Combinatorial Mutations of lac Repressor

STABILITY OF MONOMER-MONOMER INTERFACE IS INCREASED BY APOLAR SUBSTITUTION AT POSITION 84*

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Jeffry C. Nichols and Kathleen S. Matthews‡

From the Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251

To examine the monomer-monomer subunit interface in the lac repressor, a mutation that generates dimeric protein (deletion of C-terminal amino acids to disrupt the dimer-dimer interface) has been combined with amino acid substitutions that alter the monomer-monomer interface (substitution at Lys84 or Tyr282). Dimeric proteins with significantly increased stability to urea denaturation were formed by the introduction of the apolar amino acids Ala or Leu in lieu of Lys84 in concert with the deletion of 11 C-terminal amino acids. K84A/−11 deletion protein retained wild-type affinity for operator DNA, while K84L/−11 deletion protein displayed operator affinity similar to its parent tetramer. To assess further the influence of monomer-monomer interface stability on assembly and DNA binding, triple mutants were generated with Y282D, an alteration that disrupts assembly completely in the wild-type background. The triple mutants were dimeric, but they exhibited diminished dimer stability to urea denaturation and decreased operator affinity compared with the double mutations. These results demonstrate directly the stabilizing influence of apolar substitution at position 84 on the monomer-monomer interface.

High affinity binding of repressor to operator sequences within the lac operon inhibits transcription of mRNA for the lactose metabolic genes (1). A conformational change in response to binding inducer sugars, including a metabolite of lactose that serves as the in vivo inducer, results in diminished affinity for the operator sites and a consequent increase in lac mRNA transcription (1–3). Each monomer of the homotetrameric lac repressor (4, 5) is composed of 360 amino acids (6–9). The repressor contains two operator binding sites and four inducer binding sites (2, 10–15) and is divided into two domains, one involved primarily in DNA binding (amino acids −1–59) and the other in assembly and sugar binding (amino acids −60–360) (14–29). A hinge region, susceptible to protease digestion (19), connects these two domains and not only provides a physical connection for potential communication between the two ligand binding sites but also is involved directly in DNA binding (15). The binding functions of the protein are not only linked through the monomer structure but also are determined by the oligomeric and conformational states of the protein (14, 15, 18, 19, 21, 30–32). Monomeric mutant repressors generated by mutations at Tyr282 do not bind operator DNA (20, 33, 34), while dimeric mutant proteins are unable to form bidentate complexes with DNA (25, 26).

The recently solved x-ray crystallographic structures of the repressor core domain (amino acids 59–360) and intact protein (14, 15) and experimental results on a variety of mutant proteins (20, 28, 31, 32, 34, 35) demonstrate that monomer-monomer association to dimers involves multiple side chain interactions across both N- and C-subdomains within the core region (Fig. 1). These amino acids are spread over a significant portion of the primary sequence of the repressor protein. Lys84 and Tyr282 are each located in this interface and are positioned to make major energetic contributions to the association (14, 15, 35). However, the specific effects of side chain alterations along this interface on the stability of assembly have not been explored previously.

In contrast to the monomer-monomer interface, the assembly of dimers to tetramer involves a short segment at the C terminus of the protein that forms a four-helix bundle (14, 15). Mutations or deletions in the C-terminal region have demonstrated directly the requirement for the leucine heptad repeat motif found in this region for dimer-dimer assembly (25–27, 29). The importance of oligomerization to operator binding has been studied using the dimeric products of both missense and deletion mutations in the heptad repeat region (25, 27, 29, 36, 37). The observed operator binding affinity of these dimeric proteins was 10–100-fold lower than that for wild-type protein due to a linked equilibrium between monomer association to dimer and dimer-operator binding (36, 38). Although the assembled dimer presumably presents the same face to the operator DNA sequence as the tetramer (14, 15), the dimeric mutants dissociate more readily into monomers in the absence of the C-terminal coiled-coiled region, and the monomer binds with only very low affinity to DNA (20, 33, 34). Thus, the apparent $K_d$ does not reflect the intrinsic DNA binding capacity of dimer species for which the dimer-dimer interface is destabilized (36, 38). In contrast to mutations that eliminate interaction at the C terminus, a construct in which the C terminus of the lac repressor was replaced with the GCN4 sequence that generates a very stable parallel coiled-coil structure (26) exhibited higher stability under denaturing conditions and wild-type DNA binding affinity, as would be anticipated for a stabilized dimer (30, 38).

