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Dimerization and DNA Binding Facilitate α-Helix Formation of Max in Solution[1]

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Max is a basic region/helix-loop-helix/leucine zipper (b/HLH/Z) protein that forms a hetero-complex with the Myc family proteins Myc, Mad, and Mxi1, and a homo-complex with itself. These complexes specifically bind to double-stranded DNA containing CACGTG sequences. Here, we report on the structural properties in aqueous solution of a 109-amino-acid protein, Max110, corresponding to the N-terminal domain of Max containing the b/HLH/Z motif (residues 2-110), as characterized by combined use of circular dichroism (CD) and sedimentation equilibrium experiments. The results showed that the α-helical content of Max110 increases with increasing protein concentration. The sedimentation equilibrium data indicated that Max110 exists as a monomer at low protein concentration, and forms a dimer at high protein concentration. Further increases in the α-helical content of Max110 occur upon addition of DNA with the CACGTG recognition sequence. Thus, dimerization and binding to DNA of Max both favor an increase of the α-helical content.

Key words: alternative splicing, circular dichroism, c-Myc, protein structure, sedimentation equilibrium.

The Myc family proteins which include c-Myc, N-Myc, L-Myc (*1*), Max (*2*), Mad (*3*), and Mxi1 (*4*) have been implicated in cell proliferation, differentiation, and neoplasia. These proteins have a basic region/helix-loop-helix/leucine zipper (b/HLH/Z) motif in common. Through the HLH/Z region, Max forms a hetero-complex with Myc family proteins and forms homo-complexes with itself. These complexes bind to duplex DNA containing a CACGTG and related core sequences (E-box) and regulate transcription of the target gene. The c-Myc-Max hetero-complex transactivates the target gene through the transactivation domain of c-Myc (*5, 6*). On the other hand, Max, Mad, and Mxi1 do not have a domain involved in transactivation, and therefore these Myc family proteins function as antagonists of Myc (*3, 4*).

The crystal structure of a complex between the b/HLH/Z region of p22Max (from 22 to 113) and a 22-base-pair duplex DNA containing the specific binding sequence CACGTG was reported (*7*). The truncated Max binds to the major groove of the CACGTG sequence as a homodimer with its basic region forming a parallel, left-handed, four-helix bundle in the HLH region, and a parallel coiled coil in the leucine zipper region. This study clearly demonstrated the structural basis of DNA binding. Structural studies of several b/Z, b/HLH, and b/HLH/Z proteins have been reported. For example, the crystal structure of the b/Z protein GCN4 exhibits the coiled coil structure required for dimerization (*8, 9*). The structure of the b/HLH protein MyoD was investigated by using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. The results indicated that the α-helical content of MyoD increases with increasing concentration of the protein or upon binding to the specific DNA recognition sequence (*10*) and that the b/HLH motif forms a left-handed, four helix bundle structure (*11*). The b/HLH/Z proteins TFEB (*12*) and USF (*13*) have also been investigated by CD spectroscopy. The α-helical content of intact USF increases upon binding to DNA. However, there have been no reports regarding the conformational changes occurring in b/HLH/Z protein Max. The first aim in this study, therefore, was to determine the structural properties of the Max b/HLH/Z domain in solution.

Max occurs mainly in two alternative forms (*2*); p22Max, which has 160 amino acid residues, and p21Max, which consists of 151 amino acid residues. p21Max formed by alternative splicing lacks 9 amino acids upstream of the basic region of p22Max, which causes a reduction of the DNA-binding activity (*14*). Max has a casein kinase II phosphorylation site upstream of the splicing region (*15*), and phosphorylation of this site also causes a reduction of the DNA-binding ability. Therefore, it is also of interest to examine the structural properties of the N-terminal region of Max.

