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Construction of a Dimeric Repressor: Dissection of Subunit Interfaces in *Lac* Repressor†

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ABSTRACT: Formation of the lactose repressor tetramer is postulated to involve two subunit interfaces, one primarily contributing to monomer–monomer assembly to dimer and the second to dimer–dimer association to tetramer. The latter interface requires a heptad repeat of three leucines at the C-terminus of *lac* repressor that is presumed to form an abbreviated coiled-coil motif [Chakerian, A. E., Tesmer, V. M., Manly, S. P., Brackett, J. K., Lynch, M. J., Hoh, J. T., & Matthews, K. S. (1991) *J. Biol. Chem. 266*, 1371–1374; Alberti, S., Oehler, S., von Wilcken-Bergmann, B., Krämer, H., & Müller-Hill, B. (1991) *New Biol. 3*, 57–62; Chen, J., & Matthews, K. S. (1992) *J. Biol. Chem. 267*, 13843–13850]. To strengthen the dimer–dimer interface, this motif was extended by the addition of one and two leucine heptad repeat units to the C-terminus by site-specific insertion mutagenesis. The tetrameric products displayed operator and inducer affinity essentially indistinguishable from the wild-type repressor. In order to probe the effect of the elongated coiled-coil on assembly of the repressor tetramer, the other of the two postulated subunit interfaces was disrupted by introducing a point mutation (Y282D) that yields a monomeric protein in the wild-type background. Both elongated mutant repressors were able to assemble into dimeric species, apparently due to the strengthened subunit association at the C-terminal region compared to the wild-type repressor. These results further confirm the role of a coiled-coil structure in the formation of tetramer in the *lac* repressor. The generation of a stable "long-axis dimer" provides strong evidence for the hypothesis that two distinctive and experimentally separable interfaces are involved in the assembly of the tetrameric repressor.

The *lac* repressor, a key regulatory protein in the *lac* operon, controls transcription of the enzymes involved in lactose metabolism in *Escherichia coli* (Miller & Reznikoff, 1980). Repressor binding to *lac* operator blocks initiation by RNA polymerase, thereby repressing expression of the lactose metabolic enzymes. The *lac* repressor, with a molecular mass of ~150 000 Da (Riggs *et al.*, 1968), functions as a tetramer of four identical subunits, each composed of 360 amino acids (Beyreuther *et al.*, 1973; Farabaugh, 1978). Extensive genetic and chemical studies have provided insight into the correlation between primary structure of this protein and its multiple functional activities (Müller-Hill, 1975; Schmitz *et al.*, 1976; Miller, 1979; Miller *et al.*, 1979; Gordon *et al.*, 1988; LeClerc *et al.*, 1988; Kleina & Miller, 1990). The protein generally can be dissected into two principal structural domains: the N-terminal 59 residues contain a helix-turn-helix motif that confers most 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; Kleina & Miller, 1990; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a). The region that encompasses Tyr^{282} appears to be essential for assembly of the protein into a tetrameric species, as substitution by amino acids other than Leu or Phe results in a monomeric product devoid of operator binding (Schmitz *et al.*, 1976; Kleina & Miller, 1990; Chakerian & Matthews, 1991). Based on a model for the core domain generated from amino acid sequence alignment with periplasmic sugar binding proteins (Nichols *et al.*, 1993) and on site-specific mutagenesis results (Chang *et al.*, 1993), Lys^{84} has been proposed to contribute to the same subunit interface as Tyr^{282}.

In addition, the C-terminal ~18 residues are crucial for the formation of tetramer from dimer (Betz, 1986; Lehming *et al.*, 1988; Mandal *et al.*, 1990; Chakerian & Matthews, 1991; Alberti *et al.*, 1991; Chakerian *et al.*, 1991; Chen & Matthews, 1992a). This region encompasses leucine heptad repeats that have been shown to contribute to tetramer formation. Mutating the leucine residues at positions 342, 349 and 356, thought to contribute to a coiled-coil structure (Alberti *et al.*, 1991; Chakerian *et al.*, 1991), or deleting the C-terminus (Chen & Matthews, 1992a) resulted in dimeric species. This presumed coiled-coil contains three heptad leucine repeats, shorter than the average length (4~5 heptad leucine repeats) of the coiled-coil or leucine zipper motifs found in other proteins, such as the *jun* family of proteins and GCN4 (Hope & Struhl, 1986; Maki *et al.*, 1987; Ryder & Nathans, 1988; Ryder *et al.*, 1988, 1989).

Attempts to define the dimensions of the tetrameric protein have been made by several investigators. Studies in dilute solutions by small-angle X-ray scattering have revealed an elongated cylindrical shape for the molecule with a long axis of 125–180 Å and a short axis of 60–90 Å (Pilz *et al.*, 1980; McKay *et al.*, 1982); the N-terminal headpieces were deduced to be located at opposite ends of the elongated molecule (McKay *et al.*, 1982). Neutron-scattering studies yielded comparable dimensions for the tetramer (Charlier *et al.*, 1980, 1981). In order to understand the assembly of the tetrameric

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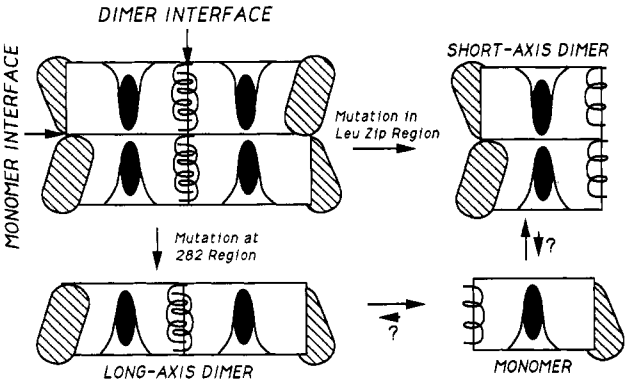

FIGURE 1: Diagrammatic illustration of the proposed assembly of tetrameric *lac* repressor. The tetrameric repressor is composed of four identical subunits, each dissected into N-terminal (hatched regions) and core domains. The inducer binding sites are represented by solid ovals, and the coiled-coil sequences by helical symbols. The two subunit interfaces are proposed to be structurally independent of each other: the monomer interface involves Tyr^{282}; the dimer interface is contributed by the C-terminal coiled-coil. Disruption of the dimer interface results in a short-axis dimer, while disruption of the monomer interface combined with elongation of the coiled-coil is proposed to elicit the formation of a long-axis dimer. In the absence of stabilization of the dimer interface, disruption of the monomer interface results in monomeric repressor (e.g., Y282D).

structure of this protein, which is essential for its regulatory function, alternative physical and genetic approaches must be employed, pending solution of the X-ray crystal structure (Pace *et al.*, 1990).

