THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 17, Issue of April 29, pp. 12482-12487, 1994
Printed in U.S.A.

Wild-type Operator Binding and Altered Cooperativity for Inducer Binding of *lac* Repressor Dimer Mutant R3*

(Received for publication, January 12, 1994)

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Substitution of the C-terminal leucine heptad repeat region of the normally tetrameric lactose repressor by the leucine heptad repeat dimerization domain of GCN4 protein resulted in cell extracts containing protein, designated R3, which behaved as a dimer based on gel retardation analysis of DNA binding (Alberti, S., Oehler, S., von Wilcken-Bergmann, B., and Müller-Hill, B. (1993) *EMBO J.* 12, 3227-3236). We have purified this R3 protein and characterized its properties in comparison with the wild-type repressor. R3 protein elutes from a molecular sieve with a Stokes radius characteristic of a dimer and a deduced molecular mass of 66 kDa. Unlike other dimeric repressors, produced by deletion or mutation in the leucine heptad repeat region, which display reduced apparent operator affinity, R3 binds to operator DNA sequences with wild-type equilibrium and kinetic properties. Although inducer affinity at neutral pH is similar for R3 and wild-type protein, at elevated pH the R3 protein undergoes a slightly smaller decrease in affinity and exhibits minimal cooperativity in sugar binding compared with the wild-type protein. Interestingly, in the presence of operator DNA, a state in which inducer binding to wild-type repressor is also of reduced affinity and slightly cooperative, R3 binding affinity is decreased to a greater extent, and the protein displays higher cooperativity than wild-type repressor. Consistent with inducer binding data in the presence of operator, the release of operator from R3 protein requires a higher sugar concentration than wild-type protein. These results are interpreted in the context of alterations involving the subunit interface which affect the allosteric behavior of the repressor protein.

The expression of proteins required for the metabolism of lactose in *Escherichia coli* is regulated by the binding of lactose repressor protein to the operator DNA sequence that lies adjacent to the promoter for the *lac* enzymes (Miller and Reznikoff, 1980). Binding to this operator DNA sequence is modulated by the interaction of *lac* repressor with inducer sugars (Miller and Reznikoff, 1980; Barkley *et al.*, 1975). The repressor protein, comprised of two principal structural domains, is a tetramer of four identical subunits which has four inducer binding sites and two DNA binding sites (Riggs and Bourgeois, 1968; Barkley *et al.*, 1975; O'Gorman *et al.*, 1980a, 1980b; Culard and Maurizot, 1981, 1982; Whitson and Matthews, 1986). Within the N-terminal 59 residues there is a helix-turn-helix motif conserved in DNA-binding proteins and for which specific interaction with operator DNA has been observed (Adler *et al.*, 1972; Lin and Riggs, 1975; Ogata and Gilbert, 1979; Lamerichs *et al.*, 1989; Lehming *et al.*, 1987, 1990; Kisters-Woike *et al.*, 1991). Residues 60–360 form a tetrameric core protein that binds to the inducer with wild-type affinity and contains the assembly determinants (Platt *et al.*, 1973; Müller-Hill, 1975; Schmitz *et al.*, 1976; Miller, 1979; Miller *et al.*, 1979; Kleina and Miller, 1990; Lehming *et al.*, 1988; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen and Matthews, 1992a).

Examination of the repressor protein by low angle x-ray and neutron scattering has demonstrated an elongated cylindrical shape for the repressor molecule (Pilz *et al.*, 1980; McKay *et al.*, 1982; Charlier *et al.*, 1980, 1981) with N termini deduced to be at opposite ends of the elongated core domain (McKay *et al.*, 1982). Two N termini at each end of the molecule are presumed to interact in a symmetric fashion with the semisymmetric operator DNA sequence (McKay *et al.*, 1982; Lamerichs *et al.*, 1989). Two experimentally separable subunit interfaces appear to be involved in the assembly of the tetrameric repressor protein as illustrated in Fig. 1*A* (Chen and Matthews, 1992a; Chen *et al.*, 1994). This separation might be anticipated based on the plane rectangular structure deduced from the examination of microcrystals (Steitz *et al.*, 1974). The region that is required for formation of "short axis" dimers in which two N termini are juxtaposed into the operator binding configuration involves polypeptide segments that encompass residues Tyr^{282} and Lys^{84} (Müller-Hill, 1975; Schmitz *et al.*, 1976; Daly and Matthews, 1986a, 1986b; Chen and Matthews, 1992b; Chang *et al.*, 1993).