The repressor protein has been found to contain discrete regions that contribute to the different properties of the protein, reflected in the structure of the protein (14, 15, 39). Because DNA binding requires the dimeric structure and bidentate loop formation involving two operator sites requires tetramer, stable assembly of the repressor is crucial to its regulatory function. However, the unique influence of alter-
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50 μg) in a total volume of 100 μl was reacted with SDS (0%, 0.01%, and 0.02%) at room temperature for 15 min. The solutions were then filtered onto nitrocellulose paper (presoaked with phosphate-buffered saline), and the proteins were reacted with B-2 antibody as described previously (40). The only difference was that the secondary antibody reaction employed a chemiluminescence kit from Amersham, with the resulting reactions visualized through exposure to x-ray film.

Trypsin Digestion—Each protein (0.25 mg/ml in 0.12 M potassium phosphate buffer, pH 7.5) was incubated with 2% (w/v) trypsin (in 0.001 M HCl) for 30 min at room temperature. For control reactions, 0.001 M HCl was added in lieu of trypsin. The reactions were stopped by the addition of 2% (w/v) phenylmethysulfonyl fluoride (in 100% ethanol). The digested proteins were then run on a 10% SDS-polyacrylamide gel, and protein products were visualized using Coomassie Blue staining.

Analytical Ultracentrifugation of Proteins—Double sector cells were used for sedimentation velocity studies, and six channel cells were used for equilibrium analysis using a Beckman XL-A analytical ultracentrifuge. In each case, a minimum of three protein concentrations was studied for each mutant. For velocity sedimentation, ~400 μl of protein (0.06–0.6 mg/ml) was loaded, the samples were centrifuged at a maximum speed of 42,000 rpm, and the cells were scanned at 280 nm. The data were analyzed using a software package provided by Beckman, and the sedimentation coefficient was determined at each concentration point using two methods: 1) the second moment algorithm (41, 42) and 2) the time derivative method (43–45). In both cases, the Svedberg values were corrected for solvent effects and viscosity (46).

For equilibrium sedimentation, the cells were loaded with ~100–110 μl of protein at varying concentrations. Samples were centrifuged at 5,000, 10,000, and 16,000 rpm, allowing the sample to reach equilibrium before increasing the speed. Using the data analysis software, plots of ln(Cv) versus r2 were fit using a least squares algorithm, where Cv is the concentration at a specific radial position, r.

Operator Binding Assay—A modified procedure of Riggins et al. (47) and Wong & Lohman (48) was used. Protein at various dilutions was mixed with 125I-end-labeled 40-base pair operator DNA (~10–12 M). After a short incubation, reactions were filtered on nitrocellulose using a 96-well plate, and the nitrocellulose was dried and exposed to a Fuji bio-imaging for visualization. The plate was read on a Fuji BAS1000 bio-imaging analyzer, and the radioactivity was quantitated using the program MacBas version 2.0 on a Macintosh computer. Free operator DNA bound to nitrocellulose was used as background in lieu of IPTG1 additions due to the very slow inducer association rate for the mutants in this study. After subtracting background intensity, the values were expressed as fractional saturation (R) and plotted against log protein concentration. The data were fit by nonlinear least squares analysis using the equation,

\[ R = \frac{[\text{protein}]}{K_D + [\text{IPTG}]} \]

utilizing the program Igor Pro on a Macintosh computer. All experiments were performed in 0.1 M potassium sulfate, 0.01 M Tris-HCl, 5% Me2SO, pH 7.5, buffer. To reduce nonspecific binding, 100 μg/ml bovine serum albumin was added to buffer used for dilutions and filtration.