We propose that the assembly of the tetrameric *lac* repressor employs two distinct subunit interfaces that are structurally independent (Figure 1): the coiled-coil region contributes to the dimer interface since disruption of this interface results in dimeric repressor (designated "short-axis dimer") (Chen & Matthews, 1992a), while the Tyr^{282}–Lys^{84} region forms the monomer interface, as mutations at Tyr^{282} yield monomeric repressors (Chakerian & Matthews, 1991; Chen & Matthews, 1992b). Based on this proposition, we hypothesized that elongation of the coiled-coil domain in *lac* repressor would result in a more stable tetramer and that a "long-axis dimer" (Figure 1) should form upon disruption of the monomer interface in this altered protein. In order to test these hypotheses, the C-terminus of the *lac* repressor has been extended with one and two additional leucine heptad repeats. Evidence is presented here that the two subunit interfaces are experimentally separable and that stabilization of the dimer interface by extending the coiled-coil motif coupled with disruption of the monomer interface by mutation at Tyr^{282} allows generation of a long-axis dimer.

MATERIALS AND METHODS

Plasmid and Bacterial Strains. Plasmid pJCl (Chen & Matthews, 1992a), containing the *lac* I gene and its promoter, was used as an expression vector for *lac* repressors and to generate single-stranded DNA as a template for site-specific mutagenesis and sequencing. Bacterial strain *E. coli* 71/18 (supE thi Δ(*lac-pro*AB)F′[*pro*AB+*lac* I^q *lac* ZΔM15]) was used as the host to prepare all the single- and double-stranded DNAs (Chakerian & Matthews, 1991), except uracil-containing ssDNAs, which were isolated from *E. coli* BW313 (a gift from Dr. T. A. Kunkel; Kunkel, 1985). The wild-type and all the mutant repressors were generated and expressed using pJCl as the parent plasmid in *E. coli* TB-1 (ara, Δ-(*lac-pro*), StrA, thi, φ80d*lac*ZΔM15r^-, m^+).

Site-Specific Mutagenesis. Oligonucleotide-directed site-specific mutagenesis was performed using pJCl according to the methods of Kunkel (1985). The mutagenic oligonucleotides ranged from 15 to 90 bases in length, were synthesized on a Biosearch 8600 DNA synthesizer, and were purified by either Sephadex G-25 (Sigma) elution or elution of the full-sized band from a polyacrylamide gel. The mutagenesis reactions were carried out according to the methods of Kunkel (1985) with the following modifications: when a long oligonucleotide (~90 bases) was used, the annealing conditions were changed to heating at 95 °C for 5 min and cooling down to room temperature in only 5–10 min; extension and ligation of the synthesized DNA strand were carried out at 37 °C for 2 h using Sequenase (version 2.0, USB) instead of Klenow fragment. At the end of the reaction, 1–5 μL of the reaction mixture were transformed into *E. coli* 71/18 competent cells followed by screening the colonies for those containing mutant DNA. The entire *lac* I gene was sequenced for each mutant to confirm the nature of mutation.

Purification of Repressors. Isolation and purification of the wild-type repressor protein from *E. coli* TB-1 cells were as described previously (Chen & Matthews, 1992a). The extension mutants LHR4 and LHR5 (for *l*eucine *h*eptad *r*epeats *4* and *5*) were purified by the same procedures. The LHR5–Y282D mutant protein was also purified by elution from the phosphocellulose column using a gradient of potassium phosphate from 0.12 to 0.3 M, although the position of the repressor peak was slightly different from that of the wild-type repressor. Purification of LHR4–Y282D mutant repressor, however, required different procedures. The repressor was eluted from the phosphocellulose column immediately after the flow-through as a second peak in 0.12 M potassium phosphate buffer. The protein was further purified by passage through a DEAE–cellulose column under the same buffer conditions.

Activity Assays. During protein isolation and purification, the activity of repressor was measured by [^{14}C]IPTG[1] binding detected by ammonium sulfate precipitation methods (Bourgeois, 1971). A 40 bp double-stranded operator DNA (sequence: 5′-TGTTGTGTGGAATTGTGAGCGGATAA-CAATTTCACACAGG-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 7.4), 0.15 M KCl, 0.1 mM DTT, 0.1 mM EDTA, and 5% DMSO (FB), with ~5×10^{-12} M [^{32}P]operator, 50 mg/L bovine serum albumin, and varying concentrations of repressor protein (O'Gorman *et al.*, 1980). Fluorescence measurements to determine inducer binding were carried out on an SLM Instruments Series 400 spectrofluorometer using a 340-nm cutoff filter (Corning) as described by Daly and Matthews (1986) in 0.01 M Tris-HCl (pH 7.5 or pH 9.2), 1 mM EDTA, 0.01 M $MgCl_2$, and 0.2 M KCl (TMS), with a protein monomer concentration of 1.5×10^{-7} M. Analysis of binding curves employed the program Igor, Version 1.2, graphing and data analysis software for the Macintosh, to generate fits to the binding equation by nonlinear least-squares analysis (see Table 1 for equation).

Gel Filtration. Gel filtration chromatography was utilized to examine the oligomeric state of the repressor proteins. Either Sephadex G-150-120 (Sigma) or Sephacryl S-200 HR (Pharmacia-LKB) was employed as matrix for the separation. The column (1.2 cm × 60 cm) was equilibrated in buffer at

[1] Abbreviations: IPTG, isopropyl β,D-thiogalactoside; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; CD, circular dichroism.

Wild-type	(340)RALADSLMQLARQVSRLESGQ(360)
LHR4	RALADSLMQLARQVSRLESVQCALRN
LHR5	RALADSLMQLARQVSRLEDKVEELLSKNYHLENE

FIGURE 2: Amino acid sequences of the proposed C-terminal coiled-coil region in the extension mutant repressors. The leucines involved in the heptad repeats are underlined.

pH 7.5 and calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and blue dextran 2000 (Pharmacia-LKB). For small zone experiments, samples were applied in a volume of 0.3–0.5 mL. For large zone experiments, the sample volumes were at least 12 mL. OD_{280} or OD_{223} was measured with LKB 4050 UV spectrophotometer for detection of protein.

Immunological Reaction. Aliquots of 2.7×10^{-4} μmol of monomer, 5.3×10^{-4} μmol of monomer, and 1.1×10^{-3} μmol of monomer of each purified protein were filtered onto nitrocellulose paper in the absence and presence of 0.02% SDS and then reacted with monoclonal antibody B-2 as described by Sams *et al.* (1985).

Circular Dichroism Measurements. Circular dichroism (CD) spectra were measured on an Aviv 62DS circular dichroism spectrophotometer. An optical cell with a light path of 10 mm was used. The spectra were measured over the range 190–260 nm at a protein concentration of 2.7 μM (monomer) in 0.12 M potassium phosphate buffer (pH 7.5).