The region that is involved in the formation of tetramer species from dimer (or in "long axis" dimer formation) is at the C terminus where the leucine heptad repeats essential for tetramer formation are found (Lehming *et al.*, 1988; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen and Matthews, 1992a; Chen *et al.*, 1994). These leucine heptad repeats are presumed to form a coiled-coil structure that results in the assembly of the tetramer species (Chakerian *et al.*, 1991; Alberti *et al.*, 1991, 1993; Chen and Matthews, 1992a; Chen *et al.*, 1994), as replacement or deletion of amino acids in this region yields short axis dimer proteins (Lehming *et al.*, 1988; Chakerian *et al.*, 1991; Alberti *et al.*, 1991; Chen and Matthews, 1992a). Alberti *et al.* (1993) have suggested that the C-terminal leucine heptad repeats in the lactose repressor form a four-helical antiparallel bundle (Fig. 1*C*). Consistent with this hypothesis, replacement of the heptad repeat region in the lactose repressor by the GCN4 leucine heptad repeat sequences, presumably able to form only dyadic structures (O'Shea *et al.*, 1991), resulted in a dimeric species as assayed by the inability of cell extracts containing this protein to generate looped complexes with operator DNA by gel mobility shift analyses (Alberti *et al.*, 1993). We

* This work was supported by National Institutes of Health Grant GM22441 and Robert A. Welch Foundation Grant C-576 (to K. S. M.), by a Graduiertenforderung Nordrhein-Westfalen stipend (to S. A.), and by a Bundesminister für Forschung und Technologie grant (to Professor Benno Müller-Hill). Facilities of the Keck Center for Computational Biology were used during this work. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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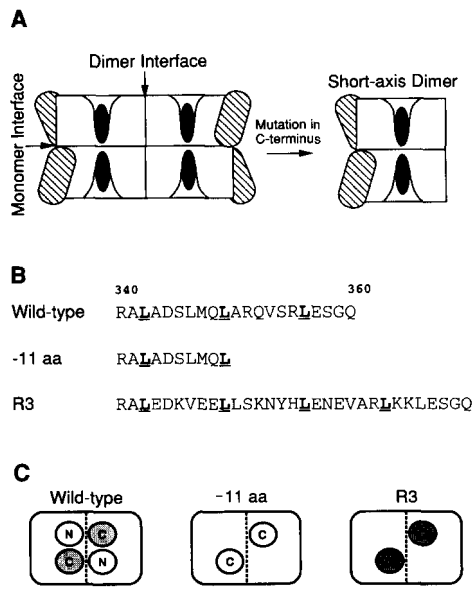

FIG. 1. *Panel A*, diagrammatic illustration of proposed assembly of dimeric and tetrameric repressors. The tetramer form of the protein is composed of four identical subunits comprised of the N-terminal (*hatched*) and core domains. The inducer binding sites are shown as *filled ovals*. The short axis dimer and tetramer structures are shown. *Panel B*, amino acid sequences of the C-terminal leucine heptad repeats in the wild-type, −11 aa, and R3 *lac* repressors. The leucines involved in the heptad repeats are in *bold* and *underlined*. The −11 aa mutant was generated by deleting the last 11 amino acids of wild-type (Chen and Matthews, 1992a). The R3 mutant bears the leucine heptad repeat sequence of GCN4 in place of the wild-type *lac* sequence (Alberti *et al.*, 1993). *Panel C*, schematic end-on view of C-terminal arrangement. The structure of the C-terminal coiled-coil region has not been determined directly in the wild-type sequences, but there is evidence that it forms a four-helical bundle (Alberti *et al.*, 1993). The dimer-dimer subunit interface is shown schematically for wild-type repressor, −11 aa, and R3 proteins. Helical regions are indicated by *circles*, with the direction of the helix denoted by *C* or *N*. For the wild-type protein, a four-helical coiled-coil bundle is shown, with an antiparallel arrangement of helices. For −11 aa, the deletion of portions of the leucine heptad repeat results in loss of interaction (*lighter shading*). For R3, the wild-type sequence is replaced with the GCN4 sequence, known to form a dyadic coiled-coil structure in parallel arrangement (O'Shea *et al.*, 1989a, 1989b, 1991). The increased strength of the subunit interface conferred by this replacement is indicated by the *darker shading* of the helical symbols.

have purified and characterized the R3 protein and confirm the deduced dimeric structure (Alberti *et al.*, 1993) and demonstrate that this protein, in contrast to the previously characterized dimers (Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Brenowitz *et al.*, 1991; Chen and Matthews, 1992a), binds to DNA with wild-type affinity and also displays alterations in the allosteric properties associated with inducer binding.