IPTG Binding—From previously reported data, proteins containing Lys48 substitutions had Kd values for IPTG binding similar to wild-type (Kd ~10–5 M) but bound to inducer much more slowly than wild type (35), precluding use of the standard IPTG binding assay. A new procedure was developed using individual reactions of 1.5 ml of protein solution at 1 × 10–7 M concentration (tetramer) with varying concentrations of IPTG. Following overnight incubation at 4°C, total fluorescence was measured using an SLM 8100 spectrofluorometer. The excitation wavelength was 285 nm directed through a filter (7-54, Corning), and emission fluorescence was measured using a 350-nm cut-off filter (O-52, Corning). Fluorescence intensity was converted to fractional saturation (R), and the data were fit by nonlinear least squares analysis using the equation,

\[ R = \frac{[\text{IPTG}]^n}{K_D^n + [\text{IPTG}]^n} \]

with the program Igor Pro, where K_D is the equilibrium dissociation constant and n is the Hill coefficient for IPTG binding. Buffers used were 0.1 M potassium sulfate, 0.01 M Tris-HCl at both pH 7.5 and pH 9.2.

1 The abbreviation used is: IPTG, isopropyl-β-D-galactopyranoside.
RESULTS AND DISCUSSION

Construction and Characterization of Mutants—Using restriction enzymes sites that flank the selected regions, the −11 deletion coding sequence was combined with the K84A, K84L, K84A/Y282D, and K84LY282D coding sequences to generate double and triple mutations. The entire coding region for these combinatorial mutants was sequenced to verify the desired changes and to eliminate the possibility of other alterations. Purification of the repressor mutants utilized the affinity of the DNA binding domain for phosphocellulose. SDS-polyacrylamide gels and Western blotting indicate high purity of the purified mutant proteins (Fig. 2). The yield for triple mutant proteins was lower than that for double mutant proteins, the first suggestion that the triple mutant proteins were less stable.

Antibody Binding—Antibody binding under denaturing conditions allows an indirect measurement of conformation and stability of a mutant protein as compared with wild-type repressor (40). Previous experiments have shown that native wild-type protein will react with B-2 monoclonal antibody in the presence of low concentrations of SDS, while monomeric repressor protein is reactive with this monoclonal antibody in the absence of SDS (33, 40). The results for dimeric proteins examined previously tend to fall somewhere in between these two extremes (29, 30, 37). The double mutants in this study reacted more strongly in the absence of SDS and demonstrated only partial reactivity in the presence of SDS (Fig. 3). These results indicate that the mutations at residue 84 impart conformational changes that expose the antibody epitope located in the C terminus in the dimer and make the epitope less available in the presence of SDS. The differences seen between the K84A/Y282D−11 deletion and the K84L/Y282D−11 deletion suggest that the character of the side chain at position 84 plays an important role in the conformation of the dimer that determines epitope availability. The K84A/Y282D−11 deletion is more reactive to antibody, regardless of SDS, as compared with the K84LY282D−11 deletion. This effect may be a consequence of the differential structural rigidity imparted by leucine versus alanine substitution at position 84.

Trypsin Digestion—Mild proteolysis results in the cleavage of the DNA binding domain (amino acids 1–59) from the core domain of the lac repressor (19, 52). The double and triple mutants, as well as wild type, were reacted with 2% (w/w) trypsin (Fig. 4). The results for the double and triple mutants clearly indicate their higher susceptibility to trypsin digestion compared with wild-type protein, which is digested to a single species under identical conditions. The digestion patterns for the double mutants and the K84LY282D−11 deletion are similar, while the pattern of protein fragments for the triple...
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**TABLE I**

