Subunit Dissociation Affects DNA Binding in a Dimeric *Lac* Repressor Produced by C-Terminal Deletion†

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ABSTRACT: The reduction in *apparent* operator binding affinity found for dimeric *lac* repressor proteins produced by disruption of the C-terminal coiled-coil interaction has been proposed to derive from thermodynamic linkage between dimer–monomer and protein–DNA equilibria [Brenowitz *et al.* (1991) *J. Biol. Chem. 266,* 1281]. To explore this linkage, we have employed two dimeric proteins, a deletion mutant (–11 aa) missing 11 amino acids at the C-terminus that has diminished *apparent* operator binding affinity [Chen & Matthews (1992) *J. Biol. Chem. 267,* 13843] and a mutant (R3) that binds to operator with wild-type affinity in which the C-terminal leucine heptad repeats of *lac* repressor were replaced by the GCN4 dimerization sequence [Alberti *et al.* (1993) *EMBO J. 12,* 3227; Chen *et al.* (1994) *J. Biol. Chem.* (in press)]. To avoid the complexities of working at the low concentrations of protein required by the high affinity between the monomer subunits, urea denaturation studies were undertaken to determine the free energy change(s) for dissociation and/or unfolding. Under denaturing conditions, dimer dissociation and monomer unfolding were found to be concerted processes, and the free energy change for the overall process of dimer to unfolded monomer was derived from these experiments for the two dimeric proteins. A monomeric mutant (Y282D) of the lactose repressor was examined to determine the free energy change of protomer unfolding. From the combination of these data, the K_d for –11 aa dimer dissociation was determined to be 7.7×10^{-8} M, and the corresponding value for R3 protein was 3.2×10^{-11} M. Using these dimer–monomer dissociation constants, the intrinsic equilibrium binding constant for DNA could be derived by fits to operator binding isotherms; for both –11 aa and R3, these values were very similar and are comparable to the corresponding apparent equilibrium dissociation constant for the wild-type protein under the conditions employed ($\sim 10^{-10}$ M). These data demonstrate directly that the monomer–dimer and dimer–operator equilibria are thermodynamically linked for the –11 aa dimer and presumably for other dimeric proteins that display reduced *apparent* operator binding. Linkage of protein–protein, protein–DNA, and protein–ligand binding is an essential feature of genetic regulatory proteins.

The regulatory function of *Escherichia coli lac* repressor relies on modulation of its interaction with operator DNA by inducers. As a tetramer composed of identical subunits, the *lac* repressor has four inducer binding sites and two DNA binding sites (Riggs & Bourgeois, 1968; Barkley *et al.*, 1975; O'Gorman *et al.*, 1980a,b; Culard & Maurizot, 1981, 1982; Whitson & Matthews, 1986). The protein can be dissected into two principal structural domains: the N-terminal 59 residues contain a helix–turn–helix motif that confers a large portion of the DNA binding capacity (Adler *et al.*, 1972; Platt *et al.*, 1973; Lin & Riggs, 1975; Lamerichs *et al.*, 1989), and the remaining core protein contains the inducer binding site and assembly determinants (Platt *et al.*, 1973; Müller-Hill, 1975; Schmitz *et al.*, 1976; Miller, 1979; Miller *et al.*, 1979; Lehming *et al.*, 1988; Kleina & Miller, 1990; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a). Specific interaction has been observed between two adjacent N-termini and two half-sites on a symmetric operator DNA sequence (Ogata & Gilbert, 1979; Lamerichs *et al.*, 1989; Lehming *et al.*, 1990; Kisters-Woike *et al.*, 1991).

Studies in dilute solutions by small-angle X-ray and neutron scattering have revealed an elongated cylindrical shape for the repressor molecule (Pilz *et al.*, 1980; Charlier *et al.*, 1980, 1981; McKay *et al.*, 1982) with the two pairs of N-termini deduced to be located at opposite ends of the elongated molecule (Pilz *et al.*, 1980; Charlier *et al.*, 1981; McKay *et al.*, 1982). Assembly of the tetrameric repressor involves two distinctive subunit interfaces that are experimentally separable (Chen *et al.*, 1994a), one contributed at least in part by the Tyr^{282} region (Schmitz *et al.*, 1976; Daly & Matthews, 1986) and the other by the C-terminal leucine heptad repeats (Figure 1) (Lehming *et al.*, 1988; Oehler *et al.*, 1990; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a; Alberti *et al.*, 1993). Thus, two types of dimeric *lac* repressors can be obtained, designated "short-axis" dimer and "long-axis" dimer (Chen *et al.*, 1994a). The leucine heptad repeats at the repressor C-terminus are postulated to assume a coiled-coil structure that is primarily responsible for the subunit interactions resulting in tetramer formation (Oehler *et al.*, 1990; Chakerian *et al.*, 1991; Alberti *et al.*, 1991; Chen & Matthews, 1992a; Alberti *et al.*, 1993; Chen *et al.*, 1994a). Disruption or deletion of this coiled-coil structure results in dimeric repressors (Lehming *et al.*, 1988; Oehler *et al.*, 1990; Chakerian *et al.*, 1991; Alberti *et al.*, 1991; Chen & Matthews, 1992a) that are short-axis dimers.

