The Structure and the Characteristic DNA Binding Property of the C-terminal Domain of the RNA Polymerase α Subunit from Thermus thermophilus*

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The C-terminal domain of the α subunit of the RNA polymerase (αCTD) from Escherichia coli (Ec) regulates transcription by interacting with many kinds of proteins and promoter upstream (UP) elements consisting of AT-rich sequences. However, it is unclear how this system is common in all eubacteria. We investigate the structure and properties of αCTD from an extremely thermophilic eubacterium, Thermus thermophilus (Tt). The solution structure of Tt αCTD (85 amino acids) was determined by NMR, and the interaction between Tt αCTD and DNA with different sequences was investigated by means of chemical shift perturbation experiments. The tertiary structure of Tt αCTD is almost identical with that of Ec αCTD despite 32% sequence homology. However, Tt αCTD interacts with the upstream region sequence of the promoter in the Tt 16 S ribosomal protein operon rather than the Ec UP element DNA. The upstream region sequence of Tt is composed of 25 base pairs with 40% AT, unlike the Ec UP element with 50% AT. The DNA binding site in Tt αCTD is located on the surface composed of helix 4 and the loop preceding helix 4. The electric charges on this surface are not remarkably localized like those of Ec αCTD.

A eubacterial RNA polymerase holoenzyme is composed of five subunits, αββ′σ. As seen on investigation of Escherichia coli (Ec), † core enzyme αββ′ is fully active in the polymerization of RNA. The α subunit of Ec is composed of two structural domains, the N-terminal domain and the C-terminal domain (αCTD). The Ec N-terminal domain of the α subunit of the RNA polymerase plays a key role in RNA polymerase assembly (1), and Ec αCTD is necessary for transcription regulation. Ec αCTD interacts with transcription activators, for example, cyclic AMP receptor protein (CRP) (1, 2), OmpR (3), and a transcription repressor, GalR (4). Furthermore, Ec αCTD recognizes promoter upstream (UP) elements that consist of AT-rich sequences and enhances transcription initiation (5). The solution structure of Ec αCTD was determined by NMR measurement (6), and crucial amino acid residues for interaction with factors were determined (6, 7).

However, it is unclear how these transcription regulation systems are used in all eubacteria. For example, it is unlikely that such AT-rich sequences as the Ec UP element exist in the genome of an extremely thermophilic eubacterium, Thermus thermophilus (Tt), because the average A + T content of the genome is 30% (8). We thus investigated whether Tt αCTD binds the Ec UP element sequence or the upstream region sequence of the promoter in the Tt 16 S ribosomal protein operon (TUP) by NMR measurements (9). For this purpose, we constructed an overexpression system of Tt αCTD in Ec cells. Using the recombinant protein, the higher thermal stability of Tt αCTD was shown by circular dichroic (CD) measurement, and the solution structure of Tt αCTD was determined by NMR. The interaction of Tt αCTD with a variety of DNAs at 37 and 50 °C was investigated by the chemical shift perturbation of NMR signals.

EXPERIMENTAL PROCEDURES

Preparation of the Expression Vector of Tt αCTD—Two synthesized DNA oligomers (142 base pairs each) were hybridized and used for the elongation reaction at 4 °C for 3 h with the Klenow fragment (Takara Shuzo, Kyoto, Japan). The produced DNA fragment encoded the amino acid sequence of Tt αCTD (85 amino acids including the starting Met). However, codons in this fragment were replaced with those frequently used for the gene expression in Ec cells (The Wisconsin Package, Genetics Computer Group, Madison, WI). The fragments were amplified by polymerase chain reaction and then digested with the restriction endonucleases, NcoI and BamHI (Takara). The digested DNA fragments (CTZ) were purified by agarose electrophoresis and then inserted to the pET-15b vector (Novagen). The nucleic acid sequence of CTZ in the vector (258 base pairs) was confirmed with a DNA autosequencer (Applied Biosystems) and given GenBank™ accession number AB038061.

Expression and Purification of Tt αCTD—Ec BL21 (DE3) cells were transformed with the CTZ-inserted vector (pET-CTZ) and then grown in LB medium. Isopropyl-1-thio-β-D-galactopyranoside was added to a liquid culture to 1 mM at A600 = 0.8. After 3 h of induction the cells were harvested and stored at −80 °C until use.