| Protein                | Sedimentation coefficient | Molecular weight |
|------------------------|---------------------------|------------------|
|                        | $s_{obs}$ | $s_{pol}$ | $g(s^*)$ |                                      |
| Wild type              | 5.6       | 6.6       | 6.3       | 7.6       | 127,000 ± 4,300                      |
| K84A/−11 deletion      | 3.6       | 4.3       | 3.6       | 4.3       | 66,400 ± 3,100                       |
| K84L/−11 deletion      | 4.0       | 4.8       | 3.9       | 4.7       | 60,000 ± 2,900                       |
| K84A/Y282D/−11 deletion| 3.2       | 3.9       | 3.3       | 3.9       | 61,500 ± 4,600                       |
| K84L/Y282D/−11 deletion| 3.9       | 4.6       | 3.8       | 4.5       | 61,200 ± 1,800                       |

**TABLE II**

| Repressor protein       | Operator binding ($K_d \times 10^6 \text{ M}$) | IPTG binding ($K_d \times 10^6 \text{ M}$) |
|-------------------------|-----------------------------------------------|-------------------------------------------|
| Wild type               | 1.7 ± 0.2                                     | 1.7 ± 0.1                                 |
| K84A/−11                | 2.0 ± 0.5                                     | 2.0 ± 0.5                                 |
| K84L/−11                | 1.9 ± 0.5                                     | 1.9 ± 0.5                                 |
| K84A/Y282D/−11 deletion | 2.3 ± 0.4                                     | 2.3 ± 0.4                                 |
| K84L/Y282D/−11 deletion | 2.3 ± 0.4                                     | 2.3 ± 0.4                                 |
| −11 deletionb           | 11 ± 1                                        | 1.8 ± 0.2                                 |

* The protein was dimeric by sedimentation analysis at the concentration used for IPTG binding.

* Data from Ref. 30.

Inducer Binding—The $K_d$ for wild-type protein at pH 7.5 is $-2 \times 10^{-6} \text{ M}$, while at pH 9.2 the affinity for IPTG decreases to $-8\text{ fold}$ (54, 55). For the dimeric C-terminal deletion mutants, the IPTG binding properties were very similar to wild-type protein (29). For the parent tetrameric mutant proteins (K84A, K84L, K84A/Y282D, and K84L/Y282D), IPTG binding affinity was similar to that of wild-type protein at neutral pH, but with no significant decrease in affinity at elevated pH levels. However, because the rate of inducer binding for the Lys84 mutants was diminished more than 100-fold, a new procedure was developed to examine IPTG binding by fluorescence for the double and triple mutants in this study. Individual reaction mixtures at a protein concentration (tetramer) of $1 \times 10^{-7} \text{ M}$ containing differing amounts of IPTG were incubated overnight at 4 °C. To ensure the viability of this procedure, wild-type protein was analyzed alongside the other mutant proteins. Table II summarizes the binding affinity for this set of proteins, and the individual binding curves can be seen in Fig. 5. Although affinity measurements for the wild type are consistent with previous data, the Hill coefficients for wild-type protein did not correspond to previously determined values, perhaps due to the long incubation times; these values are not reported. The similarity of the derived binding affinities of the double and triple mutants at neutral pH to wild type indicates that disruption of either or both subunit interfaces does not markedly affect sugar binding affinity, consistent with previous studies (29, 34). In the double mutants examined, affinity for the inducer is decreased to $-2\text{ fold}$ at pH 9.2; the added stability provided by apolar substitution in the N-subdomain subunit interface (see Fig. 1) may preclude the conformational changes with pH values that generate diminished affinity for the inducer, as observed for the parent tetrameric protein. In contrast, at elevated pH the triple mutants exhibit lower affinity for IPTG, and the pH-associated shift is greater than wild-type or the parent tetramer for the K84A/Y282D/−11 deletion protein. Disruption of the C-subdomain interface coupled with apolar substitution at position 84 may provide the requisite flexibility for pH-induced structural shifts. The effect of pH on the inducer binding behavior of the double and triple mutants correlates loosely with the stability of the monomer-monomer interface.