MATERIALS AND METHODS

Plasmid and Bacterial Strains—Plasmid pJC1 (Chen and Matthews, 1992a), containing the wild-type or −11 aa[1] deletion mutant *lacI* gene and its promoter, was used as an expression vector for the repressor proteins. Plasmid pMLR-R3 encoding the *lac* repressor mutant R3 was used as an expression vector for the R3 protein (Alberti *et al.*, 1993). Bacterial strain *E. coli* TB-1 (ara, Δ (*lac-pro*), StrA, thi, $\phi 80dlacZ\Delta M15r^-$, 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 and Matthews, 1992a). The R3 mutant protein expressed in TB-1 cells was purified by fractionation with ammonium sulfate at 37% saturation, followed by passage through a phosphocellulose column in 0.12 M potassium phosphate buffer (pH 7.5) with 0.3 mM dithiothreitol and 5% glucose. The activity-containing fractions were collected and combined following elution of the protein with the same buffer. The protein was only partially purified by the phosphocellulose chromatography, in part because of the low level of expression from this construct. Further purification was achieved by elution from a gel filtration column containing Sephadex G-150 equilibrated with various buffers depending on the experiments to be carried out with the purified samples.

Operator Binding Assays—A 40-bp double-stranded operator DNA (sequence: 5′-TGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGG-3′) labeled at the 5′-end with ^{32}P was used to determine operator binding constants by nitrocellulose filter binding methods (Riggs *et al.*, 1968). The assay to determine repressor-operator binding constants was carried out at room temperature in 0.01 M Tris-HCl (pH 7.5), 0.15 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 5% dimethyl sulfoxide (FB buffer), with 2×10^{-12} M ^{32}P-labeled operator, 50 mg/liter bovine serum albumin, and varying concentrations of repressor protein (O'Gorman *et al.*, 1980a). Binding curves were fit using the program Sigmaplot 4.0 by nonlinear least squares analysis to binding Equation 1:

$$R = [P]/(K_d + [P]) \quad \text{(Eq. 1)}$$

where R is the fraction of bound complexes within each solution calculated by $R = \text{cpm}/\text{cpm}_{max}$, [P] is the protein concentration in dimer, and K_d is the apparent dissociation constant in dimer concentration.

The effect of IPTG on operator-protein complexes was examined in FB buffer at room temperature, with both operator and protein (dimer) concentrations at 6×10^{-10} M and varying IPTG concentrations ranging from 0.1 μM to 2 mM.

The dissociation rate constants of the repressor-operator complexes were also determined by filter binding methods (Riggs *et al.*, 1970). Repressor and ^{32}P-labeled 40-mer operator (1:1 ratio) were equilibrated in FB buffer at 0 °C for 15 min, with a dimer concentration of 2×10^{-10} M. At zero time, a 200-fold excess of nonlabeled operator DNA over protein was added. Aliquots of 0.5 ml were withdrawn at various times and filtered. The half-life, $t_{1/2}$, was derived from a plot of $\log(RO/RO_0)$ *versus* time, where RO_0 corresponds to the cpm retained at zero time, and RO to the cpm at time t; both are corrected with background counts obtained by adding excess IPTG (final concentration 2 mM) to the complex solution. The dissociation rate, k_d, was then calculated from Equation 2.

$$k_d = \ln 2/t_{1/2} \quad \text{(Eq. 2)}$$

IPTG Binding Assays—During protein isolation and purification, the activity of repressor was measured by [^{14}C]IPTG binding detected by ammonium sulfate precipitation methods (Bourgeois, 1971). Fluorescence measurements to determine inducer binding were carried out on an SLM Aminco 8100 Series 2 spectrofluorometer (SLM Instruments, Inc.) with excitation at 285 nm using a 340-nm cut-off filter (Corning) for emission as described by Daly and Matthews (1986b) in 0.01 M Tris-HCl (pH 7.5 or pH 9.2), 1 mM EDTA, 0.01 M $MgCl_2$, 0.2 M KCl (TMS), with a protein monomer concentration of 1.5×10^{-7} M. For the experiments performed in the presence of operator, 0.2 or 0.8 μM 40-bp operator was incubated with the protein for 10 min prior to the IPTG titrations, and the buffer pH was 7.5. Analysis of binding curves employed the program Igor (version 1.2) using nonlinear least squares analysis to generate fits to binding Equation 3:

$$R = [\text{IPTG}]^n/(K_d^n + [\text{IPTG}]^n) \quad \text{(Eq. 3)}$$

where R is the fractional saturation of intensity change, K_d is the equilibrium dissociation constant, and n is the Hill coefficient.