Equilibrium Sedimentation. For equilibrium sedimentation experiments, protein samples in 0.12 M potassium phosphate buffer (pH 7.5) with or without 0.1 mM DTT were loaded at different concentrations ranging from 2.1 to 26.7 μM (monomer). Double-sector charcoal-filled Epon or Kel-F-coated centerpieces (12 mm) and Sapphire windows were used in an AN-F rotor at 26 000 rpm at 20 °C. Optical density at 280, 233, or 230 nm was used to monitor protein concentration, while optical density at 500 nm served as the baseline. The attainment of equilibrium was recognized when the concentration distribution in the cell did not change with time. The collected data were expressed as a plot of c vs $r^2/2$, where c is the protein concentration at r, the distance from the center of rotation, and were analyzed by a nonlinear least-squares approach with various assumed models. The NONLIN program (Johnson *et al.*, 1981) was kindly provided by the Analytical Ultracentrifugation Facility at the University of Connecticut.

RESULTS

Generation and Characterization of Two C-Terminal Extension Mutants of lac Repressor. In the wild-type repressor, the C-terminal amino acid sequence from 342 to 357 is predicted to be a stretch of α-helix (Chou *et al.*, 1975; Bourgeois *et al.*, 1979) which is consistent with the proposed coiled-coil structure (Landschulz *et al.*, 1988; Alberti *et al.*, 1991; Chakerian *et al.*, 1991), while the last four amino acids are predicted to assume a β-turn conformation. The glycine at position 359 is probably most responsible for the discontinuity of the α-helical structure. In the mutant LHR4 (for "4 leucine heptad repeats"), Gly359 is converted to a valine, and a leucine is positioned to form an additional heptad repeat. There is no "helix breaker" in this stretch of amino acids; a continuous helix is expected. In the mutant LHR5, a part of the GCN4 leucine zipper coiled-coil sequence for which the structure is known (O'Shea *et al.*, 1991; Ellenberger *et al.*, 1992) is added to the end of the *lac* repressor, extending the motif by two turns of heptad repeats. Figure 2 shows the additional amino acid sequences in these two mutants. Both mutants were expressed in *E. coli* TB-1 cells, and the mutant proteins were purified by the procedures identical to those applied in purification of the wild-type repressor.

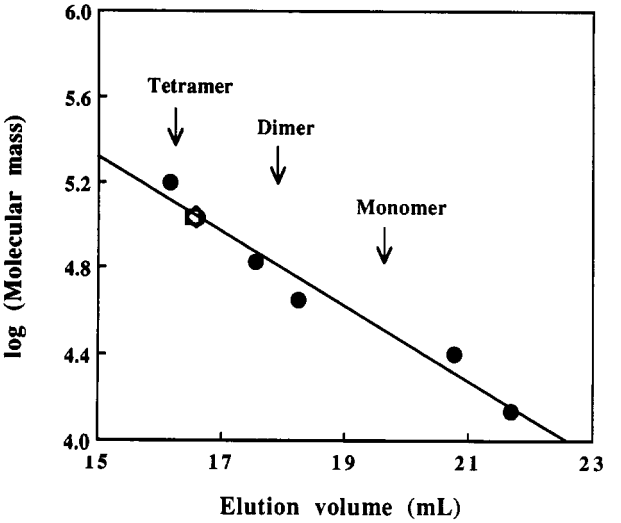

FIGURE 3: Determination of oligomeric states for LHR4 and LHR5 mutant proteins. Large zone gel-filtration chromatography was performed on a column of Sephacryl S-200 HR equilibrated with 10 mM Tris-HCl (pH 7.5) and 0.1 M K_2SO_4. The column was calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and aldolase (●). The theoretical elution volumes for tetrameric, dimeric, and monomeric repressor are marked. (△), (□), and (○) represent the elution volumes for LHR4, LHR5, and wild-type repressors, respectively.

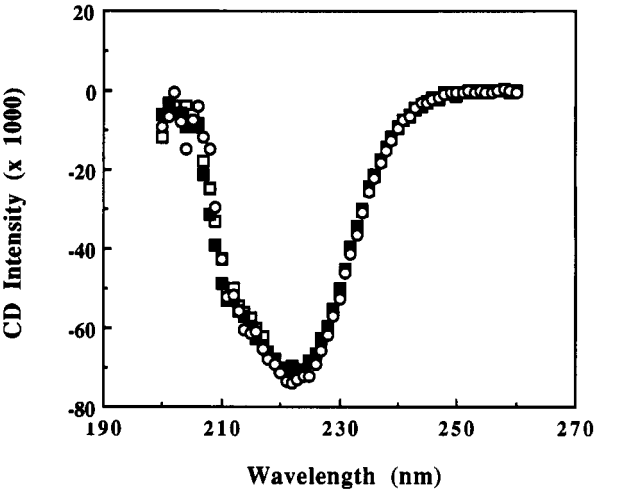

FIGURE 4: Circular dichroism spectra for the extension mutant repressors. The spectra are shown for (□) wild-type, (■) LHR4, and (○) LHR5. The protein concentration was 2.7 μM (monomer) in 0.12 M potassium phosphate buffer (pH 7.5).

Both LHR4 and LHR5 mutant proteins are tetrameric as determined by gel filtration (Figure 3). Their secondary structures were assessed by circular dichroism measurements and compared to the wild-type repressor. As shown in Figure 4, both mutant proteins have very similar CD spectra compared to the wild-type. The only expected change in the secondary structure for the extension mutants is the additional α-helix at the C-terminus, which contributes less than 2% to the total secondary structure, well beyond the sensitivity of CD detection.

The operator-binding affinities and IPTG-binding properties of the two mutant proteins are summarized in Table 1. Both mutant proteins have operator affinity comparable to the wild type. At pH 7.5, both mutant proteins display IPTG affinity

Table 1: Operator and IPTG Binding Constants for C-Terminal Extended *Lac* Repressors

repressor	wild-type	LHR4	LHR5
operator binding			
K_d ($\times 10^{11}$ M dimer)[a]	3.0 ± 0.8	5.6 ± 0.8	3.2 ± 0.6
IPTG binding at pH 7.5			
K_d ($\times 10^6$ M monomer)[b]	1.5 ± 0.2	1.5 ± 0.2	1.3 ± 0.1
Hill coefficient[b]	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
IPTG binding at pH 9.2			
K_d ($\times 10^6$ M monomer)[b]	10.0 ± 1.0	7.7 ± 1.5	17.0 ± 1.0
Hill coefficient[b]	1.6 ± 0.1	1.8 ± 0.3	1.3 ± 0.1

[a] The apparent dissociation constant for 40 bp operator DNA ($\sim 5 \times 10^{-12}$ M) was measured as described in *Materials and Methods* at room temperature. The values reported as molar concentrations of dimers are the average of at least three independent determinations. [b] Details of experimental procedures are in *Materials and Methods*. The apparent equilibrium dissociation constant (K_d) and Hill coefficient (n) are derived from fitting (Levenberg–Marquardt algorithm) 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 to the total change in fluorescence at saturating inducer concentrations. Each value shown is the average of at least three independent determinations. The dissociation constants are reported as molar concentrations of monomers.

essentially identical to the wild-type protein, with no cooperativity observed. At pH 9.2, decreased affinity as well as positive cooperativity is observed in both mutants as in the wild-type. A slightly decreased cooperativity for the LHR5 repressor is observed at elevated pH compared to the wild-type protein.