**Fig. 4. Trypsin digestion of double and triple mutant proteins.** Protein concentration was 0.25 mg/ml in 0.12 M potassium phosphate buffer, pH 7.5. Trypsin was added at a concentration of 2% (w/w). The reaction products were run on a 10% SDS-polyacrylamide gel, and the bands were visualized using Coomassie Blue stain. Odd numbered lanes were not reacted with trypsin; even numbered lanes were. Lanes 1 and 2, wild-type; lanes 3 and 4, K84A/−11 deletion; lanes 5 and 6, K84L/−11 deletion; lanes 7 and 8, K84A/Y282D/−11 deletion; lanes 9 and 10, K84L/Y282D/−11 deletion.

Mutant K84A/Y282D/−11 deletion appears to have a larger fraction of a faster migrating species (lane 8). This difference may be a consequence of lower oligomer stability in this particular mutant compared with the corresponding Leu substitution. The generally increased susceptibility of the double and triple mutants to protease digestion may derive from distortions in the hinge region and N-subdomain; these distortions may be generated by more rigid N-subdomain association that also results in increased monomer-monomer stability (see below).

Analytical Ultracentrifugation of Wild-type and Mutant Proteins—Sedimentation velocity experiments were performed to determine the Svedberg value, $s$, for the mutant and wild-type proteins. For comparison, the value of $s$ was determined and compared using two separate methods: 1) the second moment or boundary spreading method (41), which calculates $s$ (42) based on the movement of the boundary position; and 2) the time derivative method, which uses a time derivative of the concentration at each radial position to calculate an apparent sedimentation coefficient distribution pattern ($g(s^*)$) (43–45). In both cases, only those scans that had generated a depleted area between the meniscus and the boundary were used. In each case, values for $s$ were corrected for solvent density and specific volume effects. The resulting $s_{pol}$ values are shown in Table I. The corrected $s$ values for the wild-type $lac$ repressor range from $6.6 \times 10^{-13}$ (second moment) to $7.6 \times 10^{-13}$ (time derivative), in agreement with previously reported values of $-7.0 \times 10^{-13}$ (53). The values for the mutants, which are all smaller than wild-type values, indicate their dimeric state. The slight differences in the $s$ values among the mutants probably arise from either their intrinsic association properties or differences in packing of the structure.

While the sedimentation velocity results established that the mutants differ significantly in shape and size from the wild-type protein, we wished to establish the molecular weights directly by equilibrium sedimentation. The derived molecular weight values for the double and triple mutant proteins as well as wild type can be seen in Table I. These molecular weight determinations were obtained from a global fit of the data taken at varying sedimentation speeds and/or varying protein concentrations. These data provide direct evidence that the double and triple mutants exist as dimeric species.
Operator Binding—The binding of the wild-type lac repressor to a 40-base pair operator DNA sequence in 0.1 M K2SO4 buffer is 2\times 10^{-10} M (38). The operator binding affinities for dimeric proteins range from equivalent to 10–100-fold lower than wild-type protein (Table II) (29, 38). The individual binding curves for the proteins with double and triple mutations can be seen in Fig. 6. The double mutant K84A/−11 deletion binds operator comparably with the wild-type protein, as did its tetrameric parent K84A (35); these data indicate that this dimer does not dissociate to monomer under conditions used for DNA binding. The wild-type affinity for the operator occurs because interactions of the apolar amino acid alanine presumably provide energetic compensation for the loss of monomer-monomer stability normally provided by the C terminus.

Given the change to an apolar amino acid at position 84 for the K84L/−11 deletion, this protein might also be expected to bind DNA with wild-type affinity. However, this mutant binds operator DNA with ~10-fold lower affinity than wild type but with an affinity similar to that of its tetrameric parent, K84L (35). The lower affinity for both tetrameric and dimeric mutant proteins with this substitution may derive from the effect of the bulky, apolar leucine side chain at the subunit interface, resulting in alteration of the orientation and/or alignment of the N-terminal DNA-binding domains crucial for optimal DNA binding (15). However, dissociation to monomer does not appear to contribute substantially to the diminished DNA affinity for this mutant, since the K84L tetramer and K84L/−11 deletion dimer affinities for the operator are similar.

