Communication

Designed Disulfide between N-terminal Domains of Lactose Repressor Disrupts Allosteric Linkage*

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Catherine M. Falcon, Liskin Swint-Kruse‡, and Kathleen S. Matthews§

From the Department of Biochemistry & Cell Biology, Rice University, Houston, Texas 77005-1892

Substitution of Cys for Val at position 52 of the lac repressor was designed to permit disulfide bond formation between the two N-terminal DNA binding domains that comprise an operator DNA binding site. This position marks the closest approach of these domains based on the x-ray crystallographic structures of the homologous purine holorepressor-operator complex and lac repressor-operator complex (Schumacher, M. A., Choi, K.Y., Zalkin, H., and Brennan, R.G. (1994) Science 266, 769–776; Lewis, M., Chang, G., Horton, N.C., Kercher, M.A., Pace, H. C., Schumacher, M. A., Brennan, R. G., and Lu, P. (1996) Science 271, 1247–1254). The V52C mutation was generated by site-specific methods, and the mutant protein was purified and characterized. In the reduced form, V52C bound operator DNA with slightly increased affinity. Exposure to oxidizing conditions resulted in disulfide bond formation, and the oxidized protein bound operator DNA with ~6-fold higher affinity than wild-type protein. Inducer binding for both oxidized and reduced forms of V52C was comparable to wild-type lac repressor. In the presence of inducer, the reduced protein exhibited wild-type, diminished DNA binding. In contrast, DNA binding for the oxidized form was unaffected by inducer, even at 1 mM. Thus, the formation of the designed disulfide between Cys52 side chains within each dimer renders the protein-operator complex unresponsive to sugar binding, presumably by disrupting the allosteric linkage between operator and inducer binding.

The lac operon is a well-studied system for discerning principles that govern regulation of genetic expression (1, 2). Key to lac operon regulation is the lac repressor (LacI), a homotetramer of 150 kDa that binds to a specific operator sequence within Escherichia coli DNA to negatively regulate transcription of the lac operon (2–4). Each LacI monomer can be divided into distinct structural domains. The N-terminal ~60 amino acids comprise the DNA binding domain and encompass a helix-turn-helix motif common to many repressor proteins (5–7). Two pairs of N-terminal domains form two high affinity operator sites per LacI tetramer (8–10). The operator binding site itself has partial 2-fold symmetry (11), with the two “half-sites” of each operator sequence in contact with one N-terminal domain within each dimer unit (10, 12, 13). A hinge region (amino acids 50–60) tethers the N-terminal DNA binding domain to the remaining core region (amino acids 60–360) (10, 14, 15). The core domain contains the inducer binding site and the oligomeric assembly determinants (2, 10, 16–24). In the presence of inducer sugars, LacI undergoes a conformational change to a state with diminished affinity for operator but unaltered binding to nonspecific DNA sites present in much higher concentration in E. coli DNA (2, 25).

Of particular interest is how conformational shifts evoked by sugar binding are relayed from the inducer binding region to the distant DNA binding site, eventuating in release of operator DNA. The small hinge sequence that connects the N-terminal and core domains must be involved in this communication, since the x-ray crystallographic structure of the protein-operator complex reveals few direct contacts between these distinct domains (see Fig. 1) (10). Mutations in this region can abolish or enhance operator binding (23, 24, 26, 27). The crystal structure of LacI-operator DNA reveals that this hinge segment folds into an α-helix with side chains that insert into the DNA minor groove (10). In the free or inducer-bound state, this sequence is not defined crystallographically (10), and experimental evidence demonstrates that the region is unfolded in the absence of operator (28, 29).

Based on the crystal structures of the highly homologous purine repressor, PurR holorepressor-DNA (30), and LacI-DNA complexes (10), a mutation was designed at Val52 within the hinge region to generate a protein in which a disulfide bond could be formed between the N-terminal domains (Fig. 1). Consequently, the spatial relationship between the two N-terminal binding domains that form an operator binding site would be fixed. Generation of the anticipated disulfide bond in the V52C mutant protein resulted in increased affinity for operator and loss of sugar responsiveness. Thus, introduction of a disulfide between the N-terminal domains in the hinge region interrupts allosteric communication between the ligand binding domains.

