# Subunit Topography of RNA Polymerase from *Escherichia coli*. A Cross-Linking Study with Bifunctional Reagents†

Zaharia Hillel and Cheng-Wen Wu*,‡

ABSTRACT: The quaternary structures of *Escherichia coli* DNA-dependent RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) and core enzyme ($\alpha_2\beta\beta'$) have been investigated by chemical cross-linking with a cleavable bifunctional reagent, methyl 4-mercaptobutyrimidate, and noncleavable reagents, dimethyl suberimidate and *N,N'*-(1,4-phenylene)bismaleimide. A model of the subunit organization deduced from cross-linked subunit neighbors identified by dodecyl sulfate–polyacrylamide gel electrophoresis indicates that the large $\beta$ and $\beta'$ subunits constitute the backbone of both core and holoenzyme, while $\sigma$ and two $\alpha$ subunits interact with this structure along the contact domain of $\beta$ and $\beta'$ subunits. In holoenzyme, $\sigma$ subunit is in the vicinity of at least one $\alpha$ subunit. The two $\alpha$ subunits are close to each other in holoenzyme, core enzyme, and the isolated $\alpha_2\beta$ complex. Cross-linking of the "premature" core and holoenzyme intermediates in the in vitro reconstitution of active enzyme from isolated subunits suggests that these species are composed of subunit complexes of molecular weight lower than that of native core and holoenzyme, respectively. The structural information obtained for RNA polymerase and its subcomplexes has important implications for the enzyme–promoter recognition as well as the mechanism of subunit assembly of the enzyme.

DNA-dependent RNA polymerase is the key enzyme for the readout of genetic information in bacterial cells. The holoenzyme of *Escherichia coli* RNA polymerase is a protomer of mol wt about 500 000 and has a subunit composition of $\alpha_2\beta\beta'\sigma$ with the mol wt of each subunit being 40 000, 155 000, 165 000, and 90 000, respectively (Burgess, 1969; Berg and Chamberlin, 1970). The $\sigma$ subunit can be reversibly dissociated from holoenzyme to form core enzyme ($\alpha_2\beta\beta'$) (Burgess et al., 1969; Wu et al., 1975). While the $\sigma$ subunit is involved in promoter selection and specific chain initiation, core polymerase contains the basic catalytic unit for RNA chain elongation (Burgess et al., 1969; Bautz et al., 1969). The roles of the other subunits in transcription are poorly understood. The individual subunits or subunit complexes obtained by treatment of RNA polymerase with various denaturing reagents lack enzymatic activity and do not carry out any of the partial reactions involved in RNA synthesis (Ishihama, 1972; Yarbrough and Hurwitz, 1974). Under appropriate conditions, active RNA polymerase can be reconstituted from the isolated subunits (Heil and Zillig, 1970; Ishihama, 1972; Yarbrough and Hurwitz, 1974; Fukuda and Ishihama, 1974; Palm et al., 1975). In the course of reconstitution, inactive, "premature" core and holoenzyme complexes are formed, in which the arrangement of the subunits is different from that of active core and holoenzyme (Fukuda and Ishihama, 1974; Palm et al., 1975; Harding and Beychok, 1974). This indicates that proper arrangement of the subunits is necessary for the RNA polymerase activity.

A remarkable feature of bacterial transcription is that the purified bacterial polymerase is able to carry out selective transcription of certain native DNA molecules in the absence of regulatory components such as repressor or activator proteins (Chamberlin, 1974). The basic unit of selective transcription is thought to be the RNA polymerase holoenzyme. Furthermore, since in some phages the basic processes of RNA synthesis on a DNA template can be carried out by phage RNA polymerases which consist of a single polypeptide chain (mol wt ~100 000) (Chamberlin et al., 1970; Dunn et al., 1972; Maitra, 1971), the complex structure of the bacterial RNA polymerase seems to be required for regulatory rather than catalytic function. Thus, elucidation of the subunit organization of *E. coli* RNA polymerase is important for understanding the regulation of gene transcription.