The triple mutant proteins, K84A/Y282D/−11 deletion and K84L/Y282D/−11 deletion, had an observed $K_d$ ~30–60-fold lower than either wild-type protein or K84A/−11 deletion mutant protein. While the apolar K84A or K84L substitution can compensate for the −11 deletion and maintain a stable dimeric state in the double mutants, further introduction of the Y282D mutation in the C-terminal subdomain disrupts the monomer-monomer interface significantly, generating diminished operator binding. Although these proteins form dimer in the concentration range employed for sedimentation experiments, the diminished stability of the monomer-monomer interface may result in dissociation to monomer at the protein concentrations necessary to measure operator affinity.

Denaturation of Mutant Proteins—The equilibrium constant, and consequently the free energy change, between the assembled/folded and unfolded states can be obtained through the use of denaturants such as urea, guanidine hydrochloride, or temperature. Since the denaturation experimental data define an equilibrium constant, for analytical purposes the reaction must reach equilibrium, and further the reaction must be reversible (51). Using methods previously employed for lac repressor mutant proteins (37, 38), denaturation reactions in urea were allowed to reach equilibrium at room temperature for 2–24 h before fluorescence intensity measurements were taken, and it was found that the shorter time allowed equilibrium to be achieved. Reversibility was established for urea...
denaturation of the triple mutants K84A/Y282D/−11 deletion and K84L/Y282D/−11 deletion and the −11 deletion protein (38). However, denaturation of the mutant proteins K84A/−11 deletion and K84L/−11 deletion was incomplete even at the maximum urea concentrations obtainable (Fig. 7). To explore this phenomenon further, the stronger denaturant guanidine hydrochloride was employed, despite the fact that unfolding of the triple mutants and −11 deletion mutant occurred at very low concentrations in this denaturant (Fig. 7). Unfortunately, the unfolding and refolding of the more stable mutants did not follow the same pathway in guanidine hydrochloride, precluding assessment of the equilibrium constant and therefore the free energy of denaturation. Lowering the pH to 6.5 in an attempt to destabilize the double mutant proteins did not alter their behavior, and using lower pH values resulted in the precipitation of wild-type protein.

The calculated values for ΔG at zero denaturant concentration (ΔG₀) and m, which is a measure of the dependence of ΔG on denaturant concentration (51), can be found in Table III for the urea data. The data for the K84A/−11 deletion in urea were derived from the partial unfolding observed for this protein; no value could be estimated for the K84L/−11 deletion, indicating a stability of >21 kcal/mol, presuming a similar m value for this protein. The individual fits for fractional unfolding versus denaturant can be seen in Fig. 8. From the calculated ΔG₀ values, the pattern of stability of the mutant proteins, ranked from the most to the least stable, is as follows: K84L/−11 deletion > K84A/−11 deletion > −11 deletion > K84A/Y282D/−11 deletion. Not only does the apolar substitution at residue 84 strengthen the dimeric structure, but these changes generate sufficient stability to compensate for Y282D mutation, an alteration that normally disrupts the monomer-monomer interface completely to generate monomer in the wild-type background. The denaturation data provide convincing evidence for the stabilizing effect of apolar substitution at residue 84 and underscore the key contribution of the monomer-monomer interface to overall protein stability.

Conclusion—The goal of this effort was to combine mutations that affect monomer-monomer interactions (residues 84 and 282) with those that affect dimer-dimer interactions (C-terminal deletions) to examine independently the influence of substitutions in the monomer-monomer interface on protein structure and function. All mutant proteins generated were dimeric, as expected due to the requirement of the leucine heptad repeat region for tetramerization (14, 15, 24–27, 29, 56, 57). The ability of the double mutants to resist unfolding in high concentrations of urea and their high affinity operator binding provide evidence for an extremely stable dimer of lac repressor. Significantly increased stability associated with the replace-