To understand better the function-structure relationship and structural features of the N-terminal and b/HLH/Z

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Abbreviations: b, basic region; HLH, helix-loop-helix; Z, leucine zipper.

regions of Max, we prepared a 109-amino-acid residue protein, Max110, which consists of the N-terminal to b/HLH/Z regions of Max (residues 2-110) and studied the structural properties of Max110 at various protein concentrations and with various concentrations of DNA by combined use of circular dichroism (CD) and sedimentation equilibrium experiments.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The construct encoding Max110 contained b/HLH/Z for DNA binding, the casein kinase II phosphorylation site (Ser11) and the alternatively spliced segment for regulation of the DNA-binding affinity of Max (Fig. 1). Max110 DNA fragments corresponding to amino acids 1 to 110 were amplified directly from a human fetal brain cDNA library by polymerase chain reaction. The DNA fragments were cloned into a pQE expression vector (QIAGEN). The sequences of the Max110 fragments in the expression vector were confirmed and the plasmid was transformed into *Escherichia coli* SG13009 [pREP4]. Cells containing the Max110 expression vector were grown at 37°C in 1,000 ml of super medium until the absorbance at 600 nm reached 1.0. Then, isopropyl-β-D-thiogalactopyranoside (2 mM) was added to induce expression. Cells were harvested 4 h after induction by centrifugation, lysed in 20 ml of cell lysis buffer [50 mM sodium phosphate buffer (pH 6.0), 8 M urea], and incubated overnight at 4°C. The supernatant of the lysate was recovered by centrifugation, and fractionated by addition of an equivalent volume of saturated ammonium sulfate solution to 50% saturation. The supernatant fraction containing Max110 was separated and saturated ammonium sulfate solution was added to 80% saturation. The precipitates were collected by centrifugation and dissolved in 100 ml of the cell lysis buffer. The solution was applied to a column of cation exchange resin, HiTrap SP column (Pharmacia). The protein was eluted with a linear gradient of NaCl (0-0.5 M) in cell lysis buffer. Fractions containing Max110 were pooled and were applied to a gel-filtration column of Superdex 75pg (Pharmacia), pre-equilibrated with 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$. The Max110 fractions were concentrated by ultrafiltration. The concentration of the purified protein was determined from the absorbance at 275 nm using a molar absorption coefficient (ε) of 2,680 $M^{-1} \cdot cm^{-1}$ (Max110 contains two Tyr residues). The N-terminal amino acid sequence was confirmed with an automated protein sequencer (Shimadzu model PPSQ-10) based on the Edman degradation method, and showed full agreement with the published sequence (*2*), except for the first methionine (data not shown). Thus, the overexpressed Max110 was composed of 109-amino-acid residues (Ser2-Gln110). The biological activity of the recombinant Max110 was examined by electrophoretic mobility shift assay which demonstrated that the protein bound efficiently to double-stranded CM3 DNA containing the E-box sequence (data not shown). This allowed us to use the recombinant protein for further spectroscopic analyses.

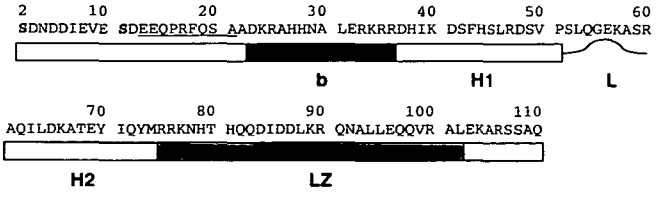

Fig. 1. **Schematic representation of Max110 protein.** Regions of the protein are indicated: b, basic region; H1, helix 1; L, loop; H2, helix 2; Z, leucine zipper. The amino acid sequence of Max110 protein used in this study is indicated on the figure with the amino acid numbering of the intact p22Max protein. The alternatively spliced region and casein kinase II phosphorylation site are indicated as underlined and boldfaced amino acid residues, respectively.

Preparation of Double-Stranded DNA—Double-stranded CM3 DNA was prepared by annealing of two oligonucleotides of the following sequences: CM3A, 5'CCCCCAACACGTGTTGCCTGA3', and CM3B, 5'TCAGGCAACACGTGTTGGGGG3' (*16*). CM3 DNA, containing a CACGTG sequence and the flanking sequences, is the same, except for the two base pairs next to the core sequence, as CM1 DNA which was originally selected as c-Myc binding DNA (*17*). These oligonucleotides were synthesized using standard phosphoramidite chemistry, and purified with OPC cartridges (Applied Biosystems). Equimolar amounts of CM3A and CM3B oligonucleotides in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ were annealed by heating at 95°C for 5 min, then cooling to 4°C.