The following procedure, except for the heat treatment, was carried out at 4 °C. Wet cells from 200 ml of culture were suspended in 50 ml of lysis buffer (50 mM Tris-HCl (pH 8.0) and 500 mM NaCl) and then sonicated on ice. The crude extract was then incubated at 60 °C for 30 min and centrifuged. The supernatant was recovered and dialyzed against 50 mM Tris buffer (pH 8.5). It was then applied to a HiTrap Q column (2-ml bed volume) (Amersham Pharmacia Biotech) and eluted with a linear gradient of sodium chloride (0–500 mM NaCl) with 50 mM Tris (pH 8.5). The fractions of Tt αCTD detected by SDS polyacrylamide gel electrophoresis were collected. They were applied to a Superdex 75 gel-filtration column (2.6 × 60 cm) (Amersham Pharmacia Biotech) with the gel-filtration buffer (50 mM KH2PO4/K2HPO4 (pH

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB038061.

‡ The atomic coordinates and structure factors (code 1DOQ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: Ec, Escherichia coli; αCTD, C-terminal domain of the α subunit of the RNA polymerase; BAF, barrier to autointegration factor; TUP, the upstream region sequence of the promoter in the 16 S ribosomal protein operon of Thermus thermophilus; Tt, Thermus thermophilus; UP, promoter upstream; rmsd, root mean square deviation.
**RESULTS**

**CD Measurements**—The UV CD patterns around 220 nm of Tt aCTD and Ec aCTD at 37 °C (Fig. 2A) show that Tt aCTD mainly consists of α-helical structures and that the α-helix content is roughly identical with that of Ec aCTD. The thermal stability of Tt aCTD was characterized by a change in ellipticity at 222 nm from 20 to 90 °C (Fig. 2B). The (θ)222 values at 90 °C of Tt aCTD and Ec aCTD were around −0.8 × 10^4 (degree cm^2 dmol^−1). Because the (θ)222 value of a typical random structure is about 0.3 × 10^4 (degree cm^2 dmol^−1) (18), both aCTDs were not denatured completely at 90 °C. However, the partial denaturation at 70 °C of each aCTD was obvious compared with that of Tt aCTD.

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Fig. 3. $^1$H–$^{15}$N HSQC spectrum of $Tt$ αCTD at 0.2 mM and 37 °C and representation of the chemical shift perturbations. Red arrows indicate the directions of the chemical shift perturbations of the HSQC signals of Gly293, Gly295, and Ser298 on the addition of DNA fragments. Blue squares (a to g) show the positions of the chemical shifts of Gly293, Gly295, and Ser298 under the following conditions; a, NON (0.2 mM); b, SUB (0.2 mM); c, UP (0.2 mM); d, 46 (0.2 mM); e, TUP (0.2 mM); f, TUP (0.3 mM); and g, TUP (0.4 mM).

Table I

Structural statistics for the 20 structures of lowest energy obtained from 100 starting structures

None of these structures exhibited distance violations greater than 0.3 Å or dihedral angle violations greater than 5 °.

| Distance Restraints | Number |
|---------------------|--------|
| NOE                  | 734    |
| Intraresidue         | 342    |
| Sequential (1 $<|i-j|<4$) | 170    |
| Medium range (4 $<|i-j|$) | 107    |
| Hydrogen bonds       | 38     |
| Dihedral angle ($\phi$) restraints (%) | 43     |
| Ramachandran plot (%) residues 256–309 | 77     |
| Most favorable region |        |
| Additionally allowed region | 23     |
| Generously allowed region | 0      |
| Disallowed region    | 0      |

Average rmsd from experimental distance restraints (Å)

- All (647) $= (2.57 \pm 0.00) \times 10^{-2}$
- All (43) $= (4.01 \pm 1.67) \times 10^{-1}$

Average rmsd from idealized covalent geometry

- Bonds (1118) $= (2.41 \pm 0.00) \times 10^{-3}$
- Angles (2059) $= (4.85 \pm 0.02) \times 10^{-1}$
- Improper (523) $= (3.82 \pm 0.02) \times 10^{-1}$

Average rmsd of atomic coordinates between 20 structures, residues 256–309

- Backbone heavy atoms (Å) $= 0.61$
- All heavy atoms (Å) $= 1.15$

* For each of the 19 hydrogen bonds, there are two distance restraints.

* The program PROCHECK NMR was used to assess the quality of the structures (21).