The extension mutants are therefore comparable to the wild-type protein in their binding properties and structures. It is almost impossible to determine directly the expected tighter dimer–dimer association, since the wild-type repressor is already very tightly associated. In a large zone gel filtration experiment, the wild-type repressor was shown not to dissociate at a concentration as low as 4×10^{-10} M monomer (data not shown), indicating a dissociation constant below this value. Such tight association precludes direct study of protein dissociation in both mutants and wild-type protein by conventional methods.

LHR4–Y282D Mutant Is a Monomer, but Self-Associates at High Concentrations. As an indirect approach to study the dimer–dimer association in the extended mutants and to test the hypothesis that a long-axis dimer can be formed employing the dimer interface of increased stability, the Y282D point mutation was introduced into the LHR4 mutant by site-specific mutagenesis. The Y282D mutant of *lac* repressor has been shown previously to be a monomeric protein with no specific DNA binding affinity (Daly & Matthews, 1986; Chen & Matthews, 1992b). Purification of the mutant LHR4–Y282D required DEAE–cellulose chromatography following phosphocellulose chromatography, a procedure similar to that for purification of the Y282D monomeric protein (Daly & Matthews, 1986). However, this protein was slightly retarded on phosphocellulose, a characteristic also observed for the short-axis dimeric repressors studied previously (Chen & Matthews, 1992a), i.e., the repressor eluted as a second peak in the flow-through. To distinguish whether this protein was dimeric or monomeric, the purified protein was examined by gel filtration chromatography and found to be a monomer at 1×10^{-7} M, with a molecular mass of 42 kDa. This protein does not display any specific DNA affinity, as expected for a monomeric repressor.

However, the phosphocellulose elution profile for the LHR4–Y282D mutant indicated a weak phosphocellulose

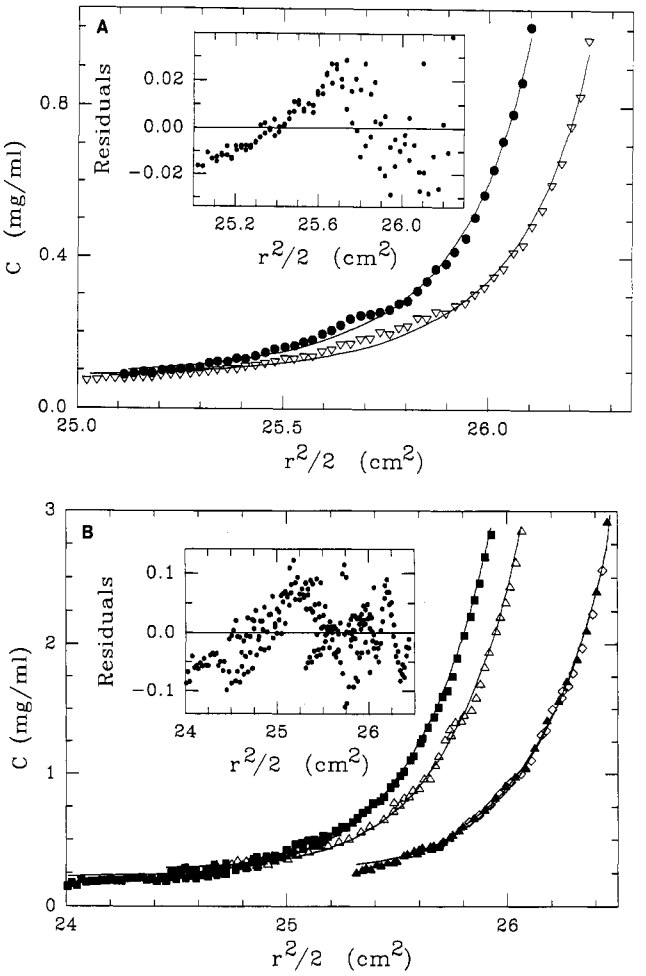

FIGURE 5: Equilibrium sedimentation data for LHR4–Y282D and Y282D mutant repressors. The equilibrium sedimentation experiments were carried out as described in *Materials and Methods*. The protein samples were loaded at concentrations of 2.7 and 10.7 μM (monomer) for LHR4–Y282D and of approximately 27 μM for Y282D. The inset in each graph shows the residuals. (A) The data points for LHR4–Y282D are shown, and the lines are the best simultaneous fit of both sets of data to a model of monomer (42.2 ± 0.2 kDa) associating to dimer with a dissociation constant of 1.3×10^{-4} M (monomer). The discontinuity is due to an aberration in the window which was amplified by using 230 nm and could not be corrected by baseline correction using the optical density at 500 nm. (B) The data points for Y282D are shown, and the lines are the best simultaneous fit of four sets of data to a model of monomer (42.0 ± 0.2 kDa) that does not undergo association.

affinity for this protein, which implied the potential for subunit association at concentrations higher than those used in gel filtration experiments. This behavior was not observed for the Y282D mutation in the wild-type background. Therefore, the oligomeric state of the double mutant at higher concentrations was investigated by equilibrium sedimentation methods. Results of this study were expressed as c vs $r^2/2$, where c is the protein concentration at r, the distance from the center of rotation (Figure 5A). The data are not compatible with the model of a single, nonassociating component. The simplest model that can fit both sets of data simultaneously is $M_1 \rightleftharpoons M_2 \rightleftharpoons M_i$, where $i > 2$ and M assumes a molecular mass of 42 200 ± 200 Da, i.e., the monomer molecular mass of the repressor. The fit to the data indicates a dimer–monomer dissociation constant of 1.3×10^{-4} M (in monomer); thus, an association to dimer is observed at protein concentrations higher than 2×10^{-5} M (monomer) for the LHR4–Y282D mutant. The formation of M_i is not sufficiently strong to permit a determination of the stoichiometry. Control ex-

