Interaction of the ω-Subunit of Escherichia coli RNA Polymerase with DNA

RIGID BODY NATURE OF THE PROTEIN-DNA CONTACT*

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The ω-subunit of Escherichia coli RNA polymerase plays an important role in the activity of many promoters by providing a direct protein-DNA contact with a specific sequence (UP element) located upstream of the core promoter sequence. To obtain insight into the nature of thermodynamic forces involved in the formation of this protein-DNA contact, the binding of the ω-subunit of E. coli RNA polymerase to a fluorochrome-labeled DNA fragment containing the rnb P1 promoter UP element sequence was quantitatively studied using fluorescence polarization. The ω dimer and DNA formed a 1:1 complex in solution. Complex formation at 25 °C was enthalpy-driven, the binding was accompanied by a net release of 1–2 ions, and no significant specific ion effects were observed. The van't Hoff plot of temperature dependence of binding was linear suggesting that the heat capacity change (ΔCp) was close to zero. Protein footprinting with hydroxyradicals showed that the protein did not change its conformation upon protein-DNA contact formation. No conformational changes in the DNA molecule were detected by CD spectroscopy upon protein-DNA complex formation. The thermodynamic characteristics of the binding together with the lack of significant conformational changes in the protein and in the DNA suggested that the ω-subunit formed a rigid body-like contact with the DNA in which a tight complementary recognition interface between ω-subunit and DNA was not formed.

A typical Escherichia coli promoter is identified by two conserved DNA sequences, the −10 and −35 regions, separated by a spacer with a consensus length of 17 bp (reviewed in Ref. 1). In addition to these core promoter elements many promoters contain an A-T-rich region located upstream of the −35 region (reviewed recently in Ref. 2). This so-called UP element was discovered first in promoters for ribosomal RNA where it was recognized that the UP element increased transcription from these promoters in a factor-independent fashion, suggesting its direct effect on RNA polymerase (3). The ω-subunit of RNA polymerase was subsequently identified as a target for a direct contact with the UP element (4). Mutations of the ω-subunit defective in UP element binding were found in the C-terminal domain of the ω-subunit, identifying two regions (vicinity of residues 265 and 298, respectively) in this domain as important for UP element recognition (5 and 6). The C-terminal DNA-binding domain of ω is attached to the N-terminal domain of the protein by a long, flexible linker (7 and 8). Site selection experiments were used to identify the consensus UP element sequence (9). Further studies revealed that the UP element consists of two subsites, the proximal and the distal site, and each of these sites binds one monomer of the ω-subunit (10). Fine mapping of the ω-subunit-UP element interactions revealed that the ω-subunit interacted with the bases in the minor groove and with the DNA backbone along the minor groove. The interactions with the major groove were found to be minimal (11).

The mechanism of UP element-induced enhancement of transcription is not completely clear. The simplest model could be that the additional contact between the UP element and the ω-subunit activates transcription by facilitating the initial recognition of promoter DNA by RNA polymerase. The data available for rnb P1 promoter show that indeed the UP element enhances recruitment of polymerase to the promoter (3, 12). However, there is also evidence that at some step downstream the initial recognition could be affected by the UP element-ω-subunit interaction (12 and 13). Moreover, evidence exists that ω-subunit-DNA interactions could inhibit promoter clearance (14) and facilitate formation of non-productive complexes (15). The effects of ω-subunit-UP element interactions on transcription in principle could be a result of the additional binding energy provided by this contact or could also involve conformational changes within the polymerase-promoter complex induced by ω-subunit-DNA contact. DNase I footprinting experiments suggested that an interaction of the ω-subunit with the UP element in the context of RNA polymerase holoenzyme and promoter involved distortions within or nearby the UP element DNA (10, 16, and 17). No information regarding the effects of DNA on an ω-subunit conformation is available. Here we show the thermodynamic and structural data suggesting that the interaction between an isolated ω-subunit with UP element-containing DNA has a rigid body character. No significant perturbation of either the DNA or the protein structure could be detected.

