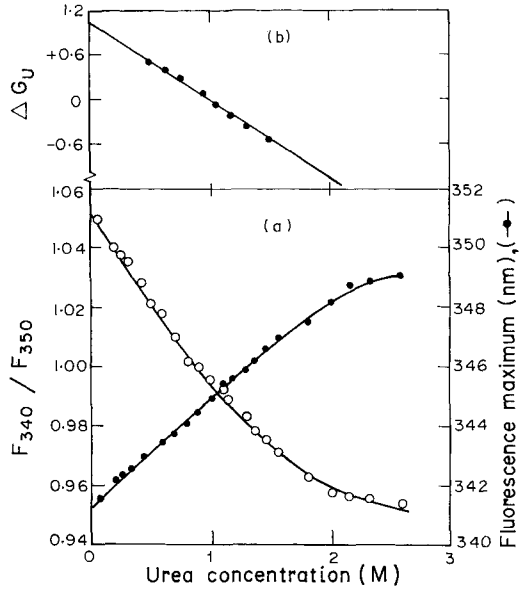

Fig. 7. Dependence of a (○) tryptophan fluorescence and (●) the emission maximum and (b) free energy of unfolding of the C-terminal domain of the λ repressor (132 – 236) on urea concentration. (a) The C-terminal domain concentration was 3 μM in 0.1 M potassium phosphate pH 8.0 at 25 °C. The excitation wavelength was 295 nm and fluorescence at 340 nm, 350 nm and emission maximum were noted. (b) The free energy of unfolding was calculated from F_{340}/F_{350}. (Numbers on the axis are kcal/mol and need to be multiplied by 4.18 to obtain values in kJ/mol).

At very high acrylamide concentrations the emission spectra reflect mostly the spectra of the most shielded tryptophan. Fig. 6 (a) shows the emission spectrum of the λ repressor at 0.5 M acrylamide (curve 3). The emission maximum is at 335 nm. We conclude that, of the three tryptophan residues in the λ repressor, at least one, and possibly two, are very exposed with emission maxima at 346.8 nm and one tryptophan is very shielded with an emission maximum at 335 nm.

Fig. 5 (b) shows the Lehrer plot for the λ repressor in 3 M urea. The initial linear portion can be extrapolated to a value of $F_o/(F_o - F) = 1.5$ at infinite acrylamide concentration. At high acrylamide concentrations deviations from linearity occur and $F_o/(F_o - F)$ approaches a value of 1. Two tryptophan residues are exposed and quenchable at relatively low acrylamide concentrations; the other tryptophan residue is somewhat less exposed. Fig. 6 (b) shows the difference spectra (curve 1 – curve 2 = curve 4) in the absence of any acrylamide (curve 1) and in 0.027 M acrylamide (curve 2). The emission maximum is at 346.2 nm, which must be composed of emission from both the exposed tryptophan residues. As shown in the inset of Fig. 5 (b), the relatively modest blue shift (approximately 1 nm) that occurs upon quenching of the two exposed tryptophan residues is an indication that the lesser exposed one is not very blue-shifted. At high acrylamide concentrations, the remaining emission spectrum is primarily from the most shielded tryptophan residue which has an emission maximum at 342.2 nm (Fig. 6b, curve 3).

Though not the only one, the most probable and straightforward explanation of the above results is that, in the absence of urea, one tryptophan residue of the λ repressor is more shielded than the other with an emission maximum of around 335 nm. Upon addition of 3 M urea, the most hidden and blue-shifted tryptophan residue becomes highly exposed and its emission maximum shifts to 342 nm or beyond.