Circular Dichroism Study—CD spectra were measured on a JASCO J-720 spectropolarimeter with 1 mm and 10 mm path-length quartz cells at 37°C. All samples were dissolved in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$. CD spectra were scanned four times for each sample, averaged, and smoothed using software provided by the manufacturer. In the experiment in which the protein concentration was varied, the CD spectrum of the buffer alone as a blank was subtracted from the raw data. In the titration experiment with DNA, the spectrum of DNA alone as a blank was measured for each DNA concentration and subtracted from the raw data. The α-helical content (f_H) was estimated from the ellipticity value at 222 nm ($[\theta]_{222}$) by using the equation:

$$f_H = -([\theta]_{222} + 2340)/30300 \quad (18).$$

Sedimentation Equilibrium—In the sedimentation equilibrium experiment, a Beckman XL-A Optima Analytical Ultracentrifuge equipped with absorbance optics and an An-60Ti rotor were used. Sample solutions of 5, 10, 25, and 50 μM Max110 in 20 mM sodium phosphate (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ were loaded in a charcoal-filled Epon six-channel center piece and spun at 18,000 rpm for 24 h at 37°C. Sedimentation data were acquired as averages of four absorbance measurements per radial position at a nominal radial spacing of 0.001 cm at 214, 225, 232, and 236 nm for 5, 10, 25, and 50 μM samples, respectively. The partial specific volume calculated from the amino acid composition at 37°C was 0.721 ml/g (*19*). The density of the solvent at 37°C was 0.993 g/ml.

The fitting equation used for the single ideal species model was:

$$A_r = A_0 \exp\{[M(1-\rho\bar{v})\ \omega^2]/2RT\}(x^2 - x_0^2) \qquad (1)$$

where A_r and A_0 are the absorbance values for a 1.2 cm path-length at radius x and at reference radius x_0, respectively, M is the gram molecular weight of particles (vari-

able), $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solvent, ω is the angular velocity of the rotor, R is the gas constant (8.314×10^7 erg·mol^{-1}·K^{-1}), and T is the temperature in degrees Kelvin. This equation was used to evaluate the average molecular mass. The fitting equation used for the monomer-dimer equilibrium model was:

$$A_r = A_{1,0}\exp\{[M(1-\rho\bar{v})\omega^2]/2RT\}(x^2-x_0^2) + A_{2,0}\exp\{[2M(1-\rho\bar{v})\omega^2]/2RT\}(x^2-x_0^2) \quad (2)$$

where $A_{1,0}$ and $A_{2,0}$ are the absorbance of monomer and dimer, respectively, for a path-length of 1.2 cm at reference radius x_0. Extinction coefficients of 1.270×10^5, 0.592×10^5, 0.238×10^5, and 0.115×10^5 M^{-1}·cm^{-1} at 214, 225, 232, and 236 nm, respectively, were used to convert the absorbance into protein concentration.

Calculation of Dissociation Constant from Circular Dichroism Data—The dissociation constant of the dimer was determined by curve-fitting of the protein concentration dependence data to the following equations using a non-linear least-squares fitting program (KaleidaGraph, Synergy Software). The ellipticity can be expressed as a function of the ellipticities of the pure monomer and dimer:

$$[\theta]_{222} = ([M]/[M]_{total})[\theta]_M + (2[D]/[M]_{total})[\theta]_D \quad (3)$$

$$[M]_{total} = [M] + 2[D] \quad (4)$$

where $[\theta]_M$ and $[\theta]_D$ represent the ellipticity values at 222 nm for the monomeric and dimeric states, respectively. $[M]$ and $[D]$ are the monomer and dimer concentrations, respectively. $[M]_{total}$ is the total protein concentration expressed in monomer units. Equation 5 can be derived from Eqs. 3 and 4:

$$[\theta]_{222} = ([\theta]_M - [\theta]_D)[M]_{total} + [\theta]_D \quad (5)$$

The dissociation constant is expressed as

$$K_d = [M]^2/[D] \quad (6)$$

where K_d is the dissociation constant of the dimer. From Eqs. 4, 5, and 6, Eq. 7 can be derived:

$$[\theta]_{222} = ([\theta]_M - [\theta]_D)\{-K_d + [K_d\,(K_d + 8[M]_{total})]^{1/2}\}/4[M]_{total} + [\theta]_D \quad (7)$$

The CD data was fitted to this equation using KaleidaGraph taking $[\theta]_M$, $[\theta]_D$, and K_d as variable parameters.