EXPERIMENTAL PROCEDURES

Materials—CPM² was from Molecular Probes (Eugene, OR). Oligonucleotides were synthesized on Applied Biosystems model 394 DNA

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2 The abbreviations used are: CPM, 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin; HMPK, heart muscle protein kinase; CD; circular dichroism, MOPS, 4-morpholinepropanesulfonic acid; bp, base pairs.

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synthesizer (Foster City, CA). All other reagents were of the highest purity commercially available.  

Expression and Purification of the ω-Subunit—The ω-subunit was expressed in E. coli strain BL21(DE3), transformed with pGEME plasmid (a gift from Dr. A. Ishihama), and purified according to the previously described procedure (24) and further purified by gel filtration with the C-terminally His6-tag and N-terminal phosphorylation site (HMPK-ω-His6) was expressed from a plasmid (a gift from R. H. Ebright) and purified as described by Tang et al. (19). Purified HMPK-ω-His6 was labeled with 32P in a 300-μl reaction mixture containing 54 μM HMPK-ω-His6, 500 units of HMPK catalytic subunit (Sigma cat. no. P2645), 0.27 μM [γ-32P]ATP (3000 Ci/mol), 10 mM Tris (pH 7.5), 100 mM NaCl, 20 mM MgCl2, and 1 mM dithiothreitol. The reaction was carried out for 1 h at 37 °C. The labeled protein was purified by metal ion affinity chromatography on Ni2+-NTA-agarose (Qiagen) (20).

Fluorescence Polarization DNA Binding Experiments—DNA binding experiments were performed with a 41-bp DNA fragment containing a UP element sequence (~70 nt ~90 nt) from the rrmB P1 promoter (GGTGGCGGCTGACAAATTATTTTAAATTTCCTCTTGTCAC) (4). DNA was labeled at the 5′-end with CPM and purified as described previously (21). Binding of an ω-subunit to the 41-bp UP element DNA fragment was monitored using a change of fluorescence polarization of CPM-labeled DNA as a signal-reporting protein-DNA complex formation (21 and 22). Fluorescence polarization measurements were performed by built T-format photon-counting fluorometer. The instrument was built using the automated KOWALA sample compartment (ISS, Inc.). The excitation was at 380 nm, and the emission was monitored using long-pass filters with a cut off wavelength of 470 nm. The measurements were performed in a 120-μl cuvette in 10 mM MOPS buffer (pH 7.0) containing 100 mM NaCl and 5% glycerol. Small aliquots of the stock solution of the ω-subunit were added to a solution of fluorochrome-labeled DNA (10 μM). After each addition the sample was incubated for 5 min, and fluorescence polarization was measured. For the measurement of salt dependence of the binding, the buffer contained either 0.05–0.2 M NaCl, or 0.05–0.2 M of sodium glutamate, or 0.05–0.2 M of KCl in place of 100 mM NaCl. Temperature dependence of the ω-subunit binding was studied from 10 to 35 °C in 10 mM MOPS buffer (pH 7.0) containing 50 μM NaCl.

Stoichiometry of the ω-subunit-DNA complex was determined by performing reverse titrations of ω-subunit-fluorochrome-labeled DNA complex with unlabeled DNA at two concentrations of the ω-subunit (20 μM and 40 μM). Small aliquots of unlabeled UP element DNA were added to a solution of 50 μM fluorochrome-labeled DNA and 20 μM (or 40 μM) ω-subunit. After each addition the sample was incubated for 10 min, and fluorescence polarization was measured. After completion of the two titration experiments, the concentrations of the competitor DNA (D1 and D2) required to produce the same signal change at the two protein concentrations used (P1 and P2) were determined for several points. These concentrations were then used to calculate the number of moles of DNA bound to the ω-subunit (v) corresponding to a particular fractional signal change according to Ref. 23 in Equation 1.

\[ v = (D_2 - D_1)(P_2 - P_1) \]  

(Eq. 1)

The degree of binding at saturation of the ω-subunit with DNA was determined by plotting v as a function of fractional signal change and extrapolating the line to the point corresponding to maximal fractional signal change.