Limited information is available concerning the quaternary structure of the enzyme. Since RNA polymerase is a large macromolecule, it is almost impossible at the present time to obtain such information by x-ray crystallography. Attempts to obtain the fine structure of the enzyme by electron microscopy have also been unsuccessful (Slayter and Hall, 1966; Colvill et al., 1966; Kitano and Kameyama, 1969; Lubin, 1969). Therefore, one has to rely upon indirect methods such as chemical cross-linking with bifunctional reagents (King et al., 1974) or partial digestion with proteolytic enzymes (Lill and Hartmann, 1975) to probe the topography of the enzyme. We have undertaken the present cross-linking study in order to obtain as complete information as possible concerning the subunit arrangement of RNA polymerase. To this end, we have carried out cross-linking of the enzyme with bifunctional reagents of varying specificity and length. Direct identification of subunit neighbors was obtained from the composition of cross-linked complexes by two-dimensional electrophoresis with an intermediate chemical cleavage of the cross-links. These results were confirmed by estimating the molecular weights of cross-linked products based on their electrophoretic mobilities on dodecyl sulfate–polyacrylamide gels. A spatial arrangement of the RNA polymerase subunits is proposed based on results obtained from cross-linking of holoenzyme,

† From the Department of Biophysics, Division of Biological Sciences, Albert Einstein College of Medicine, New York, New York 10461. *Received December 6, 1976.* This investigation was supported in part by U.S. Public Health Service Research Grant No. GM-19062 and by American Cancer Society Research Grant No. BC-94. A preliminary account of this work has been presented at the 1976 Annual Biophysical Society Meeting, Seattle, Wash. (Hillel and Wu, 1976a).

‡ Recipient of a Research Career Development Award from the National Institutes of Health.

core enzyme, and two subcomplexes of the enzyme. In addition, possible structural models for so-called "premature" core and holoenzyme are also discussed.

## Experimental Procedures

*Materials.* Dimethyl suberimidate and methyl 4-mercaptobutyrimidate were purchased from Pierce, and *N,N'*-(1,4-phenylene)bismaleimide was from Aldrich. These reagents were used without further purification. The 99% pure sodium dodecyl sulfate was obtained from Pierce and electrophoresis grade acrylamide and *N,N'*-methylenebisacrylamide were products of Polysciences.

*E. coli* B and *E. coli* K12 cells (¾ log phase) were purchased from Grain Processing. Phosphocellulose P-11 was from Reeve Angel and Ultra Pure tris(hydroxymethyl)aminomethane and urea were products of Schwarz/Mann. *N,N'*-Bis(2-hydroxyethyl)glycine (Bicine)[1] was from Sigma.

*RNA Polymerase and Its Subunits.* RNA polymerase holoenzyme was purified from *E. coli* B as described previously (Wu and Wu, 1973), or from *E. coli* K12 by the method of Burgess and Jendrisack (1975). The enzyme was 94–98% pure and contained all subunits ($\alpha$, $\beta$, $\beta'$, and $\sigma$) as shown by dodecyl sulfate–polyacrylamide gel electrophoresis in phosphate buffer. Core polymerase ($\alpha_2\beta\beta'$) and the $\sigma$ subunit were prepared from holoenzyme by the procedure of Berg et al. (1972).