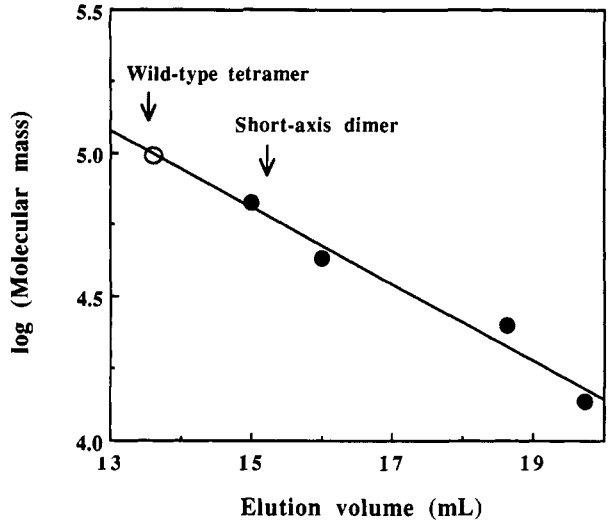

FIGURE 6: Apparent molecular mass of LHR5–Y282D determined by gel filtration. Small-zone gel filtration chromatography was performed on a column of Sephacryl S-200 HR equilibrated with 0.12 M potassium phosphate buffer (pH 7.5). The column was calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin, and bovine serum albumin (●). The elution volumes for wild-type tetramer and a short-axis dimer (–11 aa, Chen & Matthews, 1992a) are indicated. The LHR5–Y282D protein (○) eluted at a position corresponding to a molecular mass of 93 kDa. Identical results were obtained for several runs with different loading protein concentrations ranging from 1.6 to 8.8 μM (monomer).

periments were conducted with the Y282D mutant which exhibited a molecular mass corresponding to a monomer at concentrations as high as 8×10^{-5} M (Figure 5B). Therefore, the self-association of LHR4–Y282D monomer at high concentrations appears to derive from the C-terminal extension, presumably through the action of a strengthened coiled-coil interaction.

LHR5–Y282D Protein Is a Long-Axis Dimer. The Y282D point mutation was also introduced into the LHR5 mutant. This protein was purified by procedures identical to those for purification of wild-type repressor, i.e., the protein adhered to phosphocellulose in 0.12 M potassium phosphate buffer and was eluted with a gradient from 0.12 to 0.3 M potassium phosphate. The molecular mass of this protein was determined to be ~93 kDa by gel filtration over a loading concentration range from 1.6 to 8.8 μM (monomer) (Figure 6). This value falls in between the expected value for dimer (~75 kDa) and tetramer (~150 kDa). Therefore, this protein minimally shows assembly to an oligomeric state higher than monomer. However, the apparent molecular mass for a short-axis dimer (–11 aa, Chen & Matthews, 1992a) and a wild-type tetramer determined on the same gel filtration column are 65 and 100 kDa, respectively, making it difficult to deduce the exact oligomeric state of the LHR5–Y282D from its apparent molecular mass. However, a long-axis dimer, which would be an elongated molecule, would have a larger Stokes radius and elute very differently from the more spherical short-axis dimer on gel filtration. Equilibrium sedimentation methods were therefore employed to determine the molecular mass of this mutant protein independent of its shape. Five sets of data were analyzed simultaneously (Figure 7) to yield a molecular mass of 85.6 kDa. Hence, this protein is dimeric throughout the concentration range examined up to 2.6×10^{-5} M (monomer). Although further association of the dimer may occur at higher concentrations, we conclude that the LHR5–Y282D protein does not associate beyond dimer within the concentration range where its functions have been characterized.

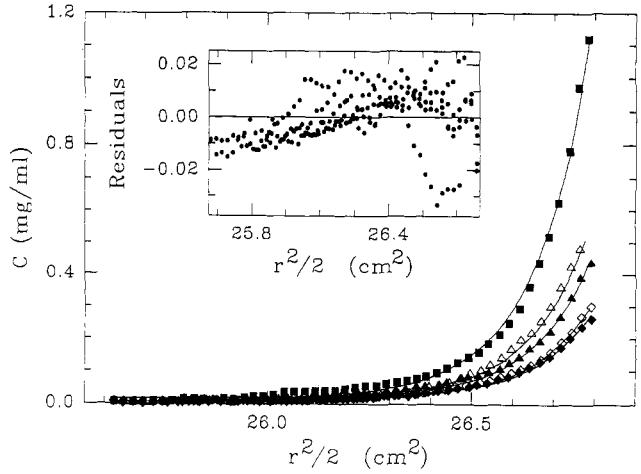

FIGURE 7: Equilibrium sedimentation data for LHR5–Y282D mutant protein. The equilibrium sedimentation experiments were carried out as described in *Materials and Methods*. The protein samples were loaded at a concentration of 2.1 μM (monomer). The data points are shown, and the lines are the best simultaneous fit of five sets of data, which correlates to a simple model consisting of a species of 85.6 ± 0.6 kDa that does not undergo dissociation or association within the concentration range examined. The inset shows the residuals.

Table 2: Hydrodynamic Properties of Wild-Type and Long-Axis Dimeric Repressors

repressor	molecular mass[a]	Stokes radius[b]	f/f_0[c]
wild type	158 000	42	1.2
LHR5–Y282D	81 900	41	1.4

[a] The molecular masses were obtained from amino acid sequences. [b] The Stokes radii were calculated from the gel filtration data assuming a linear relationship between $(-\log K_{av})^{1/2}$ and Stokes radius, where $K_{av} = (V_e - V_0)/(V_t - V_0)$ and V_e, V_0, and V_t are the elution volume of the protein, void volume, and total volume of the column, respectively. [c] f/f_0 is the frictional ratio calculated from the Stokes radius (Winzor, 1969).

Based on the gel filtration data, Stokes radii for the wild-type and LHR5–Y282D repressors were calculated to be 42 and 41 Å, respectively (Table 2). Knowing the Stokes radius and molecular weight of a protein, one can calculate the frictional ratio, f/f_0 (Winzor, 1969). It is interesting to note that the wild-type tetrameric *lac* repressor exhibits a frictional ratio of 1.2, i.e., it is slightly asymmetric in shape, a conclusion consistent with the deduced elongated cylindrical shape of the protein (Pilz *et al.*, 1980; Charlier *et al.*, 1980, 1981; McKay *et al.*, 1982). Furthermore, the calculated value of 1.4 for f/f_0 indicates that the LHR5–Y282D mutant is more asymmetric than the wild-type protein, as anticipated for this long-axis dimer. Having determined that the LHR5–Y282D mutant is not spherical, one may estimate the dimension of this molecule (Tanford, 1961). This protein can be represented by a rod-shaped molecule with a length of 215 Å, in reasonably good agreement with values of 125–180 Å reported (Pilz *et al.*, 1980; Charlier *et al.*, 1980, 1981; McKay *et al.*, 1982) for the long-axis of *lac* repressor.

