The σ Subunit Conserved Region 3 Is Part of “5’-Face” of Active Center of Escherichia coli RNA Polymerase

(Received for publication, May 31, 1994)

Konstantin Severinov, David Fenyo, Elena Severino, Arkady Mustaev, Brian T. Chait, and Seth A. Darst

From the Markey Charitable Trust, the Irma T. Hirschl Trust, and the Human Frontiers in Sciences program (to S. D.) and by National Institutes of Health Grants GM30717 (to A. G.) and RR00863 (to B. T. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Ribonucleotide analogs bound in the initiating site of Escherichia coli RNA polymerase holoenzyme in open promoter complexes were cross-linked to the β and σ70 subunits. Using limited proteolysis and chemical degradation, the cross-link site in σ70 was mapped to a segment between amino acids Glu584 and Met581 containing the C-terminal part of conserved region 3. This result, when reconciled with genetic data on the interaction of σ70 conserved regions 2 and 4 with the −35 promoter box (data not shown). We therefore reasoned that cleavage of the renatured σ subunit-dinucleotide adduct bound RNAP core enzyme (data not shown). Moreover, the resulting holoenzyme formed an open complex on the T7A1 promoter, and the dinucleotide adduct could be extended with [(α-32P]CTP, the nucleotide specific for position +3 of the promoter (Fig. 1B). Modification of σ with the ADPβS reagent was much less efficient and was only evident when the gel was overexposed. No modification of σ occurred when the AMPγS reagent was used. Similar results were obtained when RNAP in open complexes formed on the lacUV5 and the phase λ P2 promoters were labeled (data not shown).

Trypsin and CNBr cleavage were used to fragment the σ polypeptide in order to localize the cross-linking site of the ATPγS derivative. Preliminary experiments showed that the radioactive band of cross-linked σ could be excised from an SDS gel and renatured to yield active protein. The renatured σ subunit-dinucleotide adduct bound RNAP core enzyme (data not shown). Moreover, the resulting holoenzyme formed an open complex on the T7A1 promoter, and the dinucleotide adduct could be extended with [(α-32P]CTP, the nucleotide specific for position +3 of the promoter (data not shown). We therefore reasoned that cleavage of the renatured σ subunit-dinucleotide adduct would proceed just as native σ. To this end, renatured and derivatized σ subunit was mixed with unlabeled carrier σ protein purified from superproducing cells (12) for the cleavage reactions. In each case, the products of the reactions were resolved by SDS-polyacrylamide gel electrophoresis and Coomassie staining, and polypeptides containing the cross-linked adduct were visualized by autoradiography.

Trypsin degradation of the σ polypeptide proceeds in a highly ordered manner (13) and could therefore be used for cross-link mapping (Fig. 2). At low trypsin concentrations, σ was cleaved into two fragments with apparent mobilities of 65.0 and 22.5 kDa (Fig. 2A, lane 2). The 22.5-kDa fragment contained radioactivity, while the 65.0-kDa fragment did not (Fig. 2B, lanes 2–5). At higher trypsin concentrations, this band disappeared and radioactivity accumulated in a band with apparent mobil-
Priming Substrate Contact Site in RNA Polymerase σ Subunit

The pattern of trypsin degradation of native σ was identical to that of cross-linked σ (data not shown). Native σ was digested under conditions that yielded the 22.5- and 65.0-kDa mobility fragments (Fig. 2A, lane 2). The N-terminal sequence of the 65.0-kDa fragment, determined using standard methods (14), was identical to the N-terminal sequence of the intact σ subunit. The N-terminal sequence of the 22.5-kDa fragment indicated that the fragment was generated by cleavage at Arg^483, localizing the cross-link site between Thr^449 and the C terminus of σ at amino acid 613.

Next, σ was digested under conditions that yielded the 14-kDa mobility fragments. The products of the trypptic reaction were purified by fast protein liquid chromatography gel filtration on a Superose-6 column (Pharmacia Biotech Inc.) in the presence of 6 M guanidine hydrochloride (14). Well resolved peaks were collected, and guanidine hydrochloride was removed by membrane filtration on a Centricon-10 concentrator (Amicon). The purified polypeptides were then subjected to matrix-assisted laser desorption/ionization mass spectrometry (15) using a time-of-flight mass spectrometer constructed at the Rockefeller University (16, 17). Because of the low yield of the cross-linked σ, the mass spectrometric analysis was carried out on native σ (unmodified with affinity reagent). The mass spectrometric analysis revealed the presence of two polypeptides in the 14-kDa fraction, one of 10,935 ± 4 Da (fragment a) and the other of 10,263 ± 4 Da (fragment b). Based on the known specificity of trypsin cleavage, fragment a must correspond to either amino acids 1–99 or 466–562 of σ, while fragment b must correspond to either 1–93 or 487–578. Because we have already concluded that the cross-link site must be between Thr^449 and the C terminus of σ the label must be within amino acids 466–562 and/or 487–578.

