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Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor

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ABSTRACT **A 58-amino acid polypeptide containing the functional core region, the τ1 core, of the major transactivation domain of the human glucocorticoid receptor has been expressed in *Escherichia coli* and purified to homogeneity. The polypeptide retains 60–70% of the activity of the intact domain when assayed *in vivo* or *in vitro*. This report describes a structural characterization of the τ1 core peptide fragment. Circular dichroism spectroscopy shows that the τ1 core and a larger fragment encompassing the intact τ1 domain are largely unstructured in water solution under a variety of pH conditions. The τ1 core, however, acquires a significant α-helical structure when analyzed in the presence of trifluoroethanol, an agent that favors secondary structure formation in regions that have propensity for α-helical conformation. Two- and three-dimensional NMR spectroscopy of ^{15}N-labeled τ1 core, in the presence of trifluoroethanol, has allowed sequential assignment of ^{1}H and ^{15}N resonances and identification of three protein segments with α-helical character. Potentially helix-breaking proline substitutions, in proposed α-helical regions, lead to reduced activity, suggesting that α-helices are important for transactivation *in vivo*.**

The glucocorticoid receptor (GR) belongs to a family of nuclear receptors (1). Binding of glucocorticoid hormones transforms the receptor into an active species able to interact with specific DNA sequences, termed glucocorticoid response elements, located in the vicinity of target genes and subsequently to activate or repress gene activity (2). Two regions important for the post-DNA-binding transactivation potential of the receptor have been identified. In the human GR (hGR), the major transactivation domain, τ1, resides at the N terminus of the protein, whereas a minor transactivation domain, τ2, resides just C-terminal of the DNA-binding domain (Fig. 1; see ref. 3). Further deletion mutagenesis of the 185-amino acid τ1 domain has identified a fragment of 58 amino acid residues containing the functional τ1 core region, which retains 60–70% of the activity of the intact domain (4).

The τ1 domain, as well as the smaller τ1 core-containing fragment, is comparatively rich in acidic amino acids (Fig. 1; see ref. 5), suggesting that it might belong to the acidic class of activators first described by Ptashne and Gill (6). This class of activators shows very little sequence similarity between the members. Although the transactivation potential of acidic activators is related to the number of acidic residues, there is no strict correlation between negative charge and activation potential (7–11). A possible correlation between negative charge and transactivation potential for the hGR τ1 domain remains to be investigated.

Two models have been proposed for the structure of transactivation domains. Ptashne has suggested that acidic activation domains form amphipathic α-helices where the acidic

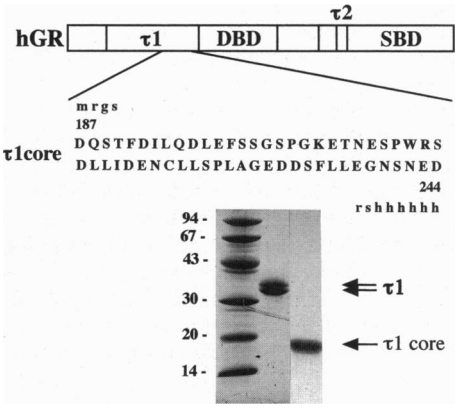

FIG. 1. General structural and functional organization of the GR and the purified τ1 and τ1 core proteins. (*Upper*) DBD, DNA-binding domain; SBD, steroid-binding domain; τ1 and τ2, major and minor transactivation domains, respectively. Amino acid sequence of the τ1 core protein is shown in single-letter code, with capital letters indicating hGR-derived amino acids. (*Lower*) Coomassie blue-stained gel showing purified τ1 and τ1 core proteins. Migration of molecular mass markers is shown (kDa).