The secondary structure of the LHR5–Y282D protein was examined by CD spectroscopy and shown to be very comparable to both the wild-type tetramer and a short-axis dimer at the same monomer concentration (Figure 8). The possible helix addition at the C-terminus in this mutant only accounts for a very small percentage of the total secondary structure that would not be detectable by the CD measurements. Full reactivity to the monoclonal antibody B-2 (Sams *et al.*, 1985) was observed for this protein in its native form (Figure 9), indicating complete exposure of the B-2 epitope that is normally

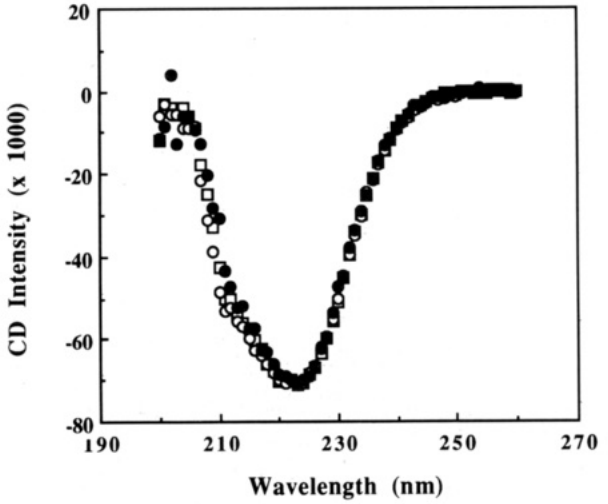

FIGURE 8: Comparison of circular dichroism spectra for the wild-type and two types of dimeric repressors. The spectra were measured in 0.12 M potassium phosphate buffer (pH 7.5) with a protein concentration of 2.7 μM (monomer). (□) Wild-type; (○) LHR5-Y282D; (●) short-axis dimer (–11 aa mutant, Chen & Matthews, 1992a).

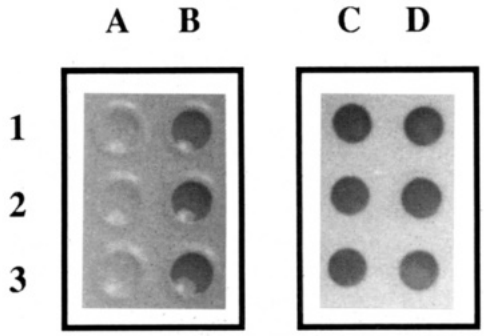

FIGURE 9: Antibody reaction with LHR5–Y282D mutant and wild-type repressors. Wild-type (A, B) and LHR5–Y282D (C, D) repressors were filtered onto nitrocellulose in a dot-blot apparatus and allowed to react with B-2 monoclonal antibody (Sams *et al.*, 1985). In A and C, repressors were filtered in their native forms, while in B and D repressors were denatured by adding 0.02% SDS. The amount of repressor protein loaded was as follows: (1) 2.7×10^{-4} μmol; (2) 5.3×10^{-4} μmol; (3) 1.1×10^{-3} μmol.

hidden in the subunit interface(s) in a tetramer or short-axis dimer (Sams *et al.*, 1985; Chakerian *et al.*, 1991; Chen & Matthews, 1992a).

In Table 3, the IPTG binding and operator binding properties of this dimer are summarized and compared to wild-type repressor, a short-axis dimer, and the Y282D monomer. At pH 7.5, the LHR5–Y282D protein binds IPTG noncooperatively with the same affinity as wild-type repressor, the short-axis dimer, and the monomer. At pH 9.2, although a slight decrease in affinity is observed, no cooperativity upon IPTG binding is detected for this dimer as found for monomeric repressor (Daly & Matthews, 1986; Chakerian & Matthews, 1991). This absence of cooperativity is consistent with the expectation for a long-axis dimer, since the subunit communication upon IPTG binding at elevated pH appears to occur primarily between the two monomers within a short-axis dimer (Chen & Matthews, 1992a). Therefore, various properties exhibited by the LHR5–Y282D mutant are consistent with those anticipated for a long-axis dimer.

Operator binding affinity that was sensitive to IPTG was displayed by this long-axis dimer, with an apparent K_d of 2.2×10^{-8} M dimer (Table 3). This DNA binding affinity was operator-dependent, as the binding was completely abolished

Table 3: Characterization of LHR5–Y282D Dimer and Comparison of Various Oligomers of *Lac* Repressor

repressor	wild type	LHR5–Y282D	–11 aa deletion[e]	Y282D[f]
DNA binding				
40 bp operator[a,b]				
K_d ($\times 10^9$ M)	0.030 ± 0.008	22 ± 2	0.63 ± 0.03	>100
40 bp hybrid operator[c]				
K_d ($\times 10^9$ M)	>100	>100		
20 bp half-operator[d]				
K_d ($\times 10^9$ M)	>100	>100		
IPTG binding at pH 7.5				
K_d ($\times 10^6$ M)[a]	1.5 ± 0.2	1.4 ± 0.3	1.8 ± 0.2	1.2
Hill coefficient[a]	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0
IPTG binding at pH 9.2				
K_d ($\times 10^6$ M)[a]	10.0 ± 1.0	2.4 ± 0.2	14.7 ± 0.5	1.7
Hill coefficient[a]	1.6 ± 0.1	0.9 ± 0.1	1.7 ± 0.3	1.0

[a] The equilibrium constants for operator and IPTG binding were determined as described in Table 1 footnotes. The values reported are the average of at least three independent determinations. K_d values for operator are in M dimer, and K_d values for inducer binding are in M monomer. [b] The DNA used was 40-bp *lac* operator. [c] The DNA used was a 40-bp hybrid containing a *lac* operator half-site and a *trp* operator half-site. [d] The DNA used was a 20-bp sequence containing the promoter proximal half-site of the *lac* operator. [e] Values for the –11 aa deletion mutant, a short-axis dimer, have been reported previously (Chen & Matthews, 1992a). [f] Values for the Y282D mutant are cited from Daly and Matthews (1986).

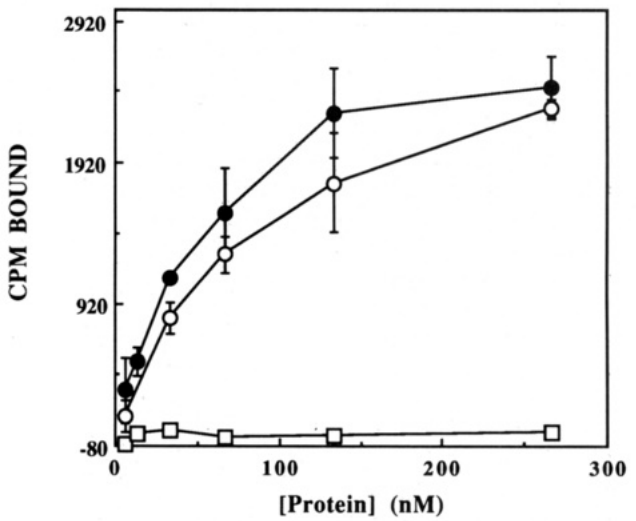

FIGURE 10: DNA titration by LHR5–Y282D. The DNA binding assay was performed as described in Materials and Methods. Binding to the 40-mer operator was examined at a DNA concentration of (●) 3×10^{-12} or (○) 1×10^{-9} M. Each set of data is the average of three independent experiments; standard deviations, where larger than symbol, are shown as error bars. No binding was observed for a 40-mer DNA with only half of the operator sequence (□).