Subunits of RNA polymerase were obtained by denaturation of holoenzyme in 7 M urea and subsequent separation on a phosphocellulose column in the presence of urea (Yarbrough and Hurwitz, 1974). The isolated $\alpha$ subunit was 93% pure as judged electrophoretically. The $\alpha_2\beta$ complex was reconstituted from a mixture of $\alpha$ and $\beta$ subunits (Palm et al., 1975) with a molar ratio of $\alpha/\beta \simeq 2$. The purity of $\alpha_2\beta$ complex was estimated to be 94% by polyacrylamide gel electrophoresis. To prepare the reconstituted enzyme complexes, either core or holoenzyme was denatured in 7 M urea for 10 min at 30 °C (Ishihama and Ito, 1972) followed by reconstitution at 0 °C (Palm et al., 1975). The cold-reconstituted or "premature" enzyme preparations were further purified by centrifugation on 12–30% linear glycerol gradients in 0.05 M Tris-acetate (pH 7.9), 0.2 M KCl, 0.01 M magnesium acetate, 0.001 M EDTA, 0.02 M mercaptoethanol at 0 °C in a SW-40 rotor (Beckman) for 28 h at 40 000 rpm or the equivalent $rpm^2$-h value. The warm reconstituted enzyme complex was obtained by incubation of the cold reconstituted preparation at 31 °C for 90 min. $s_{20,w}$ values of the reconstituted enzyme complexes were estimated using core polymerase and bovine liver catalase as standards (Martin and Ames, 1960). Enzymatic activities of the reconstituted enzyme preparations were assayed with synthetic poly[d(A-T)]·poly[d(A-T)] as template (Grand Island Biochemicals) in the presence of 0.12 M $NH_4Cl$ (Wu and Wu, 1973).

*Cross-Linking Experiments.* To prevent the aggregation of RNA polymerase which occurs under low salt conditions (Berg and Chamberlin, 1970), cross-linking reactions for holo- and core enzyme were carried out at salt concentrations higher than 0.3 M (typically 0.4 M). Isolated $\alpha$ subunit and the $\alpha_2\beta$ complex were cross-linked at lower salt concentration (0.2 M). The protein concentrations used were less than 1 mg/mL (typically 0.3 mg/mL) to favor cross-linking within protomer over that between protomers (Davies and Stark, 1970).

The reaction buffer used for cross-linking with dimethyl suberimidate (DS) was 0.05 M Bicine–NaOH (pH 8.5), $10^{-4}$ M dithiothreitol, and 0.4 M NaCl. Immediately before use, DS was dissolved in the reaction buffer (30 mg/mL) and the pH was readjusted to 8.5. Aliquots of this solution were added to proteins in the same buffer to a final reagent concentration of 3 mg/mL. After standing at room temperature for various time periods, the reaction was quenched by addition of ethanolamine-HCl (pH 8) (0.14 M final concentration). Twenty minutes later the solution was made 1% in sodium dodecyl sulfate and 2–3% in $\beta$-mercaptoethanol and dialyzed against the appropriate electrophoresis buffer.

Cross-linking with methyl 4-mercaptobutyrimidate (MMB) was performed following the procedure of Traut et al. (1973) with slight modification. The same buffer as for the DS reaction was used. The proteins were allowed to react with the reduced cross-linking reagent at a reagent concentration of 3 mg/mL for 6 min at 22 °C, and the reactions were quenched by ethanolamine as described before. After 10 min, 40 mM hydrogen peroxide was added and the reaction mixtures were incubated for 30 min at room temperature to promote cross-linking of the MMB-charged protein molecules due to disulfide bond formation betweeen the thiols of MMB. The reactions were stopped by addition of 1% sodium dodecyl sulfate. Iodoacetamide, 10 mg/mL, was added to prevent further oxidation of the thiol groups.

The cross-linking reactions with *N,N'*-(1,4-phenylene)-bismaleimide (PBM) were carried out in 0.05 M sodium phosphate buffer (pH 8), previously purged with nitrogen gas to remove oxygen. The cross-linking reagent was added from a $3 \times 10^{-2}$ M solution in dimethylformamide to give a final concentration of $10^{-4}$ M. Reaction mixtures containing reagent and protein were shaken at room temperature for 30 min. The reaction was then stopped by adding 2% $\beta$-mercaptoethanol with agitation.

Cross-linking reactions of the reconstituted enzyme complexes were carried out with DS for 60 to 90 min at 0 °C in Bicine–NaOH buffer (pH 8.5), in the presence of 0.2 M NaCl.