Gel Filtration—Gel filtration chromatography was utilized to examine the oligomeric state of the repressor proteins. Sephadex G-150–120 (Sigma) was employed as matrix for the separation. The column (1.2 × 60 cm) was equilibrated in 0.12 M potassium phosphate buffer (pH 7.5) and calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and blue dextran 2000 (Pharmacia LKB Biotechnology Inc.). Samples were applied in a volume of 0.5 ml. Absorbance at 280 nm was measured with an LKB 4050 UV spectrophotometer for detection of protein.

Antibody Binding—Aliquots of 2, 10, and 30 μg of each purified protein were filtered onto nitrocellulose paper in the absence and presence of 0.01% SDS. The protein blots were then reacted with monoclonal antibody B-2 as described by Sams *et al.* (1985).

RESULTS AND DISCUSSION

Lac Repressor R3 Mutant Is a Short Axis Dimer—In the R3 mutant, the *lac* repressor C-terminal leucine heptad repeats

[1] The abbreviations used are: aa, amino acids; bp, base pair; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

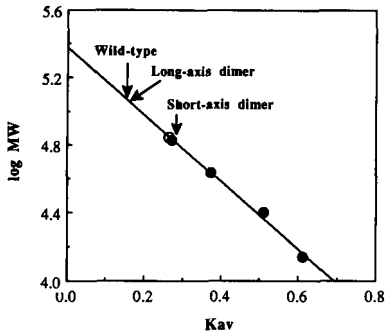

FIG. 2. **Determination of oligomeric state for R3 protein.** Gel filtration chromatography was performed on a column of Sephadex G-150 equilibrated with 0.12 M potassium phosphate buffer (pH 7.5). The column was calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin, and bovine serum albumin (●). The theoretical elution volumes for tetrameric, long axis, and short axis dimeric repressor are marked (Chen *et al.*, 1994). ○ represents the elution volume for R3 repressor. *MW*, apparent molecular weight; $K_{av} = (V_e - V_0)/V_s$, where V_e is the elution volume for each protein, V_0 is the void volume, and V_s is the volume of the stationary phase.

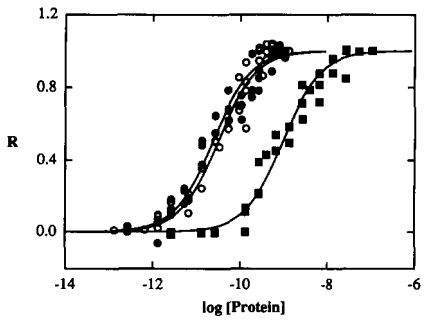

FIG. 3. **Determination of operator affinity.** Repressor binding to operator was assayed as described under "Materials and Methods" using the nitrocellulose filter binding method (Riggs *et al.*, 1968). The concentration of 40-bp ^{32}P-labeled operator DNA was 2×10^{-12} M, and the protein concentration was as indicated. Data shown are from three to four independent experiments for each protein. The curves are generated from fitting the data to Equation 1 as described under "Materials and Methods." ●, wild-type protein; ○, R3 protein; ■, −11 aa protein.

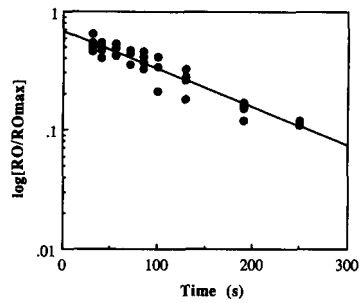

FIG. 4. **Measurement of repressor-operator dissociation rate for R3 protein.** Dissociation of repressor from operator (Riggs *et al.*, 1970) was measured as described under "Materials and Methods." Repressor dimer and 40-bp ^{32}P-labeled operator DNA at 2×10^{-10} M were equilibrated for 15 min on ice. At zero time, a 200-fold excess of nonlabeled operator DNA was added, and aliquots were filtered at the indicated times. The dissociation rate was calculated from the fit of the line using Equation 2 as described under "Materials and Methods."