Recent work reported by Müller-Hill and colleagues (Alberti *et al.*, 1993) has suggested that the leucine heptad repeats may be arranged in a 4-helical bundle structure with antiparallel orientation of helices. Substitution of the *lac* repressor C-terminal sequence with leucine heptad repeats from GCN4 resulted in a dimeric repressor as assessed by

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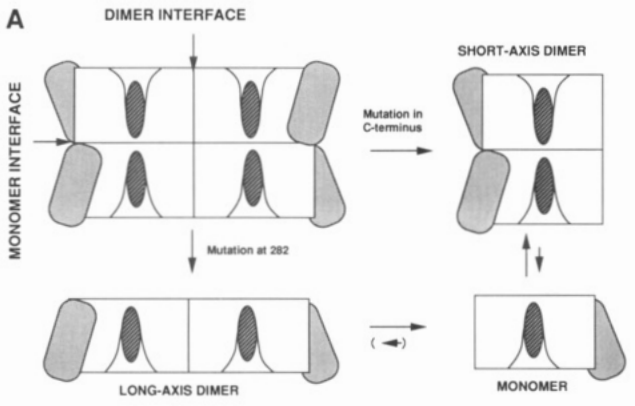

B

	340	360
Wild-type	RALADSLMQLARQVSRLESGQ	
-11 aa	RALADSLMQL	
R3	RALEDKVEELLSKNYHLENEVARLKKLESGQ	

FIGURE 1: (A) Diagrammatic illustration of proposed quaternary assembly of *Lac* repressor. The wild-type tetramer is composed of four identical subunits, each comprised by N-terminal (stippled regions) and core domains. Inducer binding sites are indicated by cross-hatching. The monomer–monomer interface involves Tyr^{282}, a site at which substitution results in monomeric repressor (Schmitz *et al.*, 1976; Chakerian & Matthews, 1991). The C-terminal region, containing leucine heptad repeats that are presumed to form a coiled-coiled domain, is required for the dimer interface, as disruption of this region results in a short-axis dimer (Lehming *et al.*, 1988; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a; Alberti *et al.*, 1993; Chen *et al.*, 1994b). Disruption of the monomer interface by mutation at Y282 combined with elongation of the coiled-coil sequence by additional leucine heptad repeats resulted in formation of a long-axis dimer (Chen *et al.*, 1994a). (B) C-Terminal sequence of wild-type, –11 aa, and R3 repressors. The leucines in heptad repeat arrangement are in boldface. The –11 aa mutant was generated by deleting the last 11 amino acids of wild-type sequence (Chen & Matthews, 1992a). The R3 mutant has the leucine heptad repeat sequence of GCN4 in place of the corresponding wild-type sequence (Alberti *et al.*, 1993).

gel-mobility shift assays of DNA binding (Alberti *et al.*, 1993) and by characterization of the purified protein (Chen *et al.*, 1994b). This protein displays altered cooperativity for inducer binding, but it binds to DNA with wild-type affinity and has a Stokes radius similar to short-axis dimers; these characteristics suggest that the ability to form a stable parallel coiled-coil structure at the C-terminus via the GCN4 sequences alters subunit communication in this protein (Chen *et al.*, 1994b). This behavior contrasts strongly to the family of dimeric repressors with deletions or substitutions at the C-terminus, which have 30- to 60-fold decreased apparent operator DNA affinity compared to the tetrameric wild-type repressor and display wild-type cooperativity for inducer binding (Chen & Matthews, 1992a).

A similar decrease in apparent DNA affinity was observed by Brenowitz and co-workers for another dimeric repressor ($lacI^{adi}$) lacking 31 amino acids at the C-terminus with 15 added missense amino acids (Lehming *et al.*, 1988; Oehler *et al.*, 1990; Brenowitz *et al.*, 1991). Brenowitz *et al.* (1991) proposed that the decreased apparent operator affinity displayed by the dimeric $lacI^{adi}$ repressor is derived from weakened monomer–monomer interaction in the dimer and consequent thermodynamic linkage of this equilibrium with dimer–operator binding; thus, the *intrinsic* affinity of operator is the same for tetrameric and dimeric repressors. Here we present strong evidence to support this hypothesis by studying two dimeric *lac* repressors differing in their C-terminal subunit interactions: the –11 aa mutant (Chen & Matthews, 1992a) which lacks the sequences that form the presumed coiled-coil structure, and the R3 mutant (Alberti *et al.*, 1993) which has the GCN4 sequence that forms a stable, dyadic, parallel coiled-coil structure.

MATERIALS AND METHODS

Plasmid and Bacterial Strains. Plasmid pJC1 (Chen & Matthews, 1992a), containing the wild-type or –11 aa mutant *lac* I gene with the I^q promoter, was used as an expression vector for the repressor proteins. Plasmid pMLR-R3 encoding the *lac* repressor mutant R3 was a generous gift from Dr. Siegfried Alberti in Prof. Benno Müller-Hill's laboratory (Alberti *et al.*, 1993). Bacterial strain *E. coli* TB-1 [ara, Δ(*lac-pro*), StrA, thi, ϕ80d*lacZ*ΔM15r$^-$, m$^+$] was used as the host to express all repressor proteins.

Purification of Repressors. Isolation and purification of the wild-type and –11 aa mutant repressor proteins from *E. coli* TB-1 cells were as described previously (Chen & Matthews, 1992a). The R3 mutant protein expressed in TB-1 cells was purified as described previously (Chen *et al.*, 1994b). Operator DNA binding activities for all proteins were >85% as measured by nitrocellulose filter binding under stoichiometric conditions.