**MATERIALS AND METHODS**

*Mutagenesis—Plasmid pJC1 (20) contains the complete LacI gene and was used as the mutagenesis vector. Oligonucleotides for mutagenesis were purchased from the Great American Gene Co. (Ramona, CA). The Chameleon double-stranded mutagenesis protocol from Stratagene (La Jolla, CA) was followed using a selection oligonucleotide that contained a 3-base pair change to alter the Val codon to a Cys codon. The oligonucleotides were used in >100-fold excess over the pJC1 concentration. The oligonucleotides were annealed to denatured plasmid DNA, and then extension and ligation were performed using T7 polymerase (New England Biolabs, Beverly, MA). T4 ligase, and single-stranded binding protein (Promega, Madison, WI). After inactivation of T4 ligase, an initial digestion with PstI was followed by transformation into XLmutS cells (Stratagene). Plasmid DNA purified from these cells using the Wizard preparation protocol (Promega) was digested with PstI and then transformed into DH5α cells (Life Technologies, Inc.). The plasmid DNA was then screened for the selection site (Xhol). Colonies were then selected for increased affinity for operator DNA binding.***
Fig. 1. Lactose repressor dimer crystal structure. The polypeptide backbone of one of the two dimers within the x-ray crystallographic structure of tetrameric lact repressor bound to operator DNA is shown in panel A (derived from Protein Data Bank file 1LBG) (10). Panel B provides a view of the helix-turn-helix DNA binding domain and hinge helix from this structure. This view is from the top surface of the protein. Panel C provides a view of the segment of the protein as shown in panel B, but rotated out of the plane of the paper. The backbone positions of Val52 in the hinge helices are highlighted as balls; the distance between the Cα for Val3 in each dimer of the protein is 5.76 Å. Only the backbone position can be shown, as side chains were not resolved in the structure complexed with DNA, and no electron density for the residues previous to position 62 are detected for free and IPTG-bound lac repressor. The Val52 side chain positions were explored using the highly homologous PurR structure to confirm that the orientation of the side chain provides the potential for disulfide bond formation for the V52C protein.

that carried the selection site were sequenced using dyeode sequencing (31). Full sequencing of the LacI gene verified the presence of only the expected mutation.

Protein Purification—For protein expression, the plasmid DNA encoding V52C was transformed into BL26 cells (BL26Blue cells from Novagen, Madison, WI, which are ompT hsdSb rK lac dcm lacI [proAB lacI 57; M15: TusTc]) (32), which had been cured of the episome that carries the lac promoter and the lac gene. The protein was purified as described previously (20). Cells frozen in lysing buffer (0.2 M Tris-HCl (pH 7.5), 0.2 M KCl, 0.01 M Mg(OAc)2, 5% glucose, and 50 μg/mL phenylmethylsulfonyl fluoride) were thawed in the presence of lysozyme (0.5 mg/mL). DNase was added, and the lysed cells were centrifuged followed by precipitation of the supernatant with 40% ammonium sulfate. The precipitate was centrifuged, and the supernatant was dialyzed overnight against 0.05 M potassium phosphate (pH 7.4), 5% glucose. The protein was loaded onto a phosphocellulose column equilibrated with the same buffer and eluted with a gradient from 0.12–0.3 M potassium phosphate (pH 7.4), 5% glucose. Fractions containing LacI activity were collected, and the protein was found to be >90% pure by SDS-PAGE. 3 Throughout purification and isolation, the protein activity was detected by the 11C-IPTG assay as described by Bourgeois (32).

Briefly, protein was mixed with [14C]IPTG, and the protein was precipitated with 70% ammonium sulfate. The precipitate was resuspended, and the supernatant was dialyzed overnight against 0.05 M potassium phosphate (pH 7.4), 5% glucose. The protein was loaded onto a phosphocellulose column equilibrated with the same buffer and eluted with a gradient from 0.12–0.3 M potassium phosphate (pH 7.4), 5% glucose. Fractions containing LacI activity were collected, and the protein was found to be >90% pure by SDS-PAGE. 3 Throughout purification and isolation, the protein activity was detected by the 11C-IPTG assay as described by Bourgeois (32).

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RESULTS AND DISCUSSION

From careful analysis of the LacI and PurR crystallographic structures (10, 30), Val52 in LacI was identified as the only potential protein-protein contact between the two hinge helices in each dimer component (Fig. 1). Fortunately, the Val52 side chains could be rotated so that substituted Cys side chains did not overlap with other side chains, and the distance between the Cβ atoms at position 52 in each polypeptide within a dimer unit was optimal for disulfide formation. Therefore, the codon for this position was altered to encode Cys using double-nucleotide mutagenesis. The entire sequence of the DNA encoding the protein was determined to ensure that the designed change was the only alteration present. The V52C protein was purified and found to form a dimer under oxidizing conditions (Fig. 2); the extent of dimer formation was >90%. Under reducing conditions, the same sample co-migrated with the monomer of wild-type LacI. Thus, a disulfide bond that links two monomers is able to form uniquely in the V52C protein and can be reduced effectively.