TABLE I
Operator binding constants for wild-type, −11 aa, and R3 lac repressors

Repressor	Apparent K_d[a]	Dissociation rate constant[b]
	M × 10^{11} dimer	*s^{-1}*
Wild-type	1.9 ± 0.1	0.010 ± 0.001
−11	95.7 ± 2.3	0.014 ± 0.001
R3	3.3 ± 0.3	0.008 ± 0.001

[a] The apparent dissociation constant for the 40-bp operator DNA (~2×10^{-12} M) was measured as described under "Materials and Methods" at room temperature. The values are reported as molar concentrations of dimers and are the average of at least three independent determinations.

[b] The dissociation rate constants were measured as described under "Materials and Methods" at 0 °C. The values for wild-type and −11 aa were reported previously (Chen and Matthews, 1992a); wild-type dissociation measured in parallel with R3 was similar to previous measurements. The value for R3 is the average of four independent determinations.

are replaced with the heptad repeats involved in dimer formation in the GCN4 protein (Alberti *et al.*, 1993). Fig. 1*B* shows the C-terminal sequence of R3 and those of wild-type and −11 aa (Chen and Matthews, 1992a) for comparison. Because of the low expression levels of the R3 gene from the plasmid (Alberti *et al.*, 1993; Lehming *et al.*, 1987), purification of the R3 protein required an additional procedure following the conventional chromatographic separation on phosphocellulose which suffices for purification of wild-type or dimeric repressors from the pJC1 vector (Chen and Matthews, 1992a). The phosphocellulose elution profile of R3 protein is, however, very similar to that of −11 aa protein, with the repressor activity eluting as a second peak in the flow-through using 0.12 M potassium phosphate buffer. This elution pattern is consistent with a dimeric structure for the protein (Chen and Matthews, 1992a). Further purification was achieved by elution from a Sephadex G-150 column. Protein samples thus obtained were at least 90% pure as estimated by silver staining of SDS-polyacrylamide gels following electrophoresis.

Based on the elution volume of R3 derived from gel filtration chromatography, the Stokes radius and apparent molecular mass of 66 kDa are almost identical to the values for −11 aa protein (Fig. 2), a typical short axis dimer (Chen and Matthews, 1992a), whereas a long axis dimer displays a much larger Stokes radius (Chen *et al.*, 1994). Therefore, we conclude that the R3 mutant is a short axis dimer (see Fig. 1). However, unlike short axis dimers derived from deletion and substitution in this region which result from abolition of the C-terminal coiled-coil interaction, R3 dimer formation presumably occurs because of alterations in the arrangement of the C-terminal domain (Fig. 1*C*). The structures formed by leucine heptad repeat sequences appear to be malleable to some degree based on the character of the amino acid side chain at specific positions; for example, changes in the GCN4 sequence can yield coiled-coils of two, three, and four polypeptide segments (Harbury *et al.*, 1993). Presuming that the arrangement favored in the wild-type tetramer for the C-terminal domain is an antiparallel four-helical bundle structure (Alberti *et al.*, 1993), the introduction of the GCN4 sequence, which forms a stable parallel two-helical coiled-coil (O'Shea *et al.*, 1989a, 1989b, 1991), must result in an alternate oligomeric arrangement in which the dimer is stabilized and tetramer cannot form.

R3 Has Wild-type Operator Binding Affinity—The operator binding affinity of the R3 protein was assessed by filter binding methods (Riggs *et al.*, 1968). The equilibrium and kinetic dissociation constants for operator binding are comparable for R3 and wild-type repressors, whereas a decreased affinity is observed for the dimers generated by deletion of or substitution in the leucine heptad repeat region (Figs. 3 and 4; Table I; Chakerian *et al.*, 1991; Chen and Matthews, 1992a). The significant difference in operator binding between the two dimeric proteins, R3 and −11 aa, which has ~30-fold lower affinity, is very intriguing. Both dimers have one pair of N termini which must