by either replacing one of the two half-sites on the operator DNA with nonspecific sequence (data shown in Figure 10 as open squares) or deleting one of the half-sites (Table 3). This activity was, however, unanticipated since the monomeric repressor does not have any specific DNA binding affinity (Daly & Matthews, 1986), and it is generally believed that a pair of side-by-side N-termini is required for binding the symmetric operator molecule (Ogata & Gilbert, 1979; Lamerichs *et al.*, 1989; Lehming *et al.*, 1990; Kisters-Woike *et al.*, 1991). In a long-axis dimer, the two N-termini are at the distal ends of the molecule, unlikely to be positioned to bind effectively to the two adjacent half-sites of the operator. In addition, specific affinity to a half-site operator DNA was not observed for this long-axis dimer or for the wild-type tetramer. To test the possibility that the observed operator affinity for the long-axis dimer may be due to a small

percentage of tetramer formed, the operator binding assay was carried out at two different operator concentrations: 3×10^{-12} and 1×10^{-9} M dimer. If the dimeric species of LHR5–Y282D is responsible for the observed operator affinity, the binding curves at these two conditions should be identical, since both operator concentrations are sufficiently low compared to the apparent K_d. On the other hand, if the observed binding at low operator concentration is due to the existence of a small amount of tetramer, the binding curve at an operator concentration of 1×10^{-9} M should shift to higher protein concentrations since the operator concentration exceeds the tetramer binding K_d (3.0×10^{-11} M dimer) by 30-fold. As shown in Figure 10, only a minor shift was observed, consistent with the conclusion that the long-axis dimer displays a specific affinity, albeit low, for operator.

DISCUSSION

Existence of the long-axis dimer provides supportive evidence for the role of the postulated coiled-coil motif in the formation of tetrameric *lac* repressor. In addition, this novel dimeric structure confirms the proposed mode of subunit assembly (Figure 1) that involves two distinctive subunit interfaces that are experimentally separable, one contributed at least in part by the Tyr^{282} region and the other by the C-terminal leucine heptad repeats. The long-axis assembly mode of the LHR5–Y282D protein is confirmed by the similarity of inducer binding parameters at neutral pH and the absence of cooperativity upon inducer binding at elevated pH, since subunit communication with regard to inducer occupancy occurs predominantly within each short-axis dimer (Chen & Matthews, 1992a). Work reported after submission of this manuscript by Müller-Hill and colleagues (Alberti *et al.*, 1993) has suggested that the leucine heptad repeats may be arranged in a four-helical bundle structure with an anti-parallel orientation of helices and that the helices may take on alternate orientations (parallel leucine-zipper-type structure or anti-parallel four-helical bundle) depending on the sequence of amino acids. Our results indicate that the extensions to four and five heptad repeats result in dyadic coiled-coil interactions, although the parallel *vs* anti-parallel orientation is not discernible in these experiments.

The long-axis dimer reacts fully with the epitope for B-2 monoclonal antibody (Figure 9) located between residues 281 and 328 (Sams *et al.*, 1985; Chen & Matthews, 1992a). This epitope is fully protected in wild-type tetramer, partially available in short-axis dimers, and fully reactive in the Y282D monomer. Thus, as would be anticipated, the long-axis dimer exposes a region protected in both wild-type tetramer and short-axis dimers of the repressor, providing access to the B-2 epitope located in the monomer interface. The similarity among the secondary structures and inducer binding at neutral pH of different oligomers indicates that assembly does not influence folding patterns for the monomer subunit (Chen & Matthews, 1992a,b). Operator affinity of *lac* repressor is presumed to be conferred by the binding of two adjacent N-terminal headpieces to the two half-sites of the semi-symmetric operator DNA (Ogata & Gilbert, 1979; Lamerichs *et al.*, 1989; Lehming *et al.*, 1990; Kisters-Woike *et al.*, 1991). Consistent with this view, short-axis dimeric repressors bind the operator, albeit with lower apparent affinity (Chen & Matthews, 1992a), while monomeric repressor does not display any detectable operator specificity (Daly & Matthews, 1986). Based on this mode of binding, one would not expect that the long-axis dimer with two N-termini at the distal ends of the molecule would bind to the operator DNA specifically. The possibility that the observed operator affinity for the long-axis dimer may be attributed to the presence of a small fraction of tetramer has been eliminated by operator binding at different operator concentrations (Figure 10). One possible interpretation for the observed operator affinity would be that the basic region of the protein (upstream from the leucine heptad repeat motif) that is homologous to GCN4 and the *jun* family of proteins (Chakerian *et al.*, 1991) is exposed and positioned by long-axis dimer formation to generate a novel DNA binding site. Further experiments to explore this possibility are underway.

The success in construction of this unique oligomer, the long-axis dimer, is an excellent example of the power of site-specific mutagenesis techniques; among the numerous mutations generated in genetic studies of *lac* repressor previously (Miller *et al.*, 1970; 1979; Müller-Hill, 1975; Schmitz *et al.*, 1976; Miller, 1979; Betz, 1986; Lehming *et al.*, 1988; Kleina & Miller, 1990; Chakerian & Matthews, 1991; Chakerian *et al.*, 1991; Alberti *et al.*, 1991; Chen & Matthews, 1992a), there is none resembling this species simply because the coiled-coil motif in the native repressor is not sufficiently strong to mediate dimerization independently. The generation of stable dimers in the context of the Y282D mutation by extension of the leucine heptad repeat motif demonstrates that these extended sequences form a dyadic structure (whether parallel or anti-parallel cannot be discerned) and do not form stable four-helical bundles (Alberti *et al.*, 1993) under these conditions, since tetramer formation is not observed. The intermediate ability of the mutant protein with four heptad repeats to form a dimer and the stability of the dimer formed with five heptad repeats are consistent with the hypothesis that a longer coiled-coil should result in a strengthened interaction in this region. Although these results do not allow us to deduce the structure for the leucine heptad repeat in wild-type tetramer, it is apparent that increased stability of the coiled-coil region is not sufficient to promote assembly to tetramer in the context of a weakened interface involving the Y282 region. The production of stable long-axis dimer demonstrates the separability of subunit interfaces in the repressor and the essential role of the leucine heptad repeat motif in generating the oligomeric structure crucial to the regulatory function of this protein.