Operator Binding Assays and Data Analysis. A 95 bp double-stranded operator DNA was employed in the operator binding analysis. This fragment was isolated from the plasmid pLA322-8 by treatment with *Eco*RI and *Bam*HI (Manly & Matthews, 1984) and was labeled at the *Eco*RI end with [^{32}P] by fill-in reactions. This labeled DNA was used to study operator binding of the repressors. The assay to determine repressor–operator binding equilibrium constants was carried out at room temperature in 0.01 M Tris-HCl (pH 7.5) and 0.1 M K_2SO_4, with 2×10^{-11} M [^{32}P]operator, 100 μg/mL bovine serum albumin, 2 μg/mL calf thymus DNA, and varying concentrations of repressor protein. Samples were loaded on 8% polyacrylamide gels containing TAE (0.04 M Tris–acetate, pH 8.0, 0.001 M EDTA), and electrophoresis was conducted at 90 V for 1.5 h. Quantitation of the repressor–DNA complex band and free DNA band following electrophoretic separation was performed on a Fuji phosphorimager. Raw data were collected as the ratio of the intensity of the bound or free DNA band to the total intensity in each lane. The raw data were analyzed mathematically to confirm the apparent end points of the titration curves (Brenowitz *et al.*, 1990). The end points obtained by this analysis did not differ from those derived from direct inspection of the binding curves. The data were then converted to fraction bound or unbound DNA (R) and fit to various models using the program Sigmaplot 4.0 by nonlinear least-squares analysis. Binding curves assuming a simple equilibrium were fit to eq 1 (DNA bound) and eq 2 (free DNA), where K_d is the apparent

$$R = [P]/(K_d + [P]) \quad (1)$$

$$R = K_d/(K_d + [P]) \quad (2)$$

equilibrium dissociation constant and [P] is the total protein concentration in dimer.

When assuming a linkage between the dimer dissociation ($2M \rightleftharpoons M_2$) and operator binding ($M_2 + O \rightleftharpoons M_2O$) equilibria, the following equations were employed:

$$K_1 = [M]^2/[M_2] \quad (3)$$

$$K_2 = [M_2][O]/[M_2O] \quad (4)$$

$$[P] = [M]/2 + [M_2] + [M_2O] \quad (5)$$

where K_1 is the dimer–monomer dissociation constant, K_2 is the dimer–operator dissociation constant, [P] is the total protein concentration in dimer, and [M], [M_2], [M_2O], and [O] are the concentrations of monomer, dimer, bound dimer, and free operator, respectively. The following eqs are derived from eqs 3–5 and were used to analyze the bound DNA and free DNA data, respectively:

$$R = (((K_1 + 16[P])^{0.5} - K_1^{0.5})/4)^2/ (((((K_1 + 16[P])^{0.5} - K_1^{0.5})/4)^2 + K_2) \quad (6)$$

$$R = K_2/((((K_1 + 16[P])^{0.5} - K_1^{0.5})/4)^2 + K_2) \quad (7)$$

IPTG-Binding Assays. During protein isolation and purification and for assessment of denaturation, the activity of repressor was measured by [^{14}C]IPTG binding detected by ammonium sulfate precipitation methods (Bourgeois, 1971).

Denaturation of Protein. The denaturation experiments were conducted in 0.1 M K_2SO_4 and 0.01 M Tris-HCl (pH 7.5) using urea as the denaturant. Ultrapure urea (Gibco BRL) was used to make a 7 M stock solution by adding 0.143 M K_2SO_4 and 0.014 M Tris-HCl (pH 7.5). This stock solution was made daily and filtered before use. Completion of the denaturing reactions was ensured by incubation at room temperature for 2–3 h. The denatured samples were examined by the following: (1) fluorescence intensity measurements made on an SLM AMINCO 8100 Series 2 spectrofluorometer with excitation at 285 nm using a 340-nm cutoff filter (Corning) to measure emission; (2) circular dichroic measurements made on an Aviv 62DS CD spectrometer (Dr. James C. Lee's laboratory, University of Texas Medical Branch at Galveston, Texas).

Unfolding Data Analysis. The unfolding data obtained from spectroscopic measurements were treated by linear extrapolation of the pre- and posttransition states (Greene & Pace, 1974; Pace, 1975) and converted to the form of fraction unfolded (F_u), using the equation

$$F_u = (Y_f - Y)/(Y_f - Y_u)$$

where Y_f and Y_u are intensities of the folded and unfolded states, respectively. The data were fit using a nonlinear least-squares program (Sigmaplot 4.0) to either a model assuming a two-state transition from repressor dimer to unfolded monomer ($M_2 \rightleftharpoons 2U$) in the cases of dimer denaturation:

$$F_u = ((K^2 + 8K[P])^{1/2} - K)/4[P]$$

or a model of monomer unfolding ($M \rightleftharpoons U$) in the case of monomer denaturation:

$$F_u = K/(1 + K)$$

where [P] is the total protein concentration in monomer, K is the equilibrium constant of unfolding which is related to the free energy change for unfolding (ΔG) by $\Delta G = -RT \ln K$. The free energy change for unfolding in the absence of denaturant ($\Delta G°$) can be derived from linear extrapolation of the free energy change of unfolding in the presence of various concentrations of denaturant (Aune & Tanford, 1969; Greene & Pace, 1974; Pace, 1975):

$$\Delta G = \Delta G° + a[\text{urea}]$$

RESULTS

Denaturation of −11 aa and R3 Dimers Is a Two-State Transition. Two dimeric repressors, −11 aa in which part of the leucine heptad repeat sequence was eliminated (Chen & Matthews, 1992a) and R3 in which the GCN4 leucine heptad repeat sequence replaced the corresponding wild-type sequence (Alberti *et al.*, 1993), were employed in this study (Figure 1). R3 repressor displays operator affinity comparable to the wild-type protein but no cooperativity for inducer binding, while −11 aa exhibits decreased apparent affinity for operator sequences and wild-type cooperativity for inducer binding (Chen & Matthews, 1992a; Chen *et al.*, 1994b). In order to compare directly subunit dissociation in these two dimers, we carried out denaturation experiments on these proteins using urea. Fluorescence intensity measurement was the primary method employed to follow the process of protein denaturation. As shown in Figure 2, the fluorescence emission spectra for the −11 aa protein at increasing urea concentrations are similar in shape and wavelength of maximum emission; these spectra differ only in intensity. Similar results were obtained for the R3 protein (data not shown). Therefore, the fluorescence emission intensity was measured by using a 340-nm cutoff filter to monitor the denaturation process. Denaturation of the proteins was at least 90% reversible, as shown in Figure 3 for the −11 aa protein, and the curves were very reproducible over time and with different protein preparations.