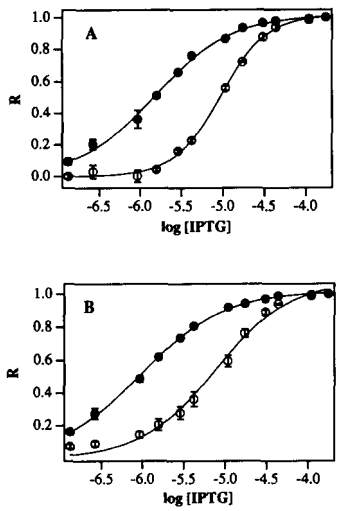

FIG. 5. **IPTG titration of R3 and wild-type repressors.** The fractional degree of saturation, *R*, was measured as described under "Materials and Methods." The values shown at each point are the average for at least three independent determinations; standard deviations greater than the radii of the data points are shown as *error bars*. The curves were generated by nonlinear least squares data fitting to Equation 3 as described under "Materials and Methods." The titrations were carried out for wild-type (*panel A*) and R3 (*panel B*) at pH 7.5 (●) and pH 9.2 (○).

form a single operator binding site (Alberti *et al.*, 1993; Chen and Matthews, 1992a). The only structural difference between these two proteins is in the C terminus, a region that does not contain DNA binding determinants (Müller-Hill, 1975; Ogata and Gilbert, 1979; Kleina and Miller, 1990). An obvious conclusion is that the apparent DNA binding difference derives from the indirect effect of subunit association. If one assumes a thermodynamic linkage between monomer-monomer association in the −11 aa dimer and its decreased apparent DNA affinity as hypothesized for the *lacI*^{adi} dimer (Brenowitz *et al.*, 1991), the wild-type affinity observed for R3 dimer suggests a strengthened subunit interaction, presumably mediated by the parallel coiled-coil structure that GCN4 sequences are known to form (O'Shea *et al.*, 1989a, 1989b, 1991). This observation of increased dimer stability has been confirmed directly by measurement of urea denaturation for this protein.[2] These data indicate that the GCN4 leucine heptad repeat sequences from each monomer within a dimer are in a spatial orientation that allows coiled-coil formation.

R3 Shows Altered Subunit Interaction—As a means to investigate further subunit interaction in R3, we examined the IPTG binding properties of this protein. Fig. 5 shows the IPTG titration curves for R3 and wild-type; the binding constants are summarized in Table II. The R3 protein has similar IPTG affinity at neutral pH compared with the wild-type. At elevated pH, the wild-type protein exhibits a lower apparent affinity and displays positive cooperativity, consistent with communication between subunits (Daly and Matthews, 1986b). We have demonstrated previously based on the data for the short axis dimers derived from deletion (Chen and Matthews, 1992a) and a long axis dimer (Chen *et al.*, 1994) that the subunit communication leading to this cooperativity is predominantly between the two monomers in a short axis dimer. For the R3 protein, although a shift of the binding curve similar to wild-type but of slightly lower magnitude is observed at elevated pH, there is no evidence of cooperativity; analysis of the curve yields a Hill coefficient of 1. The fact that subunit communication and allosteric behavior characteristic of wild-type protein and short

[2] J. Chen and K. S. Matthews, submitted for publication.

TABLE II
Inducer binding parameters for wild-type, −11 aa, and R3 lac repressors

Details of experimental procedures are described under "Materials and Methods." The apparent equilibrium dissociation constant (K_d) and Hill coefficient (n) are derived from fitting (Levenberg-Marquardt algorithm) of the data to the following equation using Igor, version 1.2: $R = [IPTG]^n/(K_d^n + [IPTG]^n)$, where R is the fractional degree of saturation, measured as the ratio of the change in fluorescence at a specific inducer concentration compared with the total change in fluorescence at saturating inducer concentrations. Each value shown is the average of at least three independent determinations. The equilibrium dissociation constants are reported as molar concentrations of monomers, as there is one inducer site per monomer.

Protein	Binding at pH 7.5		Binding at pH 9.2	
	K_d	Hill coefficient	K_d	Hill coefficient
	μM	*n*	μM	*n*
Wild-type	1.5 ± 0.2	1.0 ± 0.1	10.0 ± 1.0	1.6 ± 0.1
−11 aa	1.8 ± 0.2	1.1 ± 0.1	14.7 ± 0.5	1.7 ± 0.3
R3	0.9 ± 0.1	0.9 ± 0.1	7.7 ± 1.3	1.0 ± 0.1