RESULTS

Circular Dichroism Spectra of Max110 at Various Protein Concentrations—The concentration dependence of the far-UV CD spectra was investigated to examine the secondary structure of Max 110. In the range of 0.5–75 μM in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ at 37°C, Max110 exhibited a characteristic spectrum with double minima at 208 and 222 nm indicating the presence of α-helices (Fig. 2A) (*20*). In the range of 0.5–75 μM, the absolute value of $[\theta]_{222}$ increased from 6,100 to 17,600. The intensity of the α-helix-associated CD bands increased with increasing Max110 concentration (Fig. 2B), indicating a concentration-dependent increase in the α-helical content of Max110.

Sedimentation Equilibrium of Max110—To examine whether the change in the α-helical content dependent on the protein concentration was due to a change in the monomer-dimer or monomer-oligomer equilibrium, the sedimentation equilibrium method was applied to Max110 at 37°C. For ultracentrifugation, samples of Max110 were prepared with initial protein concentrations of 5, 10, 25, and 50 μM in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$. Centrifugation was carried out at 18,000 rpm and at 37°C for 22 h. The protein concentration at equilibrium was plotted as a function of the radial position for each concentration (Fig. 3). The weight-average molecular mass for each sample was estimated using the equation for the single ideal species model (Eq. 1), treating A_0 and M as variable parameters (Table I). The weight-average molecular mass values increased with increasing initial protein concentration and were higher than that of the monomer (12,800 Da) but lower than that of the dimer (25,600 Da). Next, the correspondence of the results to the monomer-dimer equilibrium model was examined according to Eq. 2, treating $A_{1,0}$ and $A_{2,0}$ as variable parameters and M as a constant at 12,800. The data were in agreement with the monomer-dimer equilibrium model at concentrations between 2 and 62 μM.

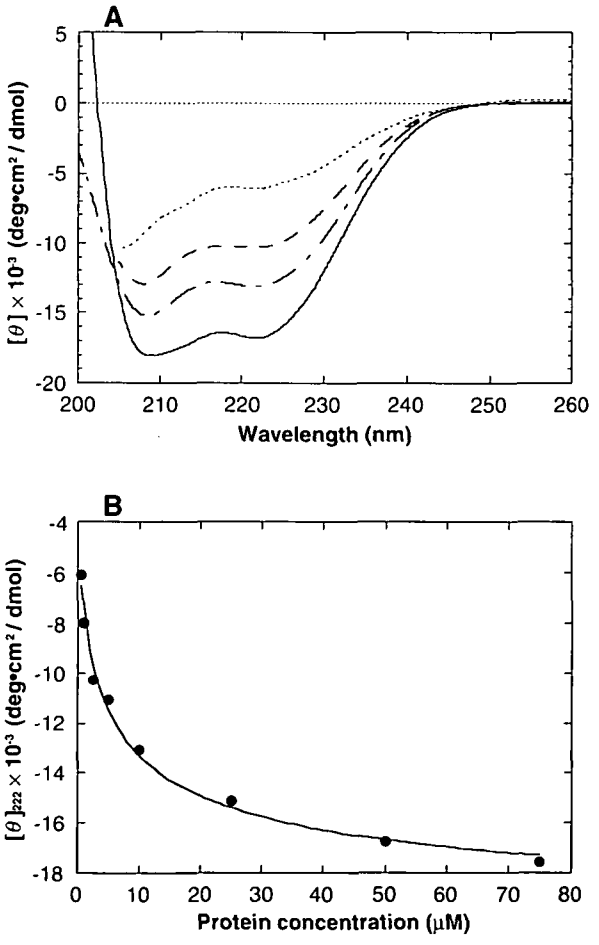

Fig. 2. **Protein concentration-dependent CD change.** (A) Far-ultraviolet CD spectra of Max110 at different protein concentrations in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ at 37°C. Protein concentrations (top to bottom): 0.5, 2.5, 10.0, and 50.0 μM. (B) CD-protein concentration profile for Max110 in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ at 37°C as monitored with the mean residue $[\theta]$ at 222 nm. The solid line represents the best-fit curve to the equation for the two-component equilibrium model.