Protein concentration dependence was observed for the denaturation of both −11 aa and R3 (Figure 4, panels A and B, respectively). The data were fit by a two-state transition model very well, as shown by the curves generated by simultaneous fits of data at different protein concentrations (Figure 4). The dependence of the process on protein concentration indicates that the dimer to monomer transition must be involved in the process being detected by fluorescence intensity measurements. However, evidence that indicates that the process monitored is not merely dimer dissociation came from monitoring IPTG binding activity at various urea concentrations (Figure 5). Dissociation from dimer to folded monomer should not result in loss of inducer binding capacity, as monomeric mutants of the repressor bind to IPTG with wild-type affinity at neutral pH (Daly & Matthews, 1986). Loss of IPTG activity that parallels the decrease of fluorescence intensity during denaturation of the −11 aa protein (Figure 5) supports the conclusion that the entire transition of dimer to unfolded monomer is being observed by the fluorescence. Thus, a multistep process is being monitored by the fluorescence intensity change: dimer dissociation and monomer unfolding, with no resolution of these two events. The more rapid loss in IPTG binding activity at low urea concentrations may indicate direct effects of urea on sugar binding, but the correspondence at higher urea concentrations is consistent with the observation of multiple equilibria by fluorescence. Circular dichroism spectra at increasing concentrations of urea are shown in Figure 6A for −11 aa protein. The helical structure that remains even at high concentrations of urea appears to correspond to the N-terminal domain, as indicated by previous studies of wild-type tetramer unfolding by urea (Schnarr & Maurizot, 1981). Loss of secondary structure monitored at 222 nm is approximately concordant with the fluorescence intensity change (Figure 6B) and consistent with concerted dissociation and unfolding processes.

R3 Is More Tightly Associated Than −11 aa. R3 protein requires a higher concentration of urea to undergo a similar

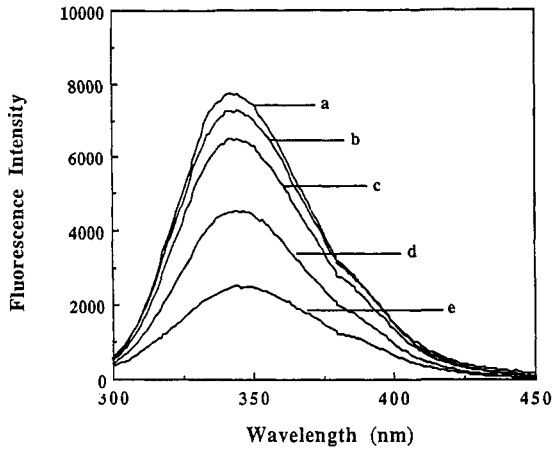

FIGURE 2: Fluorescence emission spectra for −11 aa dimer at various urea concentrations. The denaturation conditions were as described in Materials and Methods. The protein concentration was 1.3 μM monomer. The urea concentrations were (a) 0 M, (b) 2.0 M, (c) 3.0 M, (d) 3.5 M, and (e) 4.0 M.

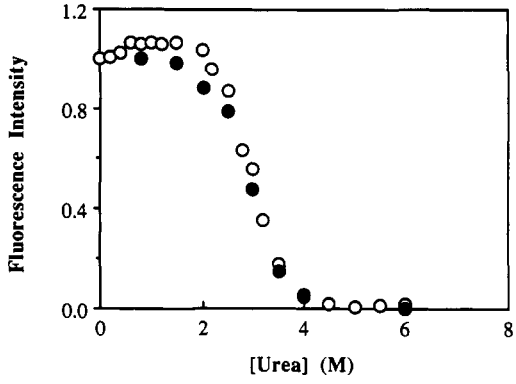

FIGURE 3: Reversibility of −11 aa denaturation. The −11 aa protein fully denatured in 6 M urea was diluted to lower urea concentrations to allow renaturation. (○) Denaturation; (●) renaturation. The protein concentration at each point was 0.26 μM monomer. The fluorescence intensity was normalized so that the intensity in the absence of urea was always "1".

fluorescence transition, suggestive that the subunits are more tightly associated than for the −11 aa deletion dimer. The difference between −11 aa and R3 denaturation is significant when curves at the same protein concentration are compared (Figure 7). The free energy changes for dimer to unfolded monomer derived from fitting the data to a two-state model for both proteins are summarized in Table 1. A substantial difference of 4.6 kcal/mol in free energy change for dissociation/unfolding is observed between these two proteins. The minor sequence difference in these two proteins should not give rise to any significant difference in the folding of their subunits except for the subunit interface; in fact, their identical capacity for IPTG binding at neutral pH indicates very similar tertiary structures in individual subunits. Therefore, we conclude that the observed difference in free energy change of dissociation/unfolding in −11 aa and R3 is mainly contributed by the difference in their subunit association rather than unfolding of individual subunits. In other words, the subunits in R3 are more tightly associated than in −11 aa.