*Polyacrylamide Gel Electrophoresis.* Proteins cross-linked with DS were electrophoresed in phosphate buffer containing sodium dodecyl sulfate as described by Shapiro et al. (1967). One-dimensional electrophoresis of PBM cross-linked products was performed, as described by Zillig et al. (1975), in linear 5–15% acrylamide-gradient slab gels using the discontinuous buffer system of Laemmli (1970).

MMB cross-linked core enzyme was analyzed on a two-dimensional gel system in which the cross-links were cleaved before electrophoresis in the second dimension. The method used was a modification of that of Wang and Richards (1974). In both dimensions, the buffer system of Laemmli (1970) was used. Electrophoresis in the first dimension was performed in a 3 mm thick, 8–9 cm long slab gel containing a 5–15% linear acrylamide gradient, topped with a 3.3% acrylamide stacking gel and ran for 800–900 V-h. After electrophoresis was completed, 6 mm wide strips containing cross-linked proteins were cut from the slab, placed in protective plastic enclosures, and dialyzed against Laemmli sample buffer (Lsb) with 3% $\beta$-mercaptoethanol for 1 h at 50 °C. This was followed by dialysis against two changes of Lsb, 45 min each, and, finally, dialysis against Lsb with $10^{-3}$ M iodoacetamide for 60 min. The gels were subsequently soaked in 20 mL of sample gel solutions (Lsb with 3.3% acrylamide, 0.045% persulfate, and 0.015% Temed) for 5 min, and then polymerized into the top of a slab gel identical with that used in the first dimension using sample

[1] Abbreviations used are: Bicine, *N,N'*-bis(2-hydroxyethyl)glycine; DS, dimethyl suberimidate; MMB, methyl 4-mercaptobutyrimidate; PBM, *N,N'*-(1,4-phenylene)bismaleimide; Lsb, electrophoresis sample buffer described by Laemmli (1970); Temed, *N,N,N',N'*-tetramethylethylenediamine.

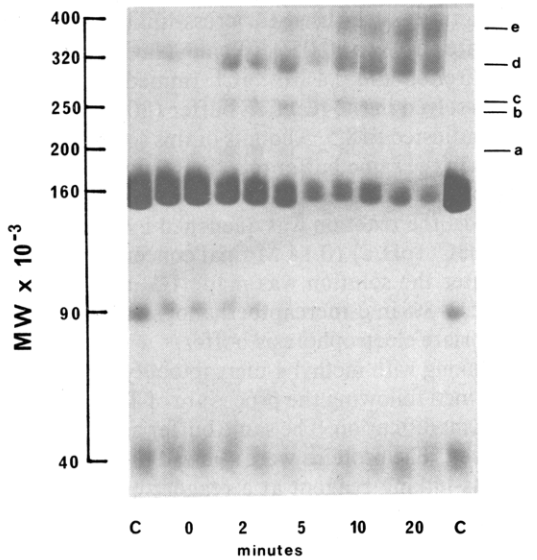


FIGURE 1: Electrophoretic patterns of holoenzyme cross-linked with dimethyl suberimidate for various time periods. Electrophoresis was performed in 3.7% polyacrylamide gels in a phosphate buffer containing sodium dodecyl sulfate in the direction of decreasing molecular weight as described under Experimental Procedures. The gels marked C at the right and left extremes are unmodified holoenzyme. The remaining gels, in pairs, represent 0-, 2-, 5-, 10-, and 20-min reaction products of holoenzyme. The cross-linked bands are labeled a to e.

gel solution containing 0.1% Temed. Electrophoresis in the second dimension was for 800–850 V-h.

All gels were stained for at least 2 h at 40 °C or >4 h at room temperature in 0.1% Coomassie Blue R solution in 5:5:1 water–methanol–acetic acid (v/v/v). Destaining was by diffusion at room temperature in the same solution lacking stain.