Protein Footprinting—Reaction mixtures contained 4–9 μM ω-subunit dimer, 1.3 mM EDTA, 0.6 mM (NH4)2Fe(SO4)2·6H2O, 1 mM H2O2, 20 mM sodium ascorbate, 10 mM MOPS-NaOH (pH 7.2), 250 mM NaCl, 10 mM MgCl2 buffer to a final volume of 10 μl. The UP element DNA was 20 μM when present. All other reagents were prepared immediately prior to use. Reactions were started by simultaneous addition of EDTA and (NH4)2Fe(SO4)2·6H2O as a freshly prepared 13.5 mM EDTA 6.6 mM (NH4)2Fe(SO4)2·6H2O mixture, H2O2, and sodium ascorbate. Reactions were terminated after 40 min by the addition of 5 μl of 3× sample buffer (150 mM Tris-HCl (pH 7.9), 36% glycerol, 12% SDS, 6% β-mercaptoethanol, and 0.01% bromophenol blue). Following electrophoresis, the gels were dried and imaged using PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Data were analyzed as described previously (24) and scanning of each lane of the gel using ImageQuant (Molecular Dynamics) yielded phosphorimager intensities plotted versus electrophoretic mobility. Corresponding peaks in the intensity plots were aligned, and the scans were corrected for gel-loading efficiencies. The electrophoretic mobility of the bands was converted to residue number using band assignments previously established for the ω-subunit (24). The final result of data analysis was presented as a difference plot showing difference of intensity in the presence and absence of DNA normalized to the intensity in the absence of DNA plotted as a function of the residue number. Data from several lanes from three independent experiments were averaged.

Circular Dichroism—CD measurements were performed on a JASCO 720 CD spectrophotometer. Spectra from 240 to 320 nm were collected in a 0.1-cm cuvette at room temperature. Samples contained a 10 μM 42-bp UP element DNA and, when present, 15 μM ω-subunit dimer in 25 mM phosphate buffer (pH 8.0) containing 0.1 M KCl. The spectrum of the buffer alone was subtracted from the spectrum of DNA alone, a spectrum of the 15 μM protein alone was subtracted from the spectrum of the protein-DNA complex.

RESULTS

Thermodynamics of the ω-Subunit-DNA Complex Formation—To gain insight into thermodynamic forces responsible for binding of the ω-subunit to the UP element DNA, quantitative binding experiments in solution were conducted. The binding was monitored by a change of fluorescence polarization of the fluorochrome-labeled 41-bp UP element DNA. Fluorescence anisotropy for the free DNA was ~0.180, and it increased to ~0.215 at high concentrations of the ω-subunit (not shown). No change of fluorescence intensity in the presence of the ω-subunit was observed. The stoichiometry of the ω-subunit-DNA complex was determined in the experiment illustrated in Fig. 1 (inset). In this experiment the degree of fluorescence signal change was related to the thermodynamically valid degree of binding by a procedure involving reverse titrations with unlabeled DNA at two different concentrations of the ω-subunit (see “Experimental Procedures”). Extrapolation of the line describing correlation between the fractional signal change and the moles of DNA bound per mole of the ω-subunit dimer formed to the point corresponding to the maximal signal change observed revealed that at saturation ~0.8 moles of DNA were bound per mole of the ω-subunit dimer, suggesting a 1:1 stoichiometry of the complex. The data shown in Fig. 1 (inset) additionally showed a simple linear relationship between the signal change and degree of binding.