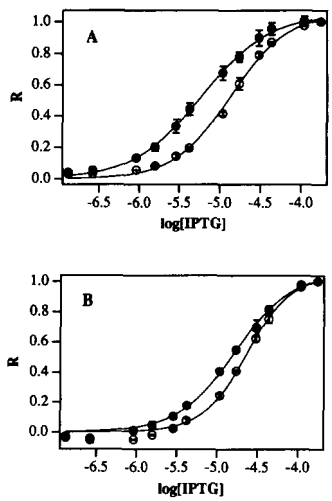

FIG. 6. **IPTG titration in the presence of operator DNA for wild-type and R3 repressors.** Titrations with IPTG were carried out at two different operator concentrations: 0.2 μM (*panel A*) and 0.8 μM (*panel B*). ●, wild-type repressor; ○, R3 protein. The data points shown are average of three independent determinations, and the curves were analyzed as described in the legend to Fig. 5.

axis deletion dimers are abolished in R3 may indicate a constraint on subunit interactions and/or orientation as a result of additional contacts between the subunits mediated by the GCN4 sequence and the consequent stronger association. A similar decrease in the cooperativity of inducer binding was observed upon modification of the repressor protein with methyl methanethiosulfonate (Daly *et al.*, 1986), which also results in protein with increased subunit affinity (Royer *et al.*, 1986).

Although the effects of high pH on inducer binding mimic the presence of operator DNA (O'Gorman *et al.*, 1980b; Daly and Matthews, 1986b), we measured directly the influence of operator DNA on inducer binding parameters (Fig. 6 and Table III). For the R3 protein, a larger decrease in affinity is found compared with the wild-type protein, and the cooperativity observed for R3 in the presence of operator is greater than wild-type repressor. The tethering of both the C-terminal domain via the GCN4 leucine heptad repeat sequence and the N termini via interaction with operator DNA results in a significant effect on the inducer binding which is not replicated by elevated pH for R3 protein. Cooperativity apparent in the presence of operator was not observed with the pH change alone. These dif-

TABLE III
Inducer binding parameters for wild-type and R3 lac repressors in the presence of operator

Details of experimental procedures are described under "Materials and Methods." The apparent equilibrium dissociation constant (K_d) and Hill coefficient (n) are derived as indicated in Table II. The buffer pH employed was 7.5. Each value shown is the average of at least three independent determinations. The dissociation constants are reported as molar concentrations of monomers.

Protein	[40-bp operator] = 0.2 μM		[40-bp operator] = 0.8 μM	
	K_d	Hill coefficient	K_d	Hill coefficient
	μM	n	μM	n
Wild-type	6.0 ± 0.9	1.1 ± 0.1	16.7 ± 1.0	1.2 ± 0.0
R3	13.7 ± 0.9	1.3 ± 0.1	23.5 ± 0.8	1.6 ± 0.1

ferences may arise from the increased stability of the subunit interface in the R3 mutant.

IPTG Induced Dissociation of Repressor-Operator Complexes—Dissociation of repressor from operator DNA at increasing sugar concentrations occurs as a consequence of the conformational change and consequent decrease in operator affinity elicited by inducer binding. For the R3 protein, as anticipated on thermodynamic grounds by the effect of operator on inducer binding, higher concentrations of inducer are required for dissociation of R3 protein from DNA than observed for the wild-type protein (Fig. 7). Dissociation of repressor from DNA by inducer binding is an essential part of the induction mechanism in the *E. coli* cell. It is apparent that this process is sensitive to changes in the subunit interactions that generate the oligomeric structure of the protein. Stabilization of the subunit interface may have significant effects on the binding properties of the protein; on the one hand, stabilization of dimers results in wild-type affinity for operator DNA, whereas on the other hand, this same stabilization alters the allosteric properties of the protein to make it less responsive to the inducer signal.

Reaction of R3 Protein with Monoclonal Antibody—The B-2 monoclonal antibody to the lactose repressor reacts with an epitope between amino acids 280 and 328 which is available fully only in the monomeric or denatured forms of the repressor and is partially exposed in dimeric repressor proteins (Sams *et al.*, 1985; Chen and Matthews, 1992a). Reaction of R3 protein with B-2 monoclonal antibody demonstrates that this epitope is partially exposed in this protein to the same extent as in the −11 aa dimer (Fig. 8), and the presence of SDS affects the extent of reaction. Partial exposure of the epitope for this antibody in R3 indicates that the extension of the C terminus does not affect availability of the target segment of the protein compared with other short axis dimers. Furthermore, since the R3 protein displays wild-type affinity for operator DNA and is a more stable dimer than those produced by deletion in the leucine heptad repeat region,[2] the partial reactivity with antibody determined for the latter proteins does not arise from dissociation of the dimer into monomer but rather appears to be derived from partial exposure of the epitope in the dimer structure.