For estimation of protein content in various electrophoretic bands, gels stained with Coomassie Blue were scanned for dye absorbance using a spectrophotometric gel scanner constructed in our laboratory. The area under each band, which represents the amount of dye bound, was measured by use of a planimeter. Using RNA polymerase subunits as standards, the areas measured by these spectrophotometric scans were found to be proportional to the amount of protein added to the gel, with an estimated error of ±11%.

## Results

*Cross-Linking with Dimethyl Suberimidate (DS).* RNA polymerase holoenzyme was cross-linked with DS at ionic strength higher than 0.25 to eliminate aggregations of the enzyme molecules (Berg and Chamberlin, 1970). The time dependence of the cross-linking reaction analyzed by electrophoresis in dodecyl sulfate–polyacrylamide gels is shown in Figure 1. It has been shown (Shapiro et al., 1967) that a linear relationship between the logarithm of molecular weight and relative electrophoretic mobility holds for proteins with molecular weights in the range of 15 000 to 165 000. We have observed that with 3.7% polyacrylamide gels the validity of this semilogarithmic relationship can be extended to a mol wt of at least 320 000 as demonstrated by the mobility of $\beta\beta'$ complex (King et al., 1974). Using gels of similar acrylamide concentrations, this linear relationship has also been applied to systems in which some components had molecular weights as high as 450 000 (Manjula and Richards, 1975). Thus, we have estimated the molecular weights of the cross-linked bands shown in Figure 1 and made the tentative identifications given in Table I. The un-cross-linked subunits of the enzyme were used as markers in the molecular weight determination. It can be seen from Figure 1 that over the 20-min period of the cross-linking reaction, the amount of cross-linked material of mol wt ≥320 000 increased with time, while the intensity of the bands with mol wt of 200 000, 245 000, and 260 000 was low and remained relatively constant after the initial increase at 2 min. This is expected since binary cross-linked subunit complexes from proteins having more than two subunits could merely be intermediates of reaction; therefore, their concentrations could have relatively low steady-state values.

TABLE I: Identification of Cross-Linked Bands Obtained with DS.

| Band | Obsd mol wt | Proposed subunit composition | Calcd mol wt |
|---|---|---|---|
| | | Holoenzyme (Figure 1) | |
| a | 200 000 | $\alpha\beta$ | 195 000 |
| | | $\alpha\beta'$ | 205 000 |
| b | 245 000 | $\alpha_2\beta$ | 235 000 |
| | | $\beta\sigma$ | 245 000 |
| c | 260 000 | $\alpha_2\beta$ | 245 000 |
| | | $\beta'\sigma$ | 255 000 |
| d | 300 000–330 000 | $\beta\beta'$ | 320 000 |
| e | 350 000–400 000 | $\alpha_2\beta\beta'$ | 400 000 |
| | | $\beta\beta'\sigma$ | 410 000 |
| | | Core Enzyme (Figure 2) | |
| f | 190 000 | $\alpha\beta$ | 195 000 |
| g | 210 000 | $\alpha\beta'$ | 205 000 |
| h | 300 000–350 000 | $\beta\beta'$ | 320 000 |
| | | $\alpha\beta\beta'$ | 360 000 |

Figure 2 shows the electrophoretic patterns of core enzyme cross-linked with DS at an ionic strength high enough so that no enzyme aggregates exist (Berg and Chamberlin, 1970). Three cross-linked bands can be identified tentatively by molecular weight estimation (see Table I). Comparison between the cross-linked bands of core enzyme (Figure 2) and holoenzyme (Figure 1) obtained under the same conditions revealed that bands of molecular weight equivalent to bands b, c, and e seen with holoenzyme are missing in core enzyme, suggesting that in holoenzyme these bands contain $\beta\sigma$, $\beta'\sigma$, and $\beta\beta'\sigma$ rather than $\alpha_2\beta$, $\alpha_2\beta'$, and $\alpha_2\beta\beta'$, respectively.