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**Fig. 6. Operator DNA binding curves of double and triple mutant proteins.** Titrations were performed as described under “Materials and Methods.” Operator DNA concentration was ~2 × 10⁻¹⁰ M, and protein concentration was varied as indicated. The line shown is the best fit to the equation R = [protein]/([protein] + Kd); the data are the average of three separate experiments with S.D. shown as error bars. A, wild-type protein; B, K84A/−11 deletion; C, K84L/−11 deletion; D, K84A/Y282D/−11 deletion; E, K84L/Y282D/−11 deletion.
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Measurement of polar amino acids involved in a salt bridge network by hydrophobic residues has also been observed in the Arc repressor protein (58). These results combine to suggest that properly positioned apolar residues may provide a significant stabilizing force for assembly and consequently for functional properties associated with the oligomeric structure. However, the specific binding characteristics generated may depend on whether the altered monomer can fold into a wild-type conformation, as illustrated by the differences observed between the K84A/211 deletion and K84L/211 deletion proteins.

The operator binding affinities for the double mutants were similar to those reported for the parent Lys84 lac repressor mutants. The replacement of Lys84 with the apolar amino acid Ala imparts to the structure both stability and preservation of wild-type DNA binding affinity in the -11 deletion background; the dissociation of dimer to monomer appears to be prevented by this apolar substitution, and the intrinsic Kd for DNA binding is observed. Complete disruption of the C-subdomain subunit interface of K84A/-11 deletion protein by substitution at Tyr282 is required to diminish the high affinity operator binding and dimer stability of this protein, although even this disruption is insufficient to preclude assembly completely. Although monomer-monomer stability appears to be increased when Lys84 is replaced with the more bulky and apolar Leu, this substitution may alter the orientation of DNA binding domains, resulting in lower affinity for operator DNA in the parent tetramer and in the dimer.

Both triple mutants are dimeric at protein concentrations in the micromolar range but apparently dissociate at lower concentrations due to the effects of Asp at position 282, a substitution that generates monomeric protein in the wild-type background (20, 28, 31, 34). This dissociation contributes at least partially to diminished affinity of the triple mutants for operator DNA. Nonetheless, the assembly of the triple mutants attests to the exceptional stabilizing influence of apolar residues at position 84. The amino acid changes in these combinatorial mutants result in relatively little influence on the in-

**TABLE III**

| Protein                  | Denaturant | ΔG° | m         |
|-------------------------|------------|-----|-----------|
| K84A/-11 deletion       | Urea       | -21 | ND*       |
| -11 deletion            | Urea       | 17.9 ± 0.2 | 6.9 ± 0.2 |
| K84L/Y282D/-11 deletion | Urea       | 15.7 ± 0.7 | 6.4 ± 0.4 |
| K84A/Y282D/-11 deletion | Urea       | 14.0 ± 0.1 | 4.6 ± 0.1 |

*Calculated from partial unfolding curves, since the completely unfolded species was not obtainable due to a limit on the maximum concentration of urea available (~10 M). The m value was not determinable from the data.

**FIG. 7.** Unfolding curves for urea and guanidine hydrochloride denaturation. Protein concentration was 10 μg/ml. Fluorescence measurements were taken at 340 nm. Data were normalized to total fluorescence change (ΔF/ΔF₀ = Δfluorescence/Δfluorescence_total). For curves where unfolding was incomplete, the final fluorescence was assumed to be 0. Shown are urea unfolding curves (A) and guanidine unfolding curves (B) for K84A/-11 deletion (△), K84L/-11 deletion (●), -11 deletion (○), K84A/Y282D/-11 deletion (■), and K84L/Y282D/-11 deletion (●).

**FIG. 8.** Individual fits for fraction of protein unfolded by urea. Protein concentration was 10 μg/ml. Curves are best fit lines generated as described under “Materials and Methods.” Fluorescence measurements were taken at 340 nm. A: -11 deletion; B: K84A/Y282D/-11 deletion; C: K84L/Y282D/-11 deletion.
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