* The precision of the atomic coordinates is defined as the average rmsd between the 20 final calculated structures and the mean coordinates.
NMR Analysis of Tt αCTD—All chemical shifts of NH, N, Ca, and Ha in the backbone were assigned, except for those of Met at the N terminus, Ala232 and Val233. Their signals were not observed in the 1H–15N HSQC spectrum (Fig. 3). The Met at the N terminus may be degraded in Ec cells, for example, by methionine aminopeptidases (19, 20). In this case, the signal of the amide proton of Ala232 would not be observed, because the rate of exchange of the amino proton at the terminus is high. That of Val233 is also weak, because the exchange rate becomes higher with the charge of the N terminus. The resonances of the amide protons of 11 residues (Leu269, Lys270, Glu272, Leu283, Leu286, Ile292, and Ile296–Glu307) remained at 37 °C for longer than 10 min after dissolution in D2O. Twenty-one resonances remained at 20 °C after 60 min. Among them, the amide protons of 19 residues (Leu259, Leu269–Glu272, Ile274, Leu280–Lys283, Ile291, Leu299, Ile302–Glu307, and Lys309) were considered to form hydrogen bonds and were used as distance restraints of hydrogen bonds for calculation of the structures of Tt αCTD. Other slowly exchanged amide protons were not assigned to specific hydrogen bonds.

Because there were no long range nuclear Overhauser enhancement signals in the Ala234–Pro246 region, the distance constraints in the Glu247–Glu315 region of Tt αCTD were analyzed for structure calculation. A summary of the structural statistics for the final set of structures of Tt αCTD (Glu247–Glu315) is given in Table I. The overlaid 20 backbone structures are shown in Fig. 4A. The averaged and energy-minimized structure was analyzed by means of the PROCHECK NMR program (21). The results showed Tt αCTD comprises Leu256–Leu259 (helical turn), Thr283–Glu297 (helix 1), Val277–Ala282 (helix 2), Leu285–Leu288 (helix 3), and Glu296–Lys309 (helix 4) (Fig. 5). From the deviations of 13Ca chemical shifts from a random coil, the secondary structure of Tt αCTD has been estimated (22). This structure was almost identical with the current result. The rmsd for the backbone heavy atoms in the Leu256–Lys309 region is 0.61 Å (Table I). Helices 1, 2, and 3 are almost perpendicular to each other. Although helix 3 is rather short according to the results with the PROCHECK NMR program, this region was determined to be a helix on comparison of the secondary structures of Tt αCTD and Ec αCTD (Fig. 5). Helix 3 is roughly antiparallel to helix 4 (Fig. 4B).

NMR Analysis of the Tt αCTD-DNA Interaction—Among the
assigned chemical shifts of the amide protons of the backbone of Tt aCTD in the 1H-15N HSQC spectrum with 0.2 mM of the protein and 37 °C, those of Gly293, Gly295, and Ser298 remarkably shifted on the addition of the DNA fragments, and their traces are shown in Fig. 3. They drifted either over 0.7 ppm along the axis of HN or over 2 ppm along the axis of N in the presence of 0.2 mM TUP. It is likely that these three amino acid residues directly interact with DNA. Although amino acid residues are Arg265, Ser266, Asn268, Leu270, Asn294, and Ser299 in Ec aCTD with DNA shown by the perturbation experiments. These amino acid residues are important for the interaction with the UP element shown by the Ala scan experiment (7).

The perturbed chemical shifts of Gly283, Gly285, and Ser288 were aligned in the order of NON < SUB and UP < −46 < TUP (0.2 to 0.4 mM) on straight lines (Fig. 3). The mode of specific binding is probably the same as that of nonspecific binding, because their shifts were aligned on the same linear lines. When the concentration of TUP was increased with a constant concentration (0.2 mM) of Tt aCTD, the observed shifts of Gly283 and Ser288 were saturated with 0.3 mM TUP (Fig. 3), indicating that most Tt aCTD formed the Tt aCTD-TUP complex under these conditions. From this titration of the chemical shifts, we could estimate the dissociation constant between Tt aCTD and TUP was 10−4 M assuming a 1:1 complex. This value is 10 times smaller than for the ordinary nonspecific interaction, as observed for Ec aCTD-SUB.2 On the other hand, no obvious difference between those of Tt aCTD-UP and Tt aCTD-SUB was observed. Ec aCTD does not exhibit meaningful affinity to UP. Because the affinity of Tt aCTD for fragment NON, which is composed of G and C, was the lowest among those of the complexes investigated in this study, Tt aCTD does not prefer just GC-rich sequences. Fragment −46 has the internal sequence of the UP element to which Ec aCTD binds with as high affinity as in the case of UP.2 Although the affinity of Tt aCTD for −46 was larger than that not only for NON but also that for UP, it was less than that for TUP.