*Cross-Linking with Methyl 4-Mercaptobutyrimidate (MMB).* The use of MMB as a cleavable cross-linking reagent was introduced by Traut et al. (1973) in unraveling the very complex structure of bacterial ribosomes. The cross-linked products of this reagent could be easily cleaved by chemical reduction thereby allowing direct identification of their composition by use of two-dimensional dodecyl sulfate–polyacrylamide gel electrophoresis. The two-dimensional electrophoretic pattern of RNA polymerase holoenzyme cross-linked with MMB and a schematic representation of the pattern are given in Figure 3 (A and B). Un-cross-linked polypeptides fall on the diagonal line. Spots 1, 2, and 3 represent the un-cross-linked $\alpha$, $\sigma$, and $\beta + \beta'$ subunits, respectively. The molecular weights of cross-linked complexes were estimated by interpolation using these un-cross-linked subunits as markers. In addition to the un-cross-linked subunits, other less intense spots seen on the diagonal line are unidentified polypeptides—either impurities or proteolytic products present in our enzyme preparations. These spots also appear on two-dimensional gels of un-cross-linked holoenzyme and amount to less than 7% of the total protein as estimated by stain densitometry. They show up clearly due to the large amount of protein (~70 μg) applied

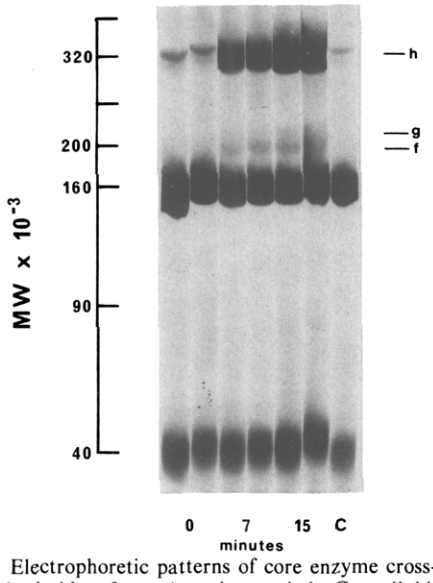


FIGURE 2: Electrophoretic patterns of core enzyme cross-linked with dimethyl suberimidate for various time periods. Cross-linking and electrophoresis were the same as in Figure 1. The gel marked C contains unmodified core enzyme. The other gels, in pairs, represent 0-, 7-, and 15-min reaction products of core enzyme. The cross-linked bands are labeled f to h.

TABLE II: Identification of Cross-Linked Complexes Obtained with MMB.

| Spot | Obsd mol wt (1st dimension) | Subunit composition (2nd dimension) | Proposed complex formula | Calcd mol wt |
|---|---|---|---|---|
| | Holoenzyme (Figure 3) | | | |
| 4 | 75 000–85 000 | $\alpha$ | $\alpha_2$ | 80 000 |
| 5 | 85 000–100 000 | $\alpha$ | $\alpha_2$ | 80 000 |
| 5 + 6 | 100 000–120 000 | $\alpha,\sigma$ | $\alpha\sigma$ | 130 000 |
| 8 + 9 + 10 | 220 000–260 000 | $\beta, \beta', \sigma$ | $\beta\sigma$ | 245 000 |
| | | | $\beta'\sigma$ | 255 000 |
| 8 + 11 | 220 000–260 000 | $\alpha, \beta, \beta'$ | $\alpha_2\beta$ | 235 000 |
| | | | $\alpha_2\beta'$ | 245 000 |
| 12 + 13 + 14 | 260 000–400 000 | $\alpha, \beta, \beta', \sigma$ | $\beta\beta'$ | 320 000 |
| | | | $\alpha\beta\beta'$ | 360 000 |
| | | | $\alpha_2\beta\beta'$ | 400 000 |
| | | | $\beta\beta'\sigma$ | 410 000 |
| | Core Enzyme (Figure 4) | | | |
| 4 | 70 000–80 000 | $\alpha$ | $\alpha_2$ | 80 000 |
| 5 | 85 000–110 000 | $\alpha$ | $\alpha_2$ | 80 000 |
| 8 + 11 | 220 000–300 000 | $\alpha, \beta, \beta'$ | $\alpha_2\beta$ | 235 000 |
| | | | $\alpha_2\beta'$ | 245 000 |
| 12 + 14 | 280 000–360 000 | $\alpha, \beta, \beta'$ | $\beta\beta'$ | 320 000 |
| | | | $\alpha\beta\beta'$ | 360 000 |
| | | | $\alpha_2\beta\beta'$ | 400 000 |
| | Core Enzyme (Figure 5) | | | |
| 4 | 75 000–80 000 | $\alpha$ | $\alpha_2$ | 80 000 |
| 7a + 7c | 190 000–205 000 | $\alpha, \beta$ | $\alpha\beta$ | 195 000 |
| 7b + 7c | 205 000–220 000 | $\alpha, \beta'$ | $\alpha\beta'$ | 205 000 |
| 12 | 260 000–330 000 | $\beta, \beta'$ | $\beta\beta'$ | 320 000 |