Thermodynamically, the free energy change for going from dimer to unfolded monomer should be the sum of the free energy changes for dimer dissociation and monomer unfolding, although these two processes are not physically uncoupled in the dimer repressors under the denaturation conditions employed. Under nondenaturing conditions, we assume that folded monomers are present and stable, since point mutations

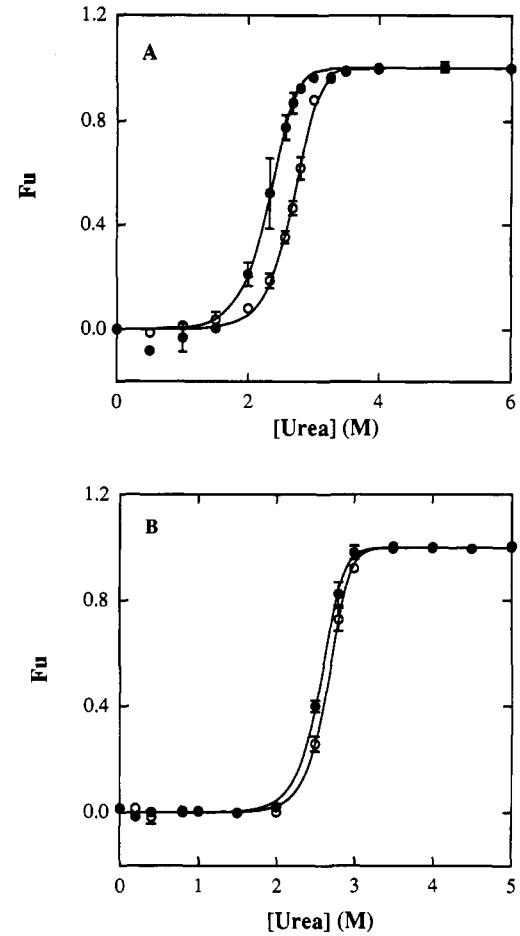

FIGURE 4: Denaturation of −11 aa and R3 repressors measured by fluorescence intensity. The experiments and data analysis were carried out as described in Materials and Methods. Each set of data was the average of 3 independent experiments; standard deviations greater than the radii of the data points are shown as error bars. The curves were generated from simultaneous fitting of the data at two protein concentrations to the two-state unfolding model. The protein concentrations in monomer are (A) 0.26 μM (●) and 1.3 μM (○) for −11 aa and (B) 0.53 μM (●) and 1.3 μM (○) for R3.

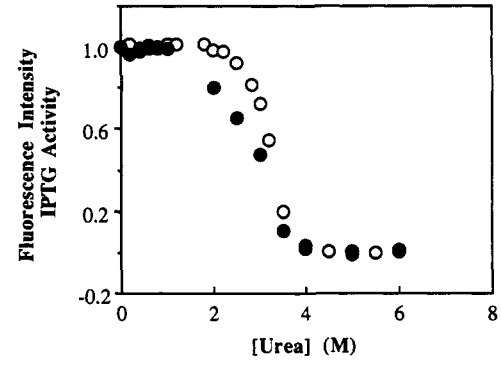

FIGURE 5: Denaturation of −11 aa measured by IPTG activity. The protein concentration was 1.3 μM monomer. IPTG activity was assayed by ammonium sulfate precipitation methods as described in Materials and Methods. Both fluorescence intensity (○) and IPTG activity (●) were normalized so that the values in the absence of urea were always "1".

can generate monomer proteins (Schmitz *et al.*, 1976; Chakerian & Matthews, 1991; Chen & Matthews, 1992b). In order to estimate the free energy change for dimer dissociation, we investigated the unfolding of one of these point mutants of *lac* repressor, Y282D (Chen & Matthews, 1992b). The Y282D protein is a monomer at concentrations up to 8×10^{-5} M (Chen *et al.*, 1994a) and is functionally

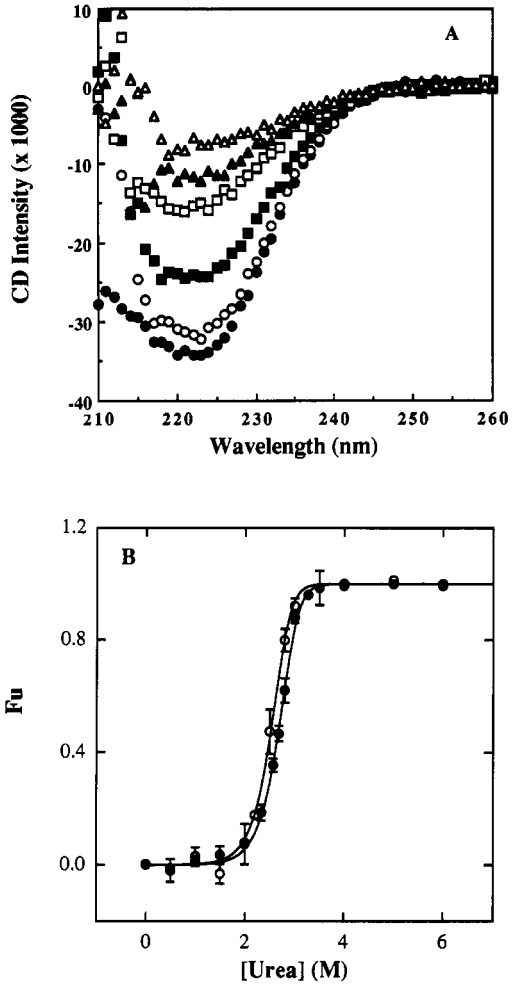

FIGURE 6: Denaturation of −11 aa monitored by circular dichroism. The protein concentration was 1.3 μM monomer. (A) CD spectra were measured at increasing concentrations of urea: (●) 0 M; (○) 2.0 M; (■) 2.5 M; (□) 3.0 M; (▲) 4.0 M; (△) 6.0 M. (B) The CD intensity at 222 nm was transformed into fraction unfolded (○) as described in Materials and Methods; the fluorescence data (●) were taken from Figure 4A. Each set of data was the average of 3 independent experiments; the standard deviations greater than the radii of the data points are shown as error bars. The curves were generated from fitting of the data to the two-state unfolding model. The free energy change obtained from CD measurements was 19.2 ± 0.8 kcal/mol, very similar to the ΔG° of 19.3 ● 1.4 kcal/mol estimated from fluorescence measurements.