residues are aligned on one side of the helix. In support of this, a peptide designed to form an amphipathic α-helix with a negatively charged surface activates transcription, whereas a putative α-helix containing the same amino acids but in a scrambled order failed to activate transcription (12). However, neither the structure of this predicted α-helix nor the importance of its presumed α-helical character was confirmed. Furthermore, neither amphipathicity, nor α-helical character appears to be important for the transactivation potential of the viral transactivator VP16 (9). It has also been proposed that acidic activators are unstructured "acidic blobs" that interact with presumed target factors purely through ionic interactions (13). This model derives from observations that progressive deletion mutants of the acidic transactivation domain from the yeast transcription factor GCN4 results in a progressive reduction in transactivation (8) and that transactivators can be encoded by random sequences of DNA (7, 10) with the only restriction being that these sequences are acidic in character. However, this model is difficult to reconcile with the lack of a strict correlation between acidity and transactivation potential and with the finding that mutation of certain nonacidic residues reduces activity (9, 11, 14).

Recently, the results of biophysical studies aimed at elucidating the structure of some acidic transactivation domains have been reported. CD and NMR spectroscopy studies of a

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Abbreviations: GR, glucocorticoid receptor; hGR, human GR; TFE, trifluoroethanol; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlated spectroscopy; HSQC, heteronuclear single-quantum correlation.

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transactivation domain from VP16 show that this domain is largely devoid of structure at neutral pH in aqueous solution (15, 16). However, the polypeptide becomes notably more structured in the presence of trifluoroethanol (TFE) (15). This agent creates a more nonpolar environment and favors secondary structure formation (refs. 17–19 and references therein). If transactivation domains mediate their effect via protein–protein interactions, they might not be experiencing an aqueous environment *in vivo* when functioning in transactivation. Studies of acidic transactivation domains from the GAL4 and GCN4 yeast transcriptional activators show structures that contain almost 100% β-sheet under slightly acidic conditions (20), conditions that might also occur during protein–protein interactions involved in transactivation. The induction of different secondary structures and the fact that these are induced under different conditions might indicate that there are different classes of acidic transactivation domains interacting with different targets.

This report describes a biophysical study of the functional core transactivation activity from the major transactivation domain of the hGR. Similar to what has recently been described for certain other acidic activators (15, 16, 20), this transactivation domain is largely unstructured at neutral pH in aqueous solution. However, under more nonpolar solvent conditions the polypeptide has significant α-helical structure, consisting of three α-helical segments. The α-helical structure appears to be relevant for activation as introduction of potentially α-helix-breaking proline residues leads to reduced activation *in vivo*.

MATERIALS AND METHODS

Plasmid. The plasmid pQE12-τ1 core expresses amino acids 187–244 of hGR fused to six C-terminal histidine residues. The plasmid pET-τ1 expresses amino acids 77–262 of the hGR fused to 10 N-terminal histidine residues. Details of the constructions are to be published elsewhere. Plasmids expressing mutated τ1 fragments in yeast are modified versions of the pRS315 phagemid vector (21). The τ1 core fragment (amino acids 187–244 of the hGR) is fused to the *Escherichia coli* LexA repressor DNA-binding domain and expressed from a hybrid GAL1PGK promoter (22). Single-stranded phagemid DNA was used as a substrate for oligonucleotide-directed *in vitro* mutagenesis by the method of Kunkel (23, 24). Mutants were identified and the complete sequence was verified by DNA sequencing.

Overexpression and Purification. The recombinant proteins were purified by nickel affinity chromatography essentially according to the instructions of the supplier (Diagen, Dusseldorf, FRG). Details of the purifications are to be published elsewhere. The τ1 core protein and the τ1 protein were estimated to be at least 95% and 90% pure, respectively, as determined from Coomassie blue staining of SDS/polyacrylamide gels. SDS/PAGE was performed as described (25). The activity of the purified proteins was tested in an *in vitro* transcription assay (4, 26).