Titration of DNA with increasing concentration of the protein produced a simple isotherm that could be fitted to a binding equation describing 1:1 complex formation (Fig. 1). When UP element-containing DNA was substituted with 41-bp random sequence DNA, much weaker binding was observed (Fig. 1, open circles). The binding was too weak for precise quantification, but a rough estimation suggested at least a 10-fold difference in the affinity between UP element DNA and random sequence DNA. Fluorescence anisotropy dilution experiments with an ω-subunit in which the emission of the single Trp residue of the protein was monitored revealed that the ω-subunit remained dimeric down to ~1 μM concentration (not shown). Taken together, the data in Fig. 1 show that the ω-subunit dimer forms a 1:1 complex with UP element DNA. No indication of the cooperativity or a different mode of binding was evident. All subsequent binding experiments were thus analyzed according to Equation 2, which describes such a simple binding reaction under conditions where the ω-subunit concentration was in excess of UP element DNA concentration.

\[ A = \Delta A_{\text{max}}K_P/(1 + K_P) \]  

(Eq. 2)

where A is the observed anisotropy, \( \Delta A_{\text{max}} \) is the maximal change of the anisotropy, K is the equilibrium association constant, and P is the total protein concentration. To gain further insight into the nature of thermodynamic forces involved in the ω-subunit-DNA complex formation, salt and temperature were used as variables in binding experiments. Entropically favorable displacement of cations condensed around the DNA molecule by the protein upon protein-DNA complex formation is frequently one of the major forces.
favorable contributions to the free energy of protein-DNA complex formation (25). The apparent equilibrium binding constant decreased with the increase of NaCl concentration. The data plotted in a form of a log-log plot could be approximated by a linear plot with a slope equal to 1.2 ± 0.2 (Fig. 2), suggesting that formation of an α-subunit-UP element DNA complex is accompanied by a net release of 1–2 ions (25). Replacing Na⁺ with K⁺ or Cl⁻ with COO⁻ (or glutamate) did not change the nature of salt dependence of $K_{app}$ (Fig. 2), suggesting that the observed salt effect is attributable to a general ion condensation phenomenon rather than to a specific cation or anion binding.

The affinity of the α-subunit for UP element DNA increased with increased temperature, suggesting that the enthalpy change accompanying protein-DNA complex formation was negative (i.e., favorable). Plotting the data in the form of the van’t Hoff plot (Fig. 3) revealed that the data could be approximated by a linear relationship; no indication of a significant curvature of the plot in the range of temperatures studied was observed. This linear behavior of the van’t Hoff plot suggested that within the range of temperatures studied, the enthalpy change for protein-DNA complex formation was independent of the temperature and thus that the heat capacity change ($\Delta C_p$) was close to zero. The linear nature of the van’t Hoff plot allowed estimation of the value of the enthalpy and entropy changes for protein-DNA complex formation. At 25 °C the binding free energy ($\Delta G^\circ$) was $-7.4 \text{ kcal/mol}$, the enthalpy ($\Delta H^\circ$) was $-18.7 \text{ kcal/mole}$, and the entropy ($\Delta S^\circ$) was $-38 \text{ cal mol}^{-1} \text{ deg}^{-1}$. Thus, the binding of the α-subunit to the UP element DNA is enthalpy driven since the enthalpy change for this reaction is favorable, whereas the entropy change is unfavorable. Extrapolation of the salt dependence of $K_{app}$ to 1 M salt allows estimation of the binding free energy at 1 M salt ($\Delta G^\circ$, 1 M NaCl). The $\Delta G^\circ$, 1 M NaCl (−2.3 kcal/mol) provides an estimate of the non-electrostatic fraction of the binding energy. It appears that only ~30% of the binding energy is non-electrostatic in nature.

Conformational Changes in the Protein and DNA upon Protein-DNA Complex Formation—We have shown previously that protein footprinting with hydroxyl radicals provides a sensitive means of detecting and mapping conformational changes in proteins induced by ligand binding (20, 26–28). The advantage of this methodology is that essentially the entire surface of the protein is scanned in a single experiment to determine whether any region of the protein was perturbed by the protein-ligand contact formation. Fig. 4A shows an example of a gel image illustrating the experiment in which the α-subunit alone and the α-subunit in the presence of UP element DNA were subjected to limited cleavage with hydroxylradicals, and the cleavage products were resolved on SDS-PAGE. The experiment was performed under single-hit conditions (29) since the majority of the protein remained uncleaved. The protein was labeled at the N terminus, therefore the relative mobility of the bands corresponding to cleavage products is proportional to the distance of the cleavage site from the C terminus. Visual inspection of the gel image reveals two regions (indicated by arrows) where significant protection from cleavage was observed in the presence of DNA. We have shown previously that it is essential to analyze the results of protein footprinting experiments in a quantitative manner to obtain objective conclusions from such
obtained for the free DNA (Fig. 5).