V52C protein was examined for ligand binding characteristics under both oxidizing and reducing conditions using glutathione as the redox agent. Buffers to maintain the reduced form contained 20 mM DTT. Reduced V52C exhibited slightly in-
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Fig. 2. SDS-polyacrylamide gel electrophoresis of wild-type LacI and oxidized and reduced forms of V52C. Oxidation/reduction reactions were carried out as described under “Materials and Methods.” Electrophoresis was performed on a 12% polyacrylamide gel in Tris-glycine buffer (25 mM Tris, 250 mM glycine (pH 8.3), 0.1% SDS) in the absence of DTT or other sulphydryl reagents. Bands were visualized by silver staining. The amount of protein loaded into each lane was approximately 4 μg. Lane 1, molecular mass markers; lane 2, V52C + 50 mM glutathione, reduced form; lane 3, wild-type LacI + 50 mM glutathione, reduced form; lane 4, V52C + 50 mM glutathione, oxidized form; lane 5, wild-type LacI + 50 mM glutathione, oxidized form.

Table I

| Protein          | Operator binding affinity<sup>a</sup> | Inducer binding affinity<sup>b</sup> |
|------------------|--------------------------------------|-------------------------------------|
|                  | [protein]/([protein] + [DNA])        | [protein]/([protein] + [DNA])        |
|                  | - IPTG                               | + IPTG                              |
| Wild-type        | 12 ± 2                               | 2.7 ± 0.5                           |
| V52C-reduced     | 5.4 ± 1                              | 2.3 ± 0.4                           |
| V52C-oxidized    | 1.9 ± 0.5                            | 1.7 ± 0.3                           |

<sup>a</sup> Values are derived from global fits to four independent experiments. Experimental details are provided in the legend to Fig. 3.

<sup>b</sup> Values are derived from global fits to data from three independent experiments using 13 IPTG concentrations ranging from <10<sup>-5</sup> M to 10<sup>-4</sup> M.

Increased affinity for operator DNA compared with wild-type protein (Table I, Fig. 3), consistent with previous studies that indicate some substitutions in this position will generate “tight binding” behavior (23, 24, 26, 27). In contrast, the oxidized form of V52C, with the disulfide linkage between two subunits, exhibited 6-fold tighter binding to operator than wild-type protein (Table I, Fig. 3). Furthermore, the formation of the disulfide linkage abolished inducer response for V52C protein, with operator binding affinity in the presence of 10<sup>-3</sup> M IPTG comparable to that in the absence of inducer (Table I, Fig. 3). In contrast, reduced V52C was inducible by IPTG to a degree similar to the wild-type protein. Thus, the formation of a disulfide bond linking the two N-terminal domains within each dimer appears to interrupt the conformational transition that elicits induction.

To determine whether the reduced V52C differed in IPTG response from the wild-type protein at differing inducer concentration, repressor-operator release in the presence of sugar was examined. The protein-operator DNA complex was formed under conditions expected to generate ~80% saturation, and the complex was then exposed to varying concentrations of inducer. Wild-type LacI exhibited a transition with a midpoint at a slightly higher IPTG concentration (data not shown). However, the disulfide form of the mutant protein was unresponsive to inducer even at high concentrations.

Since operator binding for the oxidized V52C was unresponsive to IPTG, the inducer binding capacity of these proteins was explored to establish whether disulfide bond formation disrupted the capacity of the protein to bind sugar. IPTG binding was monitored by the influence of sugar binding on the fluorescence properties of the protein, and both forms of V52C were compared with the wild-type protein. At pH 7.4, all three proteins generated similar binding curves that yielded comparable binding affinities (Table I). Thus, both oxidized and reduced V52C maintain the ability to bind inducer effectively.

The formation of the disulfide linkage involving Cys at position 52 results in a protein with enhanced DNA binding capacity and wild-type inducer binding properties, but without the ability to respond to inducer. The increased affinity observed for operator binding with the oxidized protein presumably derives, at least in part, from the decreased entropic cost in “fixing” the two N-terminal domains into the optimal orientation for operator binding. This entropic cost has been paid separately as part of the energy of forming the disulfide bond.

The ability to disconnect inducer and operator binding in V52C is of utmost interest. Structural shifts that accompany inducer binding (10) are not transmitted to the N-terminal domains when they are covalently linked by the disulfide bond. Apparently, the disulfide bond disrupts the allosteric communication completely and generates a protein that is unable to...
respond to inducer. Further examination of this mutant protein in its oxidized and reduced states may provide more detailed insight into the mechanism of allosteric communication required for induction to release the lac operator site.

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