CD Spectroscopy. CD was measured on a Jasco (Easton, MD) model J720 spectropolarimeter. Spectra were recorded in "step scan" mode, in which data readings at a rate of 2000 Hz are averaged over a time interval (typically 4 s) at each wavelength increment (typically 0.5 nm). The excitation slit width was 2 nm. Sample and optics chambers were purged with nitrogen gas at a flow rate of 15 liters/min. Sample spectra and baselines were recorded with the same 1-mm quartz glass cell. The sample temperature was not actively controlled but was found to be stable at 22°C throughout all measurements. Spectra were measured in 3 mM phosphate/1 mM dithiothreitol and, as indicated in the figures, at various pH or TFE concentrations, at a protein concentration of 100 μg/ml. Protein concentrations for the τ1 core protein were determined spectrophotometrically using the extinction coefficient ε_{280} = 6000 $M^{-1}\cdot cm^{-1}$ (calculated from tryptophan and phenylalanine absorption data). Protein concentrations for the τ1 protein were determined by the Bio-Rad protein assay using bovine serum albumin as a standard. The pH was measured directly on the samples with a pH microelectrode. The method of Manavalan and Johnson (variable selection) (27) was used to predict protein secondary structure content from CD spectra.

NMR Spectroscopy. NMR samples of the τ1 core contained ≈3 mM protein in either 10% $^2H_2O/H_2O$, 60% H_2O/40% TFE-d_3, or 60% ^2H_2O/40% TFE-d_3 with 1 mM dithiothreitol and 20 mM phosphates at pH 5.9. The ^2H_2O/TFE-d_3 sample was prepared from a lyophilized H_2O sample. All NMR samples were flushed with nitrogen prior to sealing. NMR spectra were recorded at 16°C and/or 26°C on a Varian model Unity 500 spectrometer at a magnetic field of 11.74 T. Two-dimensional homonuclear double quantum filtered correlated spectroscopy (DQF-COSY) (28), nuclear Overhauser effect spectroscopy (NOESY) (29), and clean-total correlated spectroscopy (TOCSY) (30) spectra were recorded in the hypercomplex mode (31). NOESY spectra were recorded using cross-relaxation mixing times of 75, 120, and 200 ms and clean-TOCSY spectra were recorded using isotropic mixing times of 80 ms. Heteronuclear $^{15}N^1H$ correlation spectra included heteronuclear single quantum correlation (HSQC) (32), two-dimensional heteronuclear multiple quantum correlation (HMQC)-TOCSY (33), three-dimensional ^{15}N-edited NOE-HSQC, and three-dimensional ^{15}N-edited TOCSY-HSQC. A pulsed-field gradient-selected (34) three-dimensional NOE-HSQC spectrum was also recorded on the ^2H_2O/H_2O τ1 core sample. Water suppression was achieved by presaturation of the water signal or presaturation in combination with SCUBA water suppression (35). No presaturation was used in the gradient-selected three-dimensional NOE-HSQC spectrum.

NMR spectra were processed with software from Varian (VNMR). Data processing typically involved apodization with shifted Gaussian functions in the t_2 (acquisition time) domain and sine/cosine bell functions in t_1 and baseline correction. Spin system identification and sequential resonance assignments were carried out using the homonuclear two-dimensional spectra and, especially, the three-dimensional ^{15}N NOE-HSQC and TOCSY-HSQC spectra following well established methods (36–38).

Transactivation Assays in Yeast Cells. Plasmids expressing wild-type and mutant proteins were transformed into the yeast strain W303-1A (22) containing the reporter plasmid pLG-ZLexA, where a single *E. coli* LexA repressor binding site (39) has been cloned into the *Xho* I site of pLGZ (22). The resulting reporter gene consists of a basal CYC1 promoter/LacZ fusion that is activatable by proteins binding to the LexA binding site. Yeast strains were grown and assayed for β-galactosidase expression as described (40).

RESULTS AND DISCUSSION

Purification of the τ1 Core Protein. A peptide fragment containing residues 187–244 of the hGR was expressed in *E. coli* fused to six C-terminal histidine residues (Fig. 1). The expressed protein includes 4 N-terminal and 8 C-terminal amino acid residues derived from the expression vector in addition to the 58 hGR-derived amino acids to give a 70-amino acid polypeptide. The resulting protein, the τ1 core protein, was purified by affinity chromatography on a Ni-NTA matrix and by gel filtration. An SDS gel electrophoretic analysis of the purified protein is shown in Fig. 1. The protein migrates more slowly than predicted from its calculated molecular mass (≈7.7 kDa), presumably because of its acidic character. The activity of the purified protein was confirmed in an *in vitro* transcrip-

tion assay, where the activity of the protein is assayed as its ability to squelch transcription from a DNA template (4, 26).