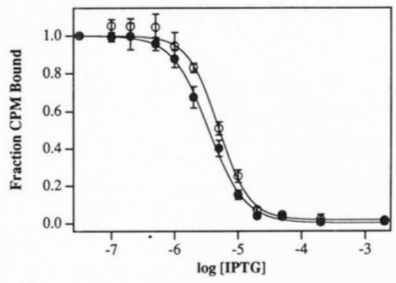

FIG. 7. **Effect of IPTG on operator binding.** ^{32}P-Labeled operator DNA and repressor dimer were mixed at concentrations 6×10^{-10} M and allowed to equilibrate at room temperature in the buffer employed for operator binding assays described under "Materials and Methods." Varying concentrations of IPTG as indicated were added to the solutions, and the mixtures were allowed to sit for an additional 15 min. The samples were then filtered and analyzed for retention of radiolabeled DNA. The data shown are the average of three independent determinations. (●), wild-type protein; ○, R3 protein.

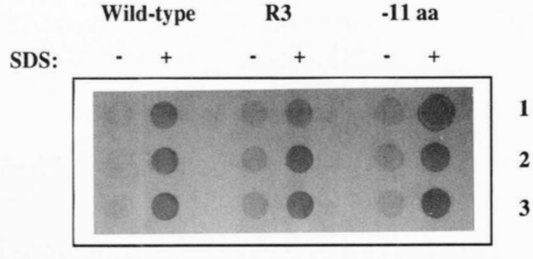

FIG. 8. **Antibody reactivity of R3 protein.** Wild-type, R3 protein, and −11 aa protein were filtered onto nitrocellulose in a dot-blot apparatus and allowed to react with B-2 monoclonal antibody (Sams *et al.*, 1985). In *−SDS lanes*, samples were filtered in their native forms; in the *+SDS lanes*, the proteins were dissociated by adding 0.01% SDS. The amount of repressor loaded was 2 μg (*lane 1*), 10 μg (*lane 2*), and 30 μg (*lane 3*). The reactivity of R3 and −11 aa dimeric proteins indicates that the residues at the C terminus do not alter the recognition of the epitope in the dimeric form.

CONCLUSION

Protein-ligand interactions and protein-DNA interactions are key processes for the regulation of transcription. Linkage between these interactions is mediated through the protein structure. Not only is the oligomeric form of most transcriptional regulators essential for DNA recognition, but allosteric linkage between ligand binding and DNA binding involves the subunit interfaces. Examples of the former linkage are dimeric derivatives of the lactose repressor which are formed by disruption of the coiled-coil structure found at the C terminus of this protein; these proteins display reduced (50~100-fold) apparent affinity to a single operator site compared with the wild-type tetramer (Brenowitz *et al.*, 1991; Chakerian *et al.*, 1991; Chen and Matthews, 1992a). This apparent decrease in affinity derives from the coupling of the dimer-monomer dissociation with the dimer-DNA binding, as indicated directly by the wild-type operator affinity found for the dimeric R3 protein in which the subunit interaction is stabilized[2] (Brenowitz *et al.*, 1991; Chen and Matthews, 1992a).

The involvement of subunit interfaces in the linkage between ligand binding and DNA binding is indicated by alterations in allostery of ligand binding in the presence of DNA in oligomeric (but not monomeric) repressor protein (Daly and Matthews, 1986a, 1986b) and in alterations in allostery by modifications that affect the subunit interface (Daly *et al.*, 1986; Daly and Matthews, 1986b; Chang *et al.*, 1993). The R3 mutant repressor, generated by replacing the C-terminal leucine heptad repeats in *lac* repressor with those from GCN4 (Alberti *et al.*, 1993), is an example of the latter effect, in which linkage between ligand binding and DNA binding is affected by alterations in the character of the subunit interface. Replacement of the leucine heptad repeat at the C terminus of the lactose repressor with the GCN4 sequence results in a stable dimeric protein with wild-type operator affinity but with altered allostery in inducer binding. This difference appears to arise from the additional stability generated by the GCN4 sequence forming a coiled-coil structure with consequent constraints on subunit interactions. From these data, it is evident that subunit association is not only necessary for formation of the DNA binding species for this regulatory protein, but the character of the subunit interface has significant consequences for the al-

losteric behavior of the protein and the associated response to sugar concentration in the cell.

Acknowledgment—We thank Professor Benno Müller-Hill for critical reading of this manuscript prior to publication.

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