The CD spectrum of the UP element DNA obtained was typical of DNA spectra free from protein interference can be obtained. DNA spectra can be obtained in the presence of the protein since molar absorption of nucleic acids are sensitive to their conformation (30), and it is possible to detect relatively small perturbations of DNA structure using this approach (31 and 32). DNA spectra can be obtained in the presence of the protein since molar absorption of DNA in the range from 240–320 nm is much higher than the protein, and by using appropriate blank sample subtraction, DNA spectra free from protein interference can be obtained. The CD spectrum of the UP element DNA obtained was typical for B-DNA (30). The spectrum obtained in the presence of the α-subunit showed no differences compared with the spectrum obtained for the free DNA (Fig. 5).

DISCUSSION

The thermodynamics of many site-specific DNA-binding proteins have been studied (33–36). Although it has been difficult to make generalized conclusions regarding the energetics of these interactions, certain correlations between the energetics and the specificity of the energetics and the distortion of DNA in protein-DNA complex formation emerged. Proteins exhibiting site-specific DNA binding activity are also able to bind more weakly to nonspecific DNA. Comparison of the thermodynamics of binding to specific and nonspecific sequences showed that specific binding most often is characterized by a large negative ΔG, whereas nonspecific binding exhibited ΔG close to zero (36). It has been proposed that a negative ΔG is the thermodynamic signature of tight site-specific binding in which a tight complementary recognition interface is formed (36). The large negative ΔG has been proposed to arise from the burial of large amounts of hydrophobic surfaces in protein-DNA complex as a result of local folding coupled to protein-DNA complex formation (37). Many protein-DNA complexes are formed with a significant distortion of the DNA. When the thermodynamics of ten protein-DNA complexes involving varying degrees of DNA distortions were examined, a striking new correlation was found (35). Formation of site-specific protein-DNA complexes involving significant distortion of DNA was entropy-driven, whereas for the complexes involving relatively undistorted DNA unfavorable entropy and favorable enthalpy was observed (35). In agreement with such correlation between the energetics and distortion of DNA in protein-DNA complex, the nonspecific DNA binding for many proteins was found to be enthalpy-driven (36). Nonspecific interactions are not expected to involve large distortion of DNA.

Interaction of the α-subunit with UP element DNA is enthalpy-driven and exhibits near zero value of ΔG. Comparison of the thermodynamic parameters describing α-UP element interaction with the described above observed for other DNA binding proteins implies that a tight complementary recognition interface is not formed, and the α-subunit-UP element contact resembles more the nonspecific rather than specific interaction. Such a thermodynamic signature seems to be consistent with a rather modest discrimination between the UP element DNA and nonspecific DNA (Fig. 1). A rather fine balance between specific and nonspecific DNA binding activity of the α-subunit is probably functionally significant. In addition to the RNA polymerase activation through UP element-α-subunit contact, interaction of the α-subunit with nonspecific DNA is likely to play a role in the activity of some transcriptional activators. It has been observed that protein-protein contact between the activator and the polymerase can induce nonspecific α-subunit-promoter DNA contact, and it is likely that these two macromolecular contacts cooperate to activate polymerase (2, 38). Due to a low affinity of the α-subunit for nonspecific DNA, we were unable to perform a protein footprinting experiment in this case, but it is very likely that the same region of α-subunit would be involved in binding to specific and nonspecific DNA.

The high entropy cost of the α-subunit-UP element complex formation can be rationalized by observing that DNA binding elements of the α-subunit are located in the C-terminal domain of the protein, which is attached to the rest of the protein by a long, flexible linker. The flexibility of the C-terminal domains of the α-subunit is reflected in the absence of the interpretable electron density corresponding to the C-terminal domains in the crystal structure of the core polymerase (39) and was also detected by NMR (8) and fluorescence experiments. The UP element was shown to contain two subsites, each of which interacted with one of the monomers in the α-subunit dimer (10). Formation of the α-subunit-UP element complex would restrict the mobility of these flexible domains resulting in large, unfavorable entropic contribution.