The dimer dissociation constant (K_d) calculated from $A_{1,0}$ and $A_{2,0}$ of each sample was 12×10^{-6} M. The correspondence of the results with monomer-dimer-trimer and monomer-dimer-tetramer equilibria was also examined. However, the estimated concentrations of trimeric and tetrameric species were nearly zero or negative. Therefore, Max110 is predominantly in an equilibrium between monomers and dimers in solution at 37°C.

Curve-Fitting of Circular Dichroism Data Used for Monomer-Dimer Equilibrium Model—On the basis of the results of the sedimentation equilibrium experiment, the CD data were fitted to the monomer-dimer equilibrium model expressed as Eq. 7, treating $[\theta]_M$, $[\theta]_D$, and K_d as variable parameters (Fig. 2B). The calculated K_d is 8×10^{-6} M, which is similar to the K_d value derived from the sedimentation equilibrium data. The calculated $[\theta]_M$ was −4,900, which corresponds to an α-helical content of 8%, which in turn implies that 9 amino acid residues are in the α-helical conformation (Table II). The $[\theta]_D$ was −20,500, which corresponds to an α-helical content of 60%

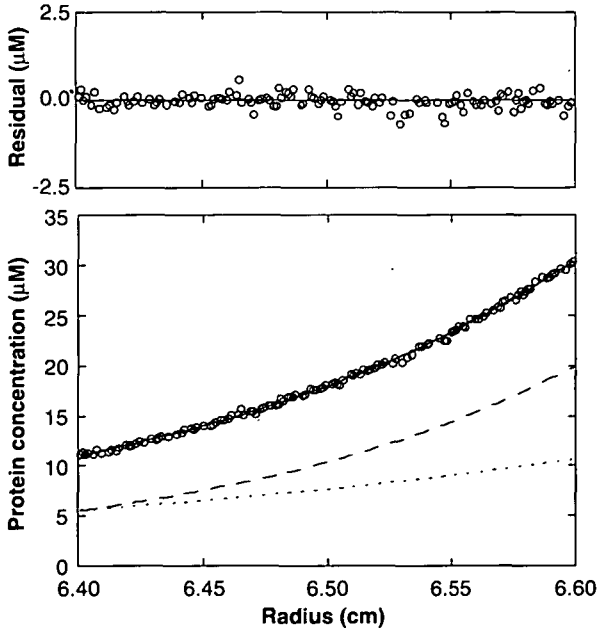

Fig. 3. **Influence of the initial protein concentration of Max110 on sedimentation equilibrium at 18,000 rpm.** The protein concentration (expressed as monomer unit concentration) calculated from the absorbance is plotted as a function of the radial position for Max110. The initial protein concentration was 25 μM. Equilibrium data at 37°C (○) were fitted to the monomer-dimer equilibrium model (solid line). The plot also shows the calculated concentrations of the monomer (dotted line) and dimer (broken line) at 37°C. Upper panel: Plot of residuals from the fit to the monomer-dimer model.

TABLE I. **Sedimentation equilibrium results of Max110 at 37°C.** Initial protein concentration is described in terms of monomer units. The weight-average molecular mass was obtained assuming no self-association by fitting the sedimentation data to the single ideal species model.

Initial protein concentration (μM)	Weight-average molecular mass (Da)
5	17,000±200
10	18,600±100
25	20,200±100
50	20,600±100

TABLE II. **Summary of the circular dichroism experiments with Max110 alone and Max110-DNA complex at 37°C.** The α-helical contents of the monomeric and dimeric Max110 were calculated from $[\theta]_M$ and $[\theta]_D$ obtained by the curve-fitting experiment. The maximum α-helical content in the presence of DNA was calculated from $[\theta]_{222}$ for the mixture of 30 μM Max110 and 30 μM double-stranded CM3 DNA.