indistinguishable from the subunits in a variety of tetrameric and dimeric repressors based on studies of ligand binding (Schmitz *et al.*, 1976; Daly & Matthews, 1986; Chen & Matthews, 1992a,b; Chen *et al.*, 1994a). This mutant protein therefore serves as a reasonable model for individual subunit unfolding in the dimer dissociation/denaturation process. Denaturation of the Y282D protein is fit by a simple monomer unfolding model very well (Figure 8), with a free energy change for unfolding of 4.8 kcal/mol (Table 1). Using this value as the free energy change for subunit unfolding for the dimers, the equilibrium constants of dimer dissociation are deduced to be 7.7×10^{-8} M for −11 aa and 3.2×10^{-11} M for R3.

Thermodynamic Linkage of Protein Dissociation and DNA Binding. According to the hypothesis of thermodynamic linkage between the dimer–operator association and monomer–monomer association (Brenowitz *et al.*, 1991), the 2000-fold higher dissociation constant for monomers in the −11 aa protein compared to the R3 protein should be sufficient to account for the lower operator affinity. In order to investigate this linkage directly, we performed operator binding assays using

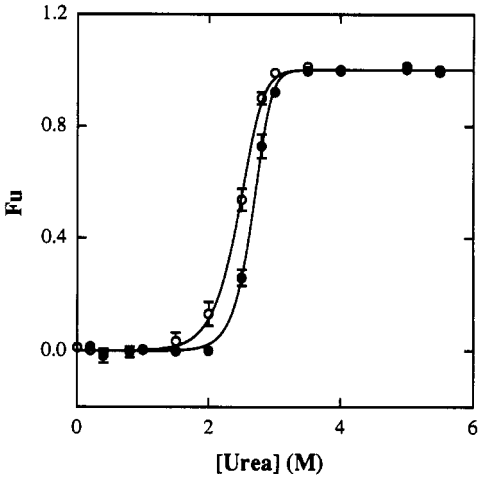

FIGURE 7: Comparison of denaturation of −11 aa and R3. The denaturation processes were monitored by fluorescence intensity, and data are presented as fractional fluorescence intensity decreases. Data for the two proteins were compared at a protein concentration of 1.3 μM monomer. Both curves were generated by fitting the data to the two-state model as described in Materials and Methods and shown in Figure 4. (○) −11 aa; (●) R3. The free energy changes thus estimated are summarized in Table 1.

Table 1: Thermodynamic Parameters Derived from Denaturation Experiments

repressor	$\Delta G^\circ_{D\text{-}U}$[a] (kcal/mol)	$\Delta G^\circ_{M\text{-}U}$[b] (kcal/mol)	$\Delta G^\circ_{D\text{-}M}$[c] (kcal/mol)	K_d[d] (M)
Y282D		4.8 ± 0.3		
−11 aa	19.3 ± 1.4		9.7	7.7×10^{-8}
R3	23.9 ± 1.0		14.3	3.2×10^{-11}

[a] The free energy change for dimer to unfolded monomer was derived from fitting the fluorescence data shown in Figure 4 to the two-state model described in Materials and Methods. [b] The free energy change for unfolding for the Y282D monomer was derived from fitting the data shown in Figure 8 to the simple monomer unfolding model described in Materials and Methods. [c] The free energy change for dimer dissociation was deduced from the difference between the free energy change for dimer to unfolded monomer and twice the free energy change for Y282D monomer unfolding. [d] The dimer dissociation constant was calculated from $\Delta G^\circ_{D\text{-}M}$ using the following function: $\Delta G^\circ = -RT \ln K_d$.

gel retardation under the same buffer conditions as employed for the denaturation experiments; i.e., 0.1 M K_2SO_4 was present in the buffer. As summarized in Table 2, the wild-type repressor binds operator with an observed K_d consistent with the ionic strength of the K_2SO_4-containing buffer (Whitson & Matthews, 1986). It should be noted that the oligomeric state of the wild-type protein at the low concentrations required for DNA binding analysis is unknown and may include dimer and/or monomer species as well as tetramer (Royer *et al.*, 1986). Detailed analysis of these potentially complex equilibria is beyond the scope of this study, and we have included the wild-type data for comparative purposes only.

The R3 protein displays an operator affinity similar to the apparent affinity for wild-type repressor, while the −11 aa protein has a 10-fold lower apparent affinity compared to those of wild-type and R3 proteins. We analyzed the operator binding data (Figure 9) taking the dimer dissociation into consideration, assuming the presence of folded monomer under the conditions of the assay, and assuming the values of dimer–monomer dissociation constants obtained by the denaturation analysis. The intrinsic operator binding constants obtained from this analysis are summarized in Table 2. For the −11 aa protein, this consideration of subunit dissociation generates an intrinsic DNA binding affinity very close to the apparent affinity observed for R3 and wild-type proteins. As expected

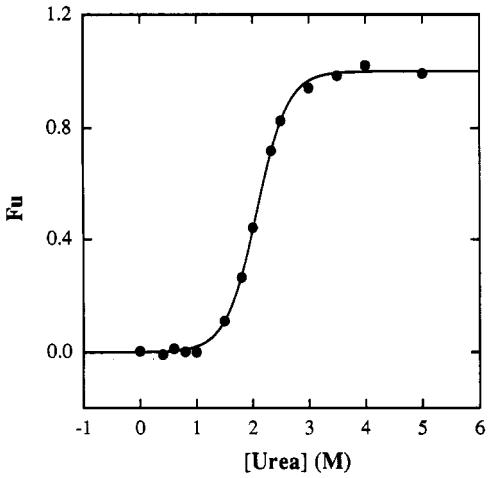

FIGURE 8: Denaturation of Y282D monomer monitored by fluorescence intensity. The experiments and data analysis were carried out as described in Materials and Methods. The data are presented as fractional fluorescence intensity decreases. The protein concentration was 0.26 μM monomer. The data shown are the combination of the results from 3 independent experiments. The curve was generated by fitting the data to the monomer unfolding model, and the ΔG° value derived is shown in Table 1.