to the gels. The almost exclusive appearance of these impurities on the diagonal line demonstrates that they are not cross-linked to RNA polymerase.

The cross-linked subunit complexes in Figure 3 were identified by the molecular weights estimated in the first dimension and the subunit compositions determined in the second dimension, as shown in Table II. The most extensive cross-linking appears to have involved the $\beta$ and $\beta'$ subunits which formed complexes whose molecular weights in the first dimension are in the range of 200 000 to 400 000 (spots 7, 8, and 12). As can be seen in Figure 3A, there is some uncertainty in the identification of the ternary and higher order cross-linked complexes due to streaking of the spots (spots 7, 8, 12, 10, and 13). However, this will not affect the conclusions we will draw below concerning the proximity relationships of the subunits since they are derived solely from the information obtained with the binary complexes.

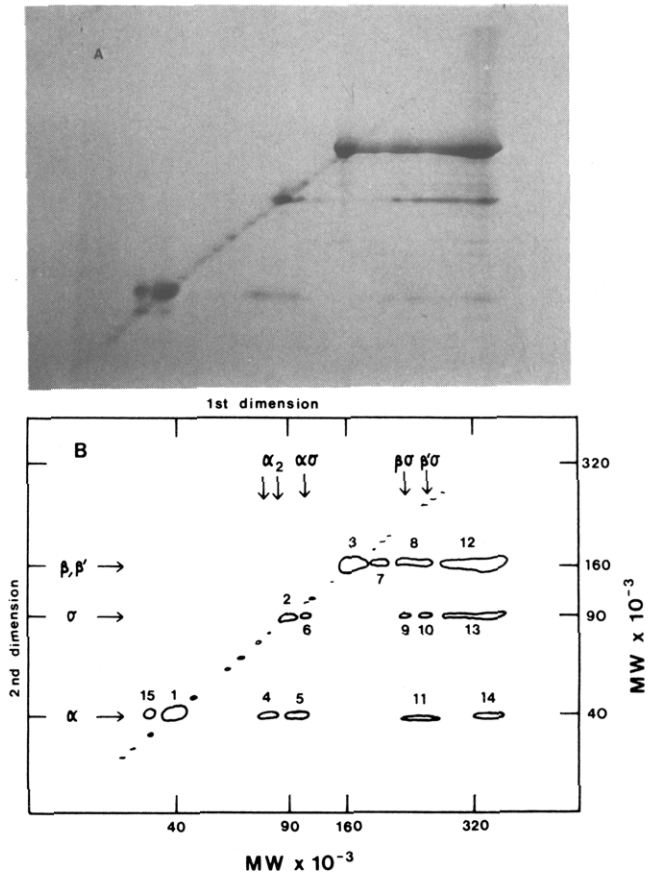


FIGURE 3: (A) Two-dimensional electrophoretic pattern of holoenzyme cross-linked with methyl 4-mercaptobutyrimidate. Holoenzyme was cross-linked with MMB and electrophoresis was performed in 5–15% acrylamide gradient gels in a discontinuous buffer system with intermediate cleavage of MMB as described under Experimental Procedures. (B) Schematic representation of the gel pattern. Electrophoresis was in the direction of decreasing molecular weight. Binary cross-linked complexes are labeled along the first dimension and un-cross-linked subunits are labeled along the second dimension.