Sample	α-helical content per monomer (%)	Calculated number of amino acid residues involved in α-helix
Max110 monomer	8	9
Max110 dimer	60	65
Max110 dimer-DNA	80	87

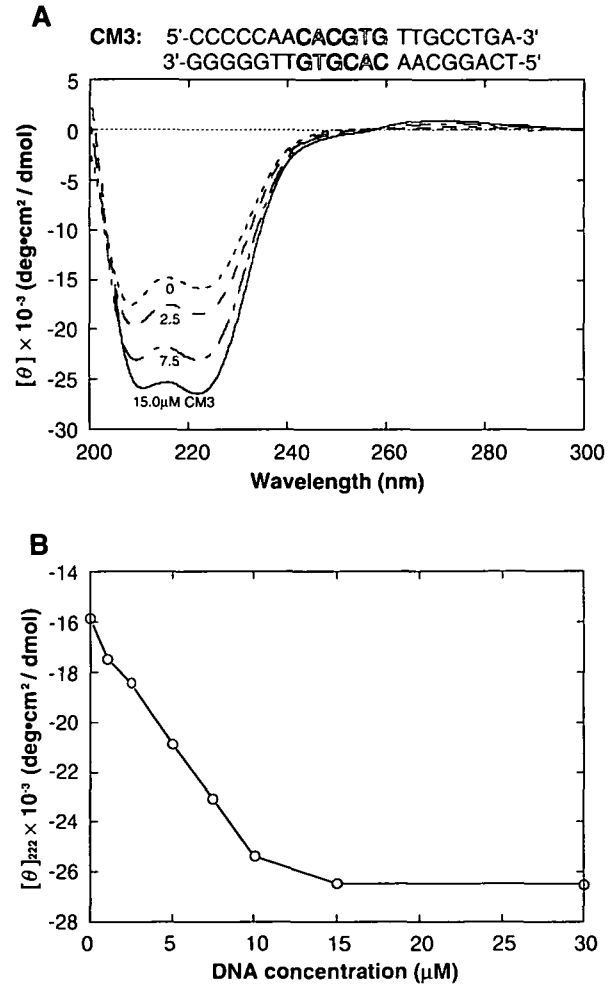

Fig. 4. **Effects of DNA concentration on CD spectra.** (A) CD spectra of Max110 (30 μM) in the presence of CM3 DNA. The sequence of the double-stranded CM3 DNA is displayed above the graph. The spectra were measured in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ at 37°C in the presence of 0, 1, 5, 10, 15, and 30 μM CM3. (B) CD-DNA concentration profile for Max110 (30 μM) as titrated with CM3 DNA and monitored with $[\theta]$ at 222 nm. CD spectra were measured in 20 mM sodium phosphate buffer (pH 6.8), 150 mM NaCl, 10 mM $MgCl_2$ at 37°C. The CD data were corrected by subtracting the $[\theta]_{222}$ of DNA alone from those of the protein-DNA mixture.

or 65 amino acid residues in the α-helix. Thus, Max110 forms an α-helical dimer from unstructured monomers at high protein concentration.

Circular Dichroism Spectra of Max110 in the Presence of DNA—CD spectra of Max110 (30 μM), in the presence of various concentrations of CM3 DNA containing one Max recognition hexamer sequence, were measured to examine the effect of DNA binding on the secondary conformation of Max110 (Fig. 4). The absolute value of $[\theta]_{222}$ showed a marked increase on addition of the DNA. The $[\theta]_{222}$ value reached a plateau at DNA concentrations around 15 μM. The observation that DNA concentration at saturation (15 μM) corresponded to half of the Max110 concentration (30 μM) suggests that the stoichiometry of the DNA binding is one Max110 dimer to one DNA duplex recognition sequence. The resulting α-helical content was 80%, which corresponds to 87-amino-acid residues in the α-helices.