The thermodynamic features of the α-subunit-UP element complex formation are also consistent with the absence of major distortions in either DNA or in the protein molecule. We have performed two additional experiments in which the conformational changes in the protein and in the DNA upon protein-DNA complex formation were probed. Protein footprinting experiments revealed no changes in the protein other than those that could be assigned to a direct protein-DNA contact (Fig. 4). CD spectroscopy showed no perturbation of DNA structure upon protein-DNA complex formation (Fig. 5). It appears,
and UP element DNA in complex with hydroxyradicals, lane $\alpha$ was loaded with the protein cleaved with hydroxyradicals in the absence of UP element DNA, and lane $\alpha$-DNA was loaded with the protein cleaved with hydroxyradicals in the presence of UP element DNA. Arrows indicate the regions where protection from cleavage was observed. The numerical scale indicates the residue numbers of the $\alpha$-subunit obtained from band assignments reported previously (24).

Panel $\alpha$ shows the results of comparing different lanes for $\alpha$-subunit alone and serves as a control to show that no artifacts are generated by the data analysis employed. Panel (DNA-$\alpha$) shows the results of comparing lanes for protein-DNA complex and protein alone.

therefore, that the $\alpha$-subunit-UP element complex formation involves rigid body interactions between the C-terminal domains of the $\alpha$-subunit and UP element DNA without significant perturbation of the structure of either the protein or the DNA component of the complex. This conclusion is consistent with both the thermodynamic characteristics of the binding as well as with direct probing of the structure of the components of the complex. The role of the $\alpha$-subunit-UP element contact formation in the context of promoter DNA has been proposed to be the enhancement of the recruitment of the polymerase to the promoter (27). A simple, rigid-body-like nature of the $\alpha$-subunit-UP element contact would be consistent with such a role.

It is interesting to observe that DNA footprinting experiments with promoter DNA containing the UP element detected perturbation of DNA structure in the vicinity of the UP element (10, 16 and 17). Our experiments with isolated UP element and isolated $\alpha$-subunit showed no perturbation of either DNA or the protein structure. It can be concluded, therefore, that the perturbation of DNA structure by the $\alpha$-subunit-UP element contact requires constraints imposed by other protein-nucleic acid interactions involved in RNA polymerase binding to promoter DNA.

FIG. 4. Protein footprinting probing of $\alpha$-subunit-UP element DNA complex. A, autoradiogram of a protein footprinting SDS-PAGE for the $\alpha$-subunit labeled at the N terminus with $^{32}$P. Lane $\alpha$-uncut was loaded with the protein not cleaved with hydroxyradicals, lane $\alpha$ was loaded with the protein cleaved with hydroxyradicals, lane $\alpha$-DNA was loaded with the protein cleaved with hydroxyradicals in the absence of UP element DNA, and lane $\alpha$-DNA was loaded with the protein cleaved with hydroxyradicals in the presence of UP element DNA. Arrows indicate the regions where protection from cleavage was observed. The numerical scale indicates the residue numbers of the $\alpha$-subunit obtained from band assignments reported previously (24). B, averaged difference plots showing results of quantitative analysis of multiple experiments identical to the one illustrated in A. Normalized difference is defined in "Experimental Procedures" and is zero when no differences in susceptibility are found, is negative when protection from cleavage is observed and is positive when hypersensitivity to cleavage is observed. Panel $\alpha$ shows the results of comparing different lanes for $\alpha$-subunit alone and serves as a control to show that no artifacts are generated by the data analysis employed. Panel (DNA-$\alpha$) shows the results of comparing lanes for protein-DNA complex and protein alone.

FIG. 5. CD spectra of UP element DNA alone (empty circles) and UP element DNA in complex with $\alpha$-subunit (filled circles).

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