The τ1 Core Protein Has α-Helix-Forming Potential. CD is sensitive to the presence of various secondary structure elements in peptides and proteins (for a review, see ref. 41). CD spectra of the τ1 core polypeptide in buffered aqueous solution (pH 6.9 and 7.7) consist of a negative absorption at 197 nm, which is characteristic of a largely unstructured peptide (Fig. 2*A*; see ref. 41). To ensure that the absence of secondary structure did not reflect only the rather small size of the τ1 core peptide, the intact 185-residue τ1 protein was purified (Fig. 1) and its secondary structure was investigated by CD. Fig. 2*A* shows that this protein is also largely unstructured under these conditions. Two acidic transactivation domains from the yeast transcription factors GAL4 and GCN4 have been shown, by CD spectroscopy, to adopt a structure that is almost 100% β-sheet in slightly acidic solutions (20). Since the τ1 core protein has an excess of acidic residues (15 acidic residues compared to 4 basic residues) and since the τ1 core protein also activates transcription in yeast cells (4), it might display the same characteristics as the two endogenous yeast transactivation domains. CD spectra of the τ1 core protein were recorded as a function of pH (Fig. 2*B*). However, the characteristics of the CD spectrum do not change significantly with pH and, specifically, there is no indication of a β-sheet-containing structure. The classical β-sheet CD spectrum exhibits a strong positive absorption at 198 nm and a weaker negative absorption at 219 nm (41). The reduced CD intensity at pH 4.4 is caused by reduced protein solubility close to the isoelectric point. It has also been suggested that transactivation domains of the acidic class may have the general property of forming amphipathic α-helices, and the results of some mutagenesis experiments are consistent with this hypothesis (12). Recent CD analysis of an artificial 15-amino acid peptide, designed to form an amphipathic α-helix and shown to activate transcription in yeast cells (12), shows that it is largely unstructured in aqueous solution but that it has α-helical potential as evidenced by the appearance of the characteristic α-helical CD spectrum in the presence of the α-helix stabilizing agent TFE (20). TFE is frequently used in studies of protein folding and is thought to stabilize α-helical structures in peptides having a propensity to form α-helices. In support of this, studies of peptides derived from proteins of known structure have shown that TFE induces α-helical structures only in peptides derived