When the perturbed signals in 1H-15N HSQC spectra were observed at 50 °C, the degrees of the perturbation were roughly the same as those at 37 °C. Those of Gly283, Gly285, and Ser288 were larger than those of others. The affinity of Tt aCTD for TUP was also recognized at 50 °C.

DISCUSSION

Comparison of the Solution Structures of Tt aCTD and Ec aCTD—The secondary structure of Tt aCTD is almost identical with that of Ec aCTD (Fig. 5). However, Ec aCTD has two helical turn regions, Pro251–Leu254 and Val257–Asp259, whereas Tt aCTD has a single helical turn, Leu257–Leu259. Besides, Ec aCTD has the C-terminal loop (Met316–Glu329), whereas Tt aCTD does not have such a loop at the C terminus. The Ala234–Pro246 region of Tt aCTD is expected to be flexible, because there were no long-range nuclear Overhauser enhancement signals in the region, and the corresponding region of Ec aCTD (Arg235–Glu248) is also flexible (23).

The backbone folding of Tt aCTD (Glu247–Glu251) is quite similar to that of Ec aCTD (Glu248–Glu252) (Fig. 6A). Comparison of the Leu256–Lys308 region of Tt aCTD with the Val257–Ser309 region of Ec aCTD gives an rmsd value of 1.70 Å for the backbone atoms despite the 32% identity of their amino acid sequence of the UP element to which CTD corresponds to the important residues for the DNA interaction in Ec aCTD (Fig. 5), their degrees of perturbation were below 0.3 ppm along the axis of HN and below 1 ppm along the axis of N in the presence of 0.2 mM TUP.

FIG. 5. Comparison of the secondary structures of Tt aCTD and Ec aCTD. The starting amino acid, Met, and Ala233 and Val234 of Tt aCTD are not contained in the sequence in this figure, because their amide proton resonances were not assigned. The secondary structure of Tt aCTD was determined with the PROCHECK NMR program (21). That of Ec aCTD was as described previously (23). The regions in blue letters form an a helix (the Leu256–Leu259 region comprises one turn of a helix called a helical turn in the text). Asterisks and dots indicate identical and similar amino acid residues, respectively. The residues are numbered from the N terminus of a full-length a subunit. Green arrows indicate the important amino acid residues for the interaction with DNA shown by the perturbation experiments. These amino acid residues are Arg265, Asn268, Cys269, and Lys297 in Ec aCTD (6), and Gly293, Gly295, and Ser298 in Tt aCTD (this study). Yellow arrows indicate Arg265, Asn268, Cys269, and Lys297 in Ec aCTD, which are important for the interaction with the UP element shown by the Ala scan experiment (7).

FIG. 6. A, comparison of the backbone of Tt aCTD with that of Ec aCTD. The backbones of Tt and Ec aCTDs are given as a blue ribbon and red ribbon, respectively. The side chains of residues Phe249, Trp251, and Ile326 in Ec aCTD form the hydrophobic core (6). B, a ribbon representation of the human BAF monomer (26). The green ribbons indicate the five a helices (H1–H5).
sequences. Helix 2 of Ec αCTD is considered to be essential for the formation of the hydrophobic core (Phe243 preceding helical turn 1 and Trp232 and Ile236 in the C-terminal loop, shown in Fig. 6A) to stabilize the protein structure; the hydrogen-deuteroion exchange rates of the amide protons in this helix were very slow (6). In Tt αCTD, however, the exchange rates in helix 2 are not slow compared with those in helix 4. This is probably due to the fact that Tt αCTD lacking the C-terminal loop allows helix 2 to expose for solvent. However, the dependence of the ellipticities of Tt and Ec αCTDs on temperature showed that the thermal stability of Tt αCTD is higher than that of Ec αCTD. Thus, the C-terminal loop of αCTD is not essential for stabilizing the protein folding. Another role of the C-terminal loop in Ec αCTD could be interaction with activator proteins, for example, OmpR (24).