We next performed CNBr cleavage of the purified, cross-linked σ subunit. Upon exhaustive treatment with CNBr, radioactivity accumulated in a single band with an apparent mobility of 8 kDa (Fig. 3A). Taking into account the presence of radioactive dinucleotide in the adduct, which adds about 1 kDa to the mass of the polypeptide itself, and also the aberrant mobility of the σ subunit and its fragments (9), the only possible fragments with such a mobility (between Thr^449 and the C terminus) are Glu^508-Met^561 (expected M, 5.8 kDa) or Asn^566-Asp^513 (expected M, 5.6 kDa). Under single-hit CNBr cleavage conditions (11), the smallest labeled band has a mobility of about 14 kDa (Fig. 3B). If the cross-link site was within Asn^566-Asp^513, the smallest labeled band would have an M, of 5.6 kDa (as seen in the Coomassie Blue-stained gel). Thus, we finally conclude that the cross-link site is within Glu^508-Met^561.

In addition to the experiments described here, we used Trp-specific BNPS-skatole (3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole) and Cys-specific 2-nitro-5-thiocyanobenzoic acid treatment to fragment the σ polypeptide (18). The results of these experiments were all consistent with and confirmed our conclusions presented above (data not shown). Thus, at least one σ subunit amino acid between Glu^508 and Met^561 is located within ~5 Å of the γ-phosphate of the priming nucleotide, which is the effective range of the probe used in this study. It should be noted that although this region of the σ subunit is close to the priming substrate, the cross-linked amino acid in this region cannot be involved in catalysis since the cross-linked adducts are still able to form at least two phosphodiester bonds (not shown). Elsewhere, we show that the β subunit Rif-region, as well as Lys^866 and His^827, are also cross-linked with the reagents used in this work (19). Hence, in the open complex the distance between these sites in the β subunit and the cross-link site in σ should not exceed ~10 Å.

The C-terminal portion of σ conserved region 3 (σ^36 amino acids 475–520) is contained within the σ^36 fragment we have identified as containing the ATP^* cross-link site. Region 3 is found in only a subset of σ factors and when present, the sequence is weakly conserved relative to conserved regions 2 and 4 (1, 2). Nevertheless, the sequence conservation observed is highly suggestive of structural and functional conservation. Also in contrast to regions 2 and 4, genetic studies to elucidate the function of region 3 have been few. A mutant E. coli σ^36 with a small deletion in region 3 (corresponding to σ^36 amino acids 503–520 in the aligned sequences) has been investigated (20). The protein, which otherwise appears to function normally, exhibits a reduced affinity for core RNAP. This is not inconsistent with our results as we have shown this region of σ^36 is close to specific sites on the β subunit.

The principal conclusion from our experiments pertinent to
the structure of RNA polymerase is that the results of our cross-link mapping, taken together with available genetic evidence on the interaction of α conserved domains 2 and 4 with the −10 and −35 promoter boxes, respectively, allow us to model the orientation of the α conserved domains in the open complex relative to promoter DNA as shown on Fig. 4. The main feature of this model is that α domains 2 and 3 are aligned “parallel” with respect to promoter DNA, but then the protein must flip back somewhere near the +1 position of the template DNA and proceed in an antiparallel orientation to the −35 promoter box. We note that such an orientation would allow an interaction between conserved regions 1 and 4 (Fig. 4), which agrees with recent findings of Dembroski et al. (21).

The −35 and −10 promoter boxes are separated by a distance of 25 base pairs or about 85 Å, assuming straight, B-form DNA. If α were spherical and had a protein density of 1.3 g/cm³, its diameter would be about 56 Å, much less than the distance it must span to interact simultaneously with the −35 and −10 promoter boxes. Our model in Fig. 4 now suggests that the distance from +1 to −35 (about 119 Å for straight, B-form DNA) must be spanned by only domains 3 and 4 (consisting of only about 18-kDa protein mass). Bending of the promoter DNA in the open complex so that the −35 promoter region is closer to +1 seems highly likely given these circumstances. Bent DNA in RNA polymerase-promoter binary complexes has previously been proposed based on gel electrophoretic experiments (22) and structural considerations (23).

REFERENCES