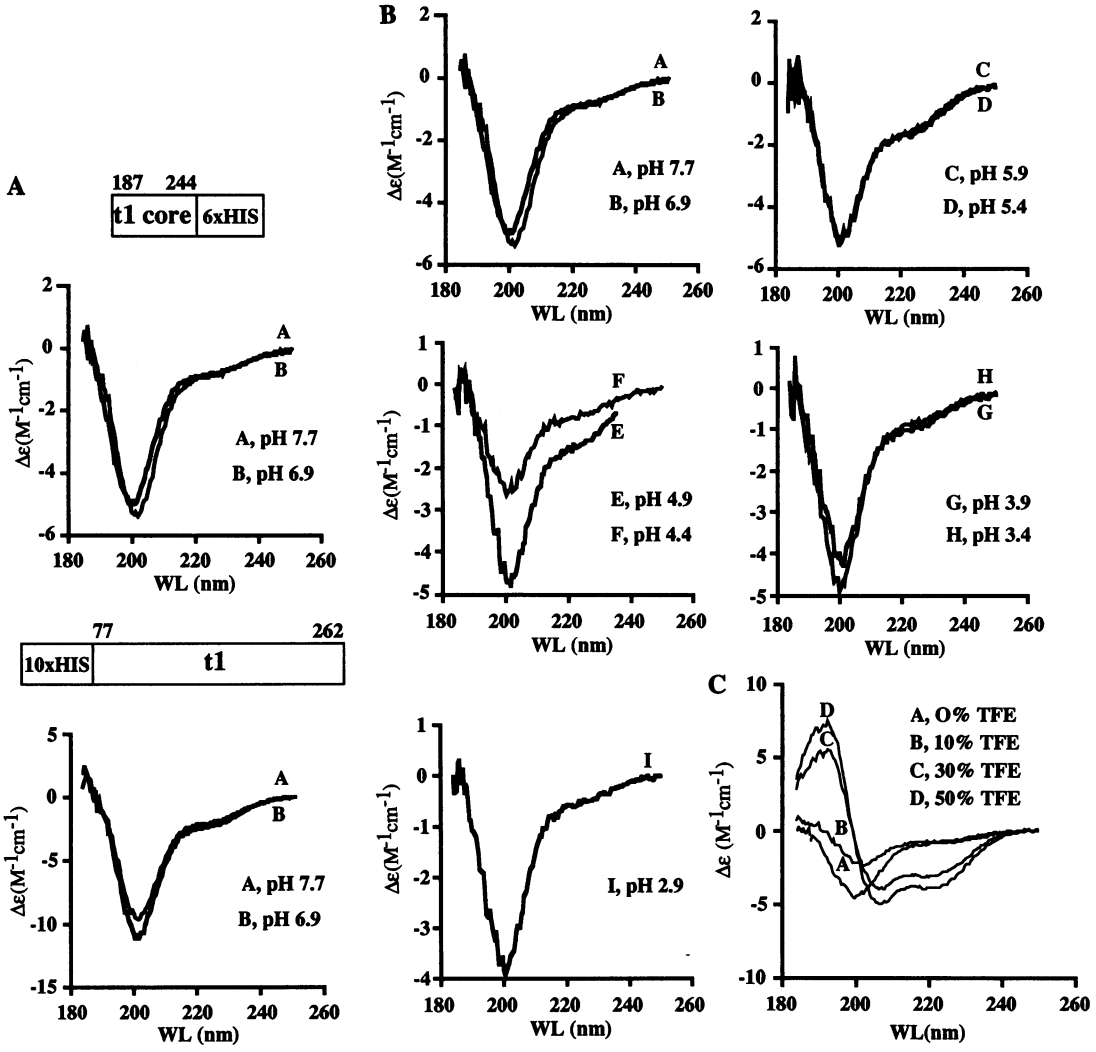

FIG. 2. The τ1 and the τ1 core protein assume random coil conformations in buffered aqueous solution. CD spectra of the τ1 core protein and the τ1 protein recorded at the indicated pH values. WL, wavelength (nm). CD is given as the differential molar extinction coefficient, Δε. The molar ellipticity, θ, can be calculated as $\theta = \Delta\varepsilon \times 3300$ (θ in units of degrees × cm^2 × $dmol^{-1}$). The minimum at 197 nm is characteristic of a mobile random coil conformation (41). (*B*) The τ1 core protein assumes a random coil conformation at a wide range of pH values. CD spectra of the τ1 core protein recorded at the indicated pH values. The reduced CD signal at pH 4.4 is due to reduced protein solubility close to the isoelectric point. (*C*) The τ1 core protein assumes an α-helical structure in the less polar helix-promoting solvent TFE. CD spectra of the τ1 core protein recorded at the indicated TFE concentrations. The maximum at 190 nm and the minima at 220 and 208 nm are characteristic of α-helical conformation (41).