Table 2: Summary of Operator Binding Data Analysis

repressor	K_{app}[a] (M)	K_1[b] (M)	K_2[c] (M)
wild-type	$(0.94 \pm 0.10) \times 10^{-10}$		
−11 aa	$(10.60 \pm 1.43) \times 10^{-10}$	7.7×10^{-8}	$(0.60 \pm 0.12) \times 10^{-10}$
R3	$(1.20 \pm 0.09) \times 10^{-10}$	3.2×10^{-11}	$(0.92 \pm 0.08) \times 10^{-10}$

[a] The apparent repressor–operator dissociation constants were determined in 0.1 M K_2SO_4 and 10 mM Tris-HCl (pH 7.5). [b] The dimer–monomer dissociation constants are derived from denaturation experiments as reported in Table 1. [c] The intrinsic repressor–operator dissociation constants are generated by fitting the operator-binding data (Figure 9) to a model describing linked dimer dissociation and DNA binding equilibria, assuming the values for dimer dissociation constants obtained from Table 1.

for the R3 protein, consideration of the monomer–dimer equilibrium does not affect the value for DNA affinity significantly, because 50% subunit association occurs at a concentration (3.2×10^{-11} M) lower than the concentration at which half-saturation of operator binding occurs (9.2×10^{-11} M).

DISCUSSION

Protein–protein interactions as well as protein–DNA interactions are fundamental for the molecular mechanism of transcriptional regulation. A thermodynamic linkage between these two types of interactions, despite structural independence of these processes, may play an essential role in many regulatory systems. As an example, although the domains for association and DNA binding are structurally independent, the monomer–dimer self-association of *gal* repressor appears to be linked directly to its operator binding as suggested by Brenowitz and colleagues based on the analysis of *gal* repressor–operator binding data (Brenowitz *et al.*, 1990). Dimeric *lac* repressors formed upon disruption of the C-terminal coiled-coil interaction generally display decreased operator binding affinity compared to that of the wild-type tetrameric repressor (Brenowitz *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a). Since these dimers retain one of the two operator binding sites in wild-type *lac* repressor (Ogata & Gilbert, 1979; Lamerichs *et al.*, 1989; Lehming *et al.*, 1990; Kisters-Woike *et al.*, 1991), the intrinsic operator affinity for the dimers would be expected to be the

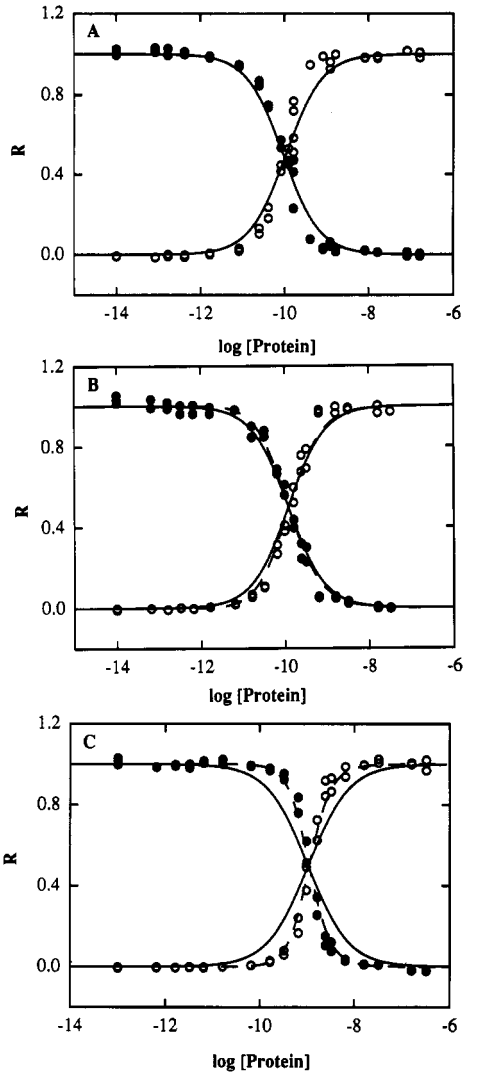

FIGURE 9: Operator binding data analysis. The operator binding experiments were carried out by gel retardation analysis in 0.1 M K_2SO_4 and 10 mM Tris-HCl (pH 7.5) as described in Materials and Methods. Protein concentrations are in the units of M dimer. The repressors are (A) wild-type, (B) R3, and (C) −11 aa. The data points shown are the combination of 4 separate determinations. (○) Protein–DNA complex; (●) free DNA. The solid curves were generated by fitting the data to a simple DNA–repressor binding model (eqs 1 and 2); the dashed curves in panels B and C show the best fits of the data to a model taking dimer dissociation into consideration (eqs 6 and 7), with dimer–monomer dissociation constants of 7.7×10^{-8} M for −11 aa, and 3.2×10^{-11} M for R3 as determined by the urea denaturation measurements (Table 1). The wild-type data were analyzed assuming a simple equilibrium; although improvement in fits might be obtained taking protein dissociation equilibria into account, the data to undertake such an analysis are not available.

same as that for the wild-type tetramer. As proposed by Brenowitz *et al.* (1991), the decreased operator affinity observed for these dimeric repressors may be derived from dimer–monomer dissociation of these proteins at the low protein concentrations in the operator binding assays to generate a thermodynamic linkage between the dimer protein dissociation and protein–DNA association. Analysis and comparison of two dimeric repressors, −11 aa and R3, have provided convincing evidence for this hypothesis.