Urea denaturation of isolated C-terminal domain (132 – 236)

In order to determine whether the transition at 2 M urea involves the hinge or C-terminal domain, we have studied the urea-induced denaturation profile of the isolated C-terminal domain (132 – 236) in 0 – 3 M urea concentrations. The emission spectrum of the C-terminal domain is somewhat red-shifted with a maximum at 342 nm. Upon addition of urea, a transition takes place which is shown in Fig. 7 (a). F_{340}/F_{350} decreases while the maximum shifts to 346 nm. The profile and nature of the transition is very similar to that of the intact repressor, indicating that the transition at low urea concentration in the intact repressor is due to one of the two tryptophan residues in the C-terminal domain. The transition, however, is shifted towards lower urea concentration when compared to the intact repressor. The plot of unfolding free energy (ΔG_u) versus urea concentrations is shown Fig. 7 (b). The calculated unfolding free energy value at zero urea concentration is 4.18 kJ/mol which is considerably lower than the corresponding transition in the free repressor.

DISCUSSION

The multiphasic denaturation of the λ repressor by urea has been studied by circular dichroic and fluorescence spectroscopic techniques. As the urea concentration was raised from 0 to 10 M, three distinct transitions were observed by different methods. These transitions are centered around 2.0, 3.5 and 7.7 M urea. The transition around 7.7 M urea is protein-concentration-dependent, characteristic of subunit dissociation. The energy transfer and polarisation studies confirm that subunit dissociation is a part of this transition. The cysteine residues, all of which are situated in the C-terminal

domain in the λ repressor, are completely unreactive even at 6 M urea, indicating that the complete C-terminal denaturation occurs at even higher urea concentrations. This is consistent with the expectation that total C-terminal domain denaturation would occur only after dissociation of dimers into monomers, since the dimerisation contacts are primarily located in the C-terminal domain region. The tryptophan fluorescence quenching and red-shift of emission maxima that occurs around 8 M urea probably indicates further unfolding of the C-terminal domain. Some residual structure probably remains in even 9 M urea, since only one of the three cysteine residues is available for the Nbs_2 reaction under this condition. The second transition centered around 3.5 M urea, which was monitored by CD, results in a major loss of secondary structure, probably α-helices, and no significant change in tryptophan fluorescence. Since the N-terminal domain contains five α-helices and no tryptophan, transition at 3.5 M urea may be due to N-terminal domain denaturation. The isolated N-terminal domain undergoes a very similar denaturation, confirming that the transition around 3.5 M urea in the intact λ repressor is due to denaturation of the N-terminal domain.

The transition centered around 2 M urea causes a change in the environment of one or more tryptophan residues. Thus the transition could represent denaturation of either the hinge or part of the C-terminal domain. Studies with isolated C-terminal domain (132–236) indicate that the transition at low urea concentration involved denaturation of part of the C-terminal domain and this denaturation does not change the reactivity of the cysteine residues. Since this transition is present in the isolated C-terminal domain, it is unlikely to be due to domain rearrangements leading to a greater exposure of a tryptophan residue. From the present data it is not possible to tell whether there is a concomitant denaturation of the hinge region.

Removal of the N-terminal domain and the hinge region results in a shift of the transition at 2 M urea to around 1 M urea. Removal of only the N-terminal domain (fragment 93–236) shifts the transition to a lower urea concentration to a lesser degree. The mid-point of the transition in fragment 93–236 is at 1.4 M urea (data not shown). This indicates that the stability of the part of the C-terminal domain that undergoes denaturation in low urea concentrations is affected by the presence of both the N-terminal domain and the hinge region. The overall emission maximum also showed a red shift upon removal of the hinge region and the N-terminal domain. The removal of these regions lowers the unfolding energy. All this is consistent with interaction of a part of the C-terminal domain with the N-terminal domain or the hinge or both. We note that DeAnda et al. (1983) have suggested an interaction of the hinge region with the C-terminal domain in the related C2 repressor from bacteriophage P22.

This suggests that the C-terminal domain may not be totally free to rotate from the N-terminal domain in the repressor which is not bound to DNA. Our recent fluorescence polarisation studies indicate that the repressor dimer tumbles at about the rate expected of its molecular mass (U. Banik and S. Roy, unpublished observation) consistent with the lack of a high degree of internal mobility. Thus, freedom of rotation of the C-terminal domain in operator-bound repressor, as hypothesized by Hochschild and Ptashne (1988), could be at the expense of the interaction energy postulated above or an operator-induced conformational change that would make the C-terminal domain move freely.

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