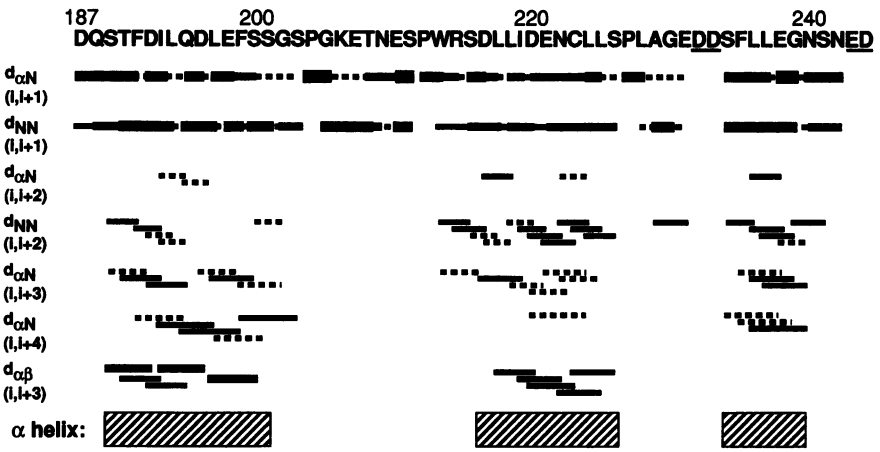

FIG. 3. The τ1 core protein has three segments with α-helix-forming propensity. Summary of $^1H^1H$ NOE connectivities for the τ1 core protein in 40% TFE. $d_{\alpha N(i,i+1)}$ represents a connectivity between Hα of residue i and NH of residue i+1. The intensity of NOE cross-peaks is indicated by the thickness of the lines. NOE assignments that are somewhat less firm due to spectral overlap are indicated by dashed lines. The strong $d_{\alpha N(i,i+3)}$ and $d_{\alpha\beta(i,i+3)}$ connectivities are characteristic for α-helical regions (36). The three α-helical regions identified here are indicated. Underlined amino acids are those for which complete backbone ^{15}N and ^1H resonance assignments could not be made.

from regions known to adopt α-helical structure in the context of an intact protein (18). CD spectra of the τ1 core in the presence of various concentrations of TFE are shown in Fig. 2*C*. The CD spectra in 30% and 50% TFE display clear α-helical character with the characteristic maximum at 190 nm and minima at 208 and 222 nm (Fig. 2*C*; ref. 41). Interestingly, closer inspection of the CD spectra in aqueous solution indicates a slight minimum at 220 nm (Fig. 2 *A* and *B*). Prediction of the secondary structure from the CD spectra using a method based on variable selection (27) produced values of 29% and 36% helix content in the presence of 30% and 50% TFE, respectively, and 10% helix in aqueous solution. Furthermore, the inclusion of low concentrations of SDS (0.5–5 mM), which is thought to induce β-sheet structure in proteins with a propensity to form β-sheet (42), did not induce any structure in the τ1 core polypeptide (data not shown). This suggests that the τ1 core peptide cannot be induced to form any structure just depending on the environment. Transactivation domains, including the τ1 core protein, might share the property of being conformationally very flexible polypeptides, which upon interaction with their target factors acquire a more rigid conformation. Depending on the actual target factor(s), different transactivation domains would then require different conditions for structural stabilization and could acquire very different structures (this study; refs. 15 and 20).

Identification of Three Segments of the τ1 Core with α-Helical Character. CD is a good method to evaluate the presence of secondary structural elements in peptides and proteins; however, it takes no account of the actual location of these structural elements within the peptide or protein. NMR spectroscopy allows such an allocation of secondary structural elements to various parts of the protein. Since the τ1 core is a relatively small and soluble protein (70 amino acids), which can be obtained in large quantities, it is suitable for a structural determination using NMR spectroscopy. Based on the results of CD analysis, NMR experiments were carried out in the presence of 40% TFE. τ1 core protein labeled with ^{15}N was produced to simplify sequential assignments and identification of secondary structure by use of ^{15}N-edited three-dimensional NMR experiments. Complete backbone ^{15}N and ^1H resonance assignments could be made for all hGR-derived amino acids except D232, D233, E243, and D244 (underlined in Fig. 3). Several medium range NOEs (Fig. 3) could be identified in two-dimensional NOESY and three-dimensional NOE-HSQC experiments (a complete list of NOEs can be obtained from the authors). Some of these NOEs are highly characteristic of α-helices (36) and define three segments of α-helical structure in the τ1 core protein (Fig. 3). These segments correspond to ≈60% of the polypeptide. However, this is not in contrast to the CD analysis, which gives a value of 36% for the total α-helical content under these conditions since α-helical segments identified by NMR might be present only during a fraction of the time. A close inspection of the first two helices reveals a high degree of similarity in the spatial arrangement of acidic and nonpolar residues. The hydrophobicity pattern also resembles that found for VP16 (14), thus implying that a combination of negative charge and nonpolar surfaces is an important feature in the interaction of τ1 with its target factor(s). We were not able to identify any long-range NOEs, and the folding of the τ1 core protein in nonpolar solvents is therefore probably restricted to the formation of secondary structure elements. The six C-terminal histidine residues do not interfere with the folding of the molecule since removal of