When a DNA, in which the central two bases (CG) of the CACGTG core sequence are replaced with GC, is used, the increase in the absolute value of $[\theta]_{222}$ on addition of DNA is considerably smaller (about 80%) with than that when CM3 is used (Horiuchi, M., unpublished data).

DISCUSSION

We examined the structural properties of the b/HLH/Z protein Max110 in solution. The α-helical content of Max110 increased with increasing protein concentration. Sedimentation equilibrium analysis revealed that this result can be explained by the monomer-dimer equilibrium of Max110. Interaction of Max110 with the CM3 DNA oligomer induced further increases in the α-helical content. Therefore, α-helix formation of Max110 is induced by two processes; dimerization and DNA binding.

The α-helical contents of other transcription factors, *i.e.* the b/HLH region of MyoD (*10*) and the b/Z region of GCN4 (*21*), have also been reported to be concentration-dependent. Similar results were also reported for the leucine zipper fragments of c-Myc, TFEB (*22*), and Max (*23*). These phenomena can be explained by conformational changes from unstructured monomers to α-helical dimers or to α-helical tetramers. Sedimentation equilibrium analysis confirmed that the change in the α-helical content of Max110 is correlated with dimerization, which facilitates α-helical conformation.

From the results of curve-fitting analysis for protein concentration dependence, the saturated α-helical content was 60% which corresponds to 65-amino-acid residues involved in the helical structure. In the crystal structure of a truncated Max (22-113)-DNA complex, helix 1 (15 amino acid residues), helix 2 (15 amino acid residues), and leucine zipper regions (28 amino acid residues) form α-helices including a total of 58 amino acid residues per monomer and the C-terminal region beyond Leu102 adopts a random coil conformation (*7*). Thus, we suggest that the α-helical regions of Max110 dimers consist of the helix 1, helix 2, leucine zipper, and another region, probably the N-terminal region preceding the basic domain. Quite recently, the crystal structure of a complex between intact p21Max and a 13-mer DNA was reported (*24*). The basic region and the preceding N-terminal region (9 amino acids shorter than that in p22Max) form α-helices in the complex.

In the presence of DNA, the saturated α-helical content of Max110 was 80%, which corresponds to 87 amino acid residues involved in the helical structure (see Table II). This implies that 29 amino acid residues are included in the α-helical conformation in addition to the H1, H2, and Z segments (total 58 amino acid residues). DNA-dependent α-helix formation within the basic region, the DNA binding domain, has been reported for other b/HLH/Z proteins (TFEB and USF), b/Z protein (GCN4), and b/HLH protein (MyoD). The basic region of Max110, which consists of 15 amino acid residues (Ala22-Arg36), also might form an α-helix.

The b/HLH proteins MyoD (*10*) and myogenin (*25*), and the b/HLH/Z proteins Myc (*26*), TFEB (*27*), and USF (*13*) have been shown to exist as tetramers in the absence of DNA. The b/HLH/Z domain of USF forms a stable tetramer in the absence of DNA when the leucine zipper motif is intact. The tetrameric USF can bind to two independent DNA sites and plays an important role in gene activation. As the amino acid sequence of Max is similar to that of USF within the b/HLH/Z region, we would expect similar oligomerization properties for the two proteins. However, the results of the present study demonstrated that Max110 exists in a monomer-dimer equilibrium which is dependent on the protein concentration. As shown by sedimentation equilibrium analysis, inclusion of terms for higher order assemblies such as tetramers did not improve the fit (Fig. 3). Therefore, the multimerization properties of Max110 are different from those of Myc, USF, and other structurally similar proteins.

The present study revealed that the α-helical content of Max110 is enhanced by two processes; dimerization and DNA binding. We also found that a part of the N-terminal region upstream of the basic region of Max110 can be incorporated into the α-helix. The structure of the Max b/HLH/Z domain and intact p21Max have been determined by X-ray crystallography (*7, 24*), but that of the N-terminal region which modulates the DNA binding ability and biological function has not been determined yet in solution. Max110 is a good model protein for elucidation of the role of the N-terminal region of b/HLH/Z proteins.

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