When the tertiary structure of Tt αCTD was analyzed with DALI (version 2.0) (25), the similarity was indicated with that of the human barrier to autointegration factor (BAF) (26) (Z score, 3.1) and that of Ec αCTD (Z score, 7.6). BAF is an 89-residue protein, and it is composed of five helices. The result with DALI showed that the structures of helix 1-loop-helix 2 and that of helix 3-loop-helix 4 in Tt αCTD correspond roughly to that of helix 2-loop-helix 3 and that of helix 4-loop-helix 5 in BAF, respectively. The arrangement of the helix-loop-helix regions of Tt αCTD also corresponds roughly to that of BAF (Fig. 6B). BAF is the only protein whose folding is similar to that of eubacterial αCTD.

Interaction between Tt αCTD and DNA—The consensus UP element sequence of Ec was determined using an in vitro selection procedure (27), it being found that A tracts in the UP element are important for increasing the activity of the Ec rrnB P1 promoter (28). A tracts in DNA intrinsically cause bending of the DNA molecule (29). The UP element in Ec is AT-rich and also has a tendency to be bent (30). This bending causes narrowing of the minor groove that contributes to the affinity to Ec αCTD, as observed by NMR measurements. Furthermore, it was suggested that the activation mechanism by the interaction between αCTD and A-tract DNA should be applicable throughout eubacteria (27). If this mechanism is used in Tt cells, A tracts must be remarkably characteristic of the sequence of the genome, of which the average G + C content is 70% (8). However, Tt αCTD does not bind to Ec UP element DNA with meaningful affinity. At least in Tt, it is thought that transcriptional regulation by the interaction between αCTD and A-tract DNA does not occur.

Therefore, in what points is the mode of DNA binding of Tt αCTD different from that of Ec αCTD? In Ec αCTD, helix 1, helix 4, and the loop preceding helix 4 directly interact with DNA (6). In Tt αCTD, however, only helix 4 and the loop preceding helix 4 are involved in the interaction with DNA, and the Gly293 residue enables the loop to form a β-turn structurally (Fig. 4B). Thus, the DNA binding site in Tt αCTD is expected to form a typical phosphate-binding helix-turn-helix module that binds to DNA without sequence specificity (31).

The DNA binding surface in Ec αCTD has a positive charge because of residue Arg265 on helix 1, and residues Lys297 and Lys298 on helix 4 (Fig. 7A). These residues are involved in the DNA recognition, as indicated by the NMR experiments (6) and the Ala replacement experiments (7). In Tt αCTD, however, the three residues, Gly293, Gly295, and Ser298, were indicated to be important for the interaction in this study, and they do not have charged side chains. The amide protons in these residues, particularly Gly293 and Ser298, are expected to form hydrogen bonds with the phosphate backbone in DNA. Although the surface for these residues on Tt αCTD corresponds to the DNA binding surface on Ec αCTD, the deviation of the electric charge in this region is less because Lys297 (charged negative) in Ec structurally corresponds to Gly293 (charged negative) in Tt (Fig. 7B). Not only Gly295 in Tt but also Arg265 in Ec did not show a large shift. Thus, the interaction between Tt αCTD and DNA should not be due to the localization of the electric charge on the surface. However, it is difficult to explain why Tt αCTD has higher affinity to TUP than UP and SUB, because the nucleotide sequence of TUP is not palindromic nor AT-rich like UP.

Perturbation experiments should be carried out at 75 °C, which is the optimal growth temperature of Tt (8). However, at 60 °C, some signals of the amide protons disappeared because of their fast exchange. Besides, it is difficult to perform experiments at 75 °C, because the DNA oligomers easily melt. However, the interaction between Tt αCTD and TUP at 50 °C should have biological meaning, because the minimum growth temperature of Tt is 47 °C (8).

Some Tt promoters have upstream regions (up to 50 base pairs from the −35 region of the promoter) whose A + T contents are 42–46%, and their transcription activities in vivo...
are higher than those of other Tt promoters (32). We, however, found that the affinity of Tt aCTD for TUP is higher than that for SUB, though the former A + T content is also 40%, and the same applies to the latter. Thus, 40% A + T content of a promoter upstream region should not be sufficient for transcription activation. Although SUB was used as the control for the Ec UP element (5), TUP locates in the upstream region of the promoter of the Tt 16 S rRNA operon and corresponds to the Ec UP element, judging from the genome structure. If we can assume the biological function of TUP is similar to that of the Ec UP element, TUP might have the ability to activate the transcription of the 16 S ribosomal RNA operon by interacting with the α subunit of the RNA polymerase in Thermus thermophilus.

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