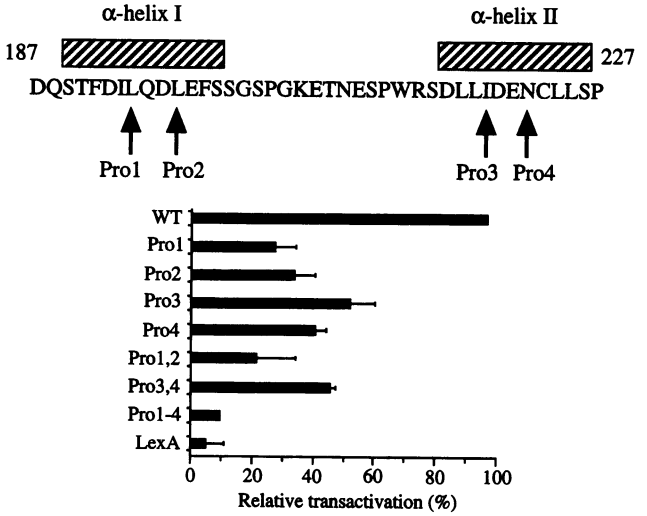

FIG. 4. Potentially helix-breaking proline residues reduce the transactivation potential of the τ1 core. The transactivation activity of proline substitution mutants in the τ1 core is shown relative to that of the wild-type nonmutated protein (WT). Positions of proline substitution mutations are indicated. Pro-1, -2 and Pro-3, -4 are double substitution mutants with two mutations in either helix. Pro-1, -4 has two proline substitutions in both helices. LexA represents the transactivation activity of the isolated LexA DNA-binding domain without any added τ1 core fragment.

these residues by carboxypeptidase A cleavage does not significantly affect the NMR spectra (data not shown).

Potentially Helix-Breaking Proline Residues Reduce the Transactivation Potential of the τ1 Core. To investigate the significance of the propensity of the polypeptide to fold into α-helices, potentially helix-breaking proline residues were introduced at two positions in the middle of helix I and helix II. Mutant τ1 core fragments were fused to the LexA repressor DNA-binding domain and the activity of the proteins was tested *in vivo* in yeast cells, where the expression of β-galactosidase is controlled by a LexA repressor binding site. The mutational analysis focused on helix I and helix II, since our previous analysis had shown that the region corresponding to these helices is most important for τ1 core-mediated activation (4). Furthermore, the first two helices have the largest number of identified NOE connectivities characteristic of α-helical structures (Fig. 3). All of the single proline substitution mutants showed reduced activity (Pro-1 to Pro-4; Fig. 4). Substitution with two proline residues in one helix leads to a slight further reduction (Pro-1, -2; Fig. 4) or no significant further reduction (Pro-3, -4; Fig. 4) in activity, suggesting that one proline substitution is enough to disrupt the formation of a helix and that the nonmutated helix still provides some activity. Substitution with two proline residues in both α-helices (Pro-1, -4; Fig. 4) leads to a greatly reduced activation potential (≈10% of the wild-type level), suggesting that the disruption of both helices is detrimental for activity. Proline substitution mutants at two positions outside the identified helices did not affect the activity of the protein (data not shown). Structural studies of mutant proteins are planned and will further establish the extent to which the identified α-helices contribute to transactivation.

Note Added in Proof. Preliminary studies of mutant proteins demonstrate a correlation between transactivation *in vivo* and α-helix forming potential *in vitro*.

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