Müller-Hill and colleagues generated the R3 mutant repressor by replacing the C-terminal leucine heptad repeats in *lac* repressor with those from GCN4, and they concluded that this mutant protein was a dimer based on the absence of looped complex formation by the mutant cell extract (Alberti

et al., 1993). Investigation of the purified R3 protein by gel-filtration chromatography indicated that this protein is a short-axis dimer (Chen *et al.*, 1994b) with the same mode of subunit assembly (Figure 1) as the −11 aa protein, one of the family of short-axis dimers (Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a). Interestingly, R3 displays an operator affinity much higher than −11 aa and the other short-axis dimers. The sequence difference between R3 and −11 aa suggests that while −11 aa dimerizes only through the Tyr^{282} interface because of deletion of the C-terminal coiled-coil sequence, the subunit interaction in the R3 dimer may occur via both the Tyr^{282} region and a dyadic C-terminal coiled-coil mediated by GCN4 sequences. On the basis of this added interaction, the R3 dimer would be expected to be more strongly associated than −11 aa protein. The $\Delta\Delta G^{\circ}_{D\text{-}U}$ for −11 aa *vs* R3 of 4.6 kcal/mol is in the lower range of estimates for the ΔG° for association of GCN4 leucine heptad repeat sequences (5–10 kcal/mol; Thompson *et al.*, 1993; O'Shea *et al.*, 1991; C. Carr and P. Kim, personal communication). The effects of the R3 mutation on cooperativity of IPTG binding at elevated pH and in the presence of operator are consistent with tighter association of monomers, which may constrain the subunits and thus influence subunit communication and interactions (Chen *et al.*, 1994b).

Measurement of dimer–monomer dissociation for the −11 aa and R3 proteins directly in solution is precluded by the low concentrations of protein required and insufficient sensitivity in protein detection. By utilizing denaturation by urea as a means to observe dissociation linked with unfolding, it has been possible to bypass these problems. Quantitative analysis of data for urea denaturation relies entirely on the assumption of linear extrapolation (Aune & Tanford, 1969; Greene & Pace, 1974; Pace, 1975) which requires ideal behavior of the protein upon denaturation. The *lac* repressor displays a certain degree of resistance to urea denaturation as monitored by circular dichroism, as we observed and as reported previously by Schnarr and Maurizot (1981); the latter have interpreted the residual circular dichroism signal as incomplete unfolding of the N-terminal domain at high urea concentrations. We have utilized fluorescence of tryptophans in the protein (W201 and/or W220) to monitor events in the core domain, which is involved in subunit assembly (Schmitz *et al.*, 1976; Daly & Matthews, 1986; Oehler *et al.*, 1990; Chakerian & Matthews, 1991; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a,b; Alberti *et al.*, 1993; Chen *et al.*, 1994a). Using this approach, dissociation/denaturation of the proteins examined is largely reversible, and the concerted process is fit well by a two-state transition model. Combining this information with data extracted from the denaturation of a monomeric repressor (Y282D) and assuming the presence of folded monomer under nondenaturing conditions, we have been able to estimate the subunit dissociation constants for these dimeric repressors.

A key factor to ensure validity of the comparison of subunit dissociation between R3 and −11 aa is the efficacy of the Y282D monomer as a model for the subunits in both dimers. Since the −11 aa protein displays an identical denaturation curve compared to the L349A mutant (Chakerian *et al.*, 1991) (data not shown), a dimeric repressor with the same DNA affinity as −11 aa and a full-length C-terminus, the deleted C-terminus does not seem to contribute significantly to the overall free energy change for dissociation/unfolding. Thus, we presume that the 10 amino acid extension of the R3 C-terminus does not play a significant role in terms of subunit unfolding. We deduce that the observed difference in denaturation between R3 and −11 aa is derived mainly from the contribution of the sequence alterations to subunit association rather than monomer folding. The estimation of subunit affinity from the urea denaturation data reveals a stronger subunit association for the R3 dimer by 2000-fold, in good agreement with the hypothesis that the decreased operator affinity of −11 aa is due to a weakened monomer–dimer association (Brenowitz *et al.*, 1991). Analysis of the operator binding data incorporating the subunit dissociation information yielded an intrinsic operator–protein dissociation constant almost identical for both proteins and corresponding closely to the apparent wild-type dissociation constant. These results confirm the thermodynamic linkage between subunit dissociation and protein–DNA equilibria in the dimeric repressor mutants.

Linkage between protein–protein, protein–DNA, and protein–ligand equilibria mediated by structurally independent domains is a requirement of many genetic regulatory systems that must be responsive to cellular and extracellular environmental signals. The behavior of two different dimeric variants of the lactose repressor explored in this study demonstrates that the parameters for intersubunit interaction have significant consequences for the functional affinity of the oligomeric form for the target site on DNA. This energetic interconnection between the multiple binding reactions in which multivalent oligomeric proteins participate determines the physiological outcome for the organism. Small changes in single parameters may have significant consequences for the regulatory function in the cell. The thermodynamic linkage between different functions that are structurally independent provides the potential for generating a broad range in cellular response. Measurement of binding parameters for specific reactions and understanding the energetic as well as structural interconnections between these processes will provide further insight into the crucial process of genetic regulation.

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