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REFERENCES

Adler, K., Beyreuther, K., Fanning, E., Geisler, N., Gronenborn, B., Klemm, A., Müller-Hill, B., Pfahl, M., & Schmitz, A. (1972) *Nature 237*, 322–328.

Alberti, S., Oehler, S., von Wilcken-Bergmann, B., Krämer, H., & Müller-Hill, B. (1991) *New Biol. 3*, 57–62.

Alberti, S., Oehler, S., von Wilcken-Bergmann, B., & Müller-Hill, B. (1993) *Embo J. 12*, 3227–3236.

Betz, J. L. (1986) *Gene 42*, 283–292.

Beyreuther, K., Adler, K., Geisler, N., & Klemm, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3576–3580.

Bourgeois, S. (1971) *Methods Enzymol. 21D*, 491–500.

Bourgeois, S., Jernigan, R. L., Szu, S. C., Kabat, E. A., & Wu, T. T. (1979) *Biopolymers 18*, 2625–2643.

Chakerian, A. E., & Matthews, K. S. (1991) *J. Biol. Chem. 266*, 22206–22214.

Chakerian, A. E., Tesmer, V. M., Manly, S. P., Brackett, J. K., Lynch, M. J., Hoh, J. T., & Matthews, K. S. (1991) *J. Biol. Chem. 266*, 1371–1374.

Chang, W.-I., Olson, J. S., & Matthews, K. S. (1993) *J. Biol. Chem. 268*, 17613–17622.
Charlier, M., Maurizot, J.-C., & Zaccai, G. (1980) *Nature (London) 286*, 423–425.
Charlier, M., Maurizot, J.-C., & Zaccai, G. (1981) *J. Mol. Biol. 153*, 177–182.
Chen, J., & Matthews, K. S. (1992a) *J. Biol. Chem. 267*, 13843–13850.
Chen, J., & Matthews, K. S. (1992b) *Gene 111*, 145–146.
Chou, P. Y., Adler, A. J., & Fasman, G. D. (1975) *J. Mol. Biol. 96*, 29–45.
Daly, T. J., & Matthews, K. S. (1986) *Biochemistry 25*, 5474–5478.
Ellenberger, T. E., Brandl, C. J., Struhl, K., & Harrison, S. C. (1992) *Cell 71*, 1223–1237.
Farabaugh, P. (1978) *Nature 274*, 765–769.
Gordon, A. J. E., Burns, P. A., Fix, D. F., Yatagai, F., Allen, F. L., Horsfall, M. J., Halliday, J. A., Gray, J., Bernelot-Moens, C., & Glickman, B. W. (1988) *J. Mol. Biol. 200*, 239–251.
Hope, I. A., & Struhl, K. (1986) *Cell 46*, 885–894.
Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. R. (1981) *Biophys. J. 36*, 575–588.
Kisters-Woike, B., Lehming, N., Sartorius, J., von Wilcken-Bergmann, B., & Müller-Hill, B. (1991) *Eur. J. Biochem. 198*, 411–419.
Kleina, L. G., & Miller, J. H. (1990) *J. Mol. Biol. 212*, 295–318.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Lamerichs, R. M. J. N., Boelens, R., van der Marel, G. A., van Boom, J. H., Kaptein, R., Buck, F., Fera, B., & Ruterjans, H. (1989) *Biochemistry 28*, 2985–2991.
Landschulz, W., Johnson, P. F., & McKnight, S. L. (1988) *Science 240*, 1759–1764.
LeClerc, J. E., Christensen, J. R., Tata, P. V., Christensen, R. B., & Lawrence, C. W. (1988) *J. Mol. Biol. 203*, 619–633.
Lehming, N., Sartorius, J., Oehler, S., von Wilcken-Bergmann, B., & Müller-Hill, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7947–7951.
Lehming, N., Sartorius, J., Kisters-Woike, B., von Wilcken-Bergmann, B., & Müller-Hill, B. (1990) *EMBO J. 9*, 615–621.
Lin, S., & Riggs, A. D. (1975) *Biochem. Biophys. Res. Commun. 62*, 704–710.
Maki, Y., Bos, T. J., Davis, C. Starbuck, M., & Vogt, P. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2848–2852.
Mandal, N., Su, W., Haber, R., Adhya, S., & Echols, H. (1990) *Genes Develop. 4*, 410–418.
McKay, D. B., Pickover, C. A., & Steitz, T. A. (1982) *J. Mol. Biol. 156*, 175–183.
Miller, J. H. (1979) *J. Mol. Biol. 131*, 249–258.
Miller, J. H., & Reznikoff, W. S., Eds. (1980) *The Operon*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, J. H., Platt, T., & Weber, K. (1970) in *The Lactose Operon* (Beckwith, J. R., & Zipser, D., Eds.) pp 343–351, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, J. H., Coulondre, C., Hofer, M., Schmeissner, U., Sommer, H., Schmitz, A., & Lu, P. (1979) *J. Mol. Biol. 131*, 191–222.
Müller-Hill, B. (1975) *Prog. Biophys. Mol. Biol. 30*, 227–252.
Nichols, J. C., Vyas, N. K., Quiocho, F. A., & Matthews, K. S. (1993) *J. Biol. Chem. 268*, 17602–17612.
Ogata, R., & Gilbert, W. (1979) *J. Mol. Biol. 132*, 709–728.
O'Gorman, R. B., Dunaway, M., & Matthews, K. S. (1980) *J. Biol. Chem. 255*, 10100-10106.
O'Shea, E. K., Klemm, J. D., Kim, P. S., & Alber, T. (1991) *Science 254*, 539–544.
Pace, H. C., Lu, P., & Lewis, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1870–1873.
Pilz, I., Goral, K., Kratky, O., Bray, R. P., Wade-Jardetzky, N. G., & Jardetzky, O. (1980) *Biochemistry 19*, 4087–4090.
Platt, T., Files, J. G., & Weber, K. (1973) *J. Biol. Chem. 248*, 110–121.
Riggs, A. D., Bourgeois, S., Newby, R., & Cohn, M. (1968) *J. Mol. Biol. 34*, 365–368.
Ryder, K., & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8464–8467.
Ryder, K., Lau, L. F., & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1487–1491.
Ryder, K., Lanahan, A., Perez-Albuerne, E., & Nathans, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1500–1503.
Sams, C. F., Hemelt, V. B., Pinkerton, F. D., Schroepfer, G. J., Jr., & Matthews, K. S. (1985) *J. Biol. Chem. 260*, 1185–1190.
Schmitz, A., Schmeissner, U., Miller, J. H., & Lu, P. (1976) *J. Biol. Chem. 251*, 3359–3366.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 306 and 345, John Wiley & Sons, New York.
Winzor, D. J. (1969) in *Physical Principles & Techniques of Protein Chemistry* (Leach, S. J., Ed.) p 469, Academic Press, New York.