Under our reaction conditions, the $\alpha$ subunit has shown relatively low reactivity with MMB. Although the $\alpha$ subunit spots derived from complexes which contain the $\alpha$ subunit (spots 4, 5, 11, and 14) are faint on gels of cross-linked enzyme, they have been reproduced in five different experiments and did not appear on gels of un-cross-linked enzyme. Spot 7 (mol wt ~190 000) is probably $\beta$ or $\beta'$ subunit derived from an $\alpha\beta$ or $\alpha\beta'$ complex. The $\alpha$ subunit spot from such a complex could be too faint to be seen in this gel.

Spot 15 is interesting because it is the only one to appear above the diagonal in the two-dimensional gel. It has an apparent mol wt of ~35 000 in the first dimension. Since this spot moved with the mol wt of $\alpha$ (40 000) in the second dimension, we propose that it is the result of intrasubunit cross-linking of

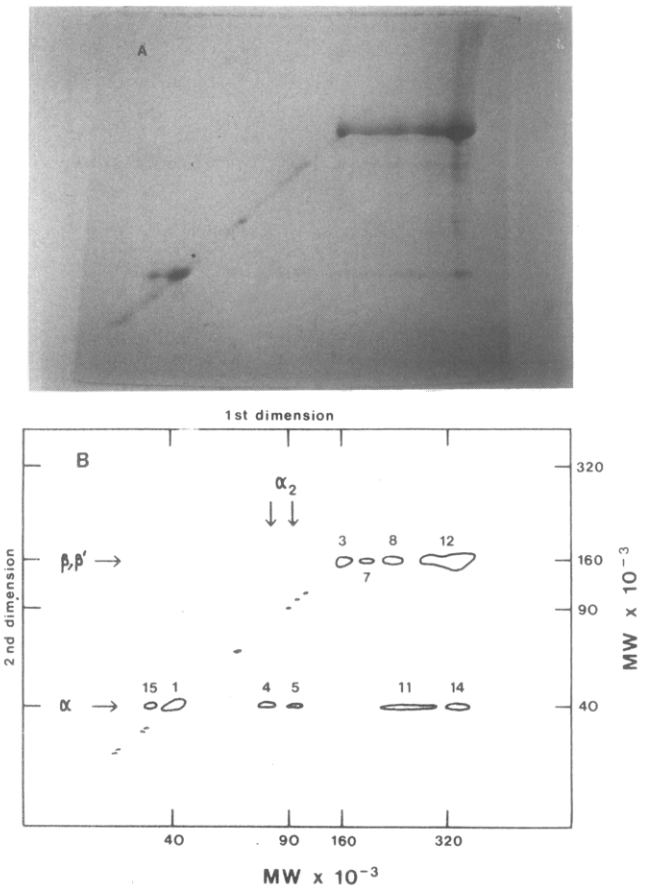


FIGURE 4: (A) Two-dimensional electrophoretic pattern of core enzyme cross-linked with methyl 4-mercaptobutyrimidate. Core enzyme was cross-linked with MMB and subjected to electrophoretic analysis as described in Figure 3A. (B) Schematic representation of the gel pattern. Binary cross-linked complexes are labeled along the first dimension and un-cross-linked subunits are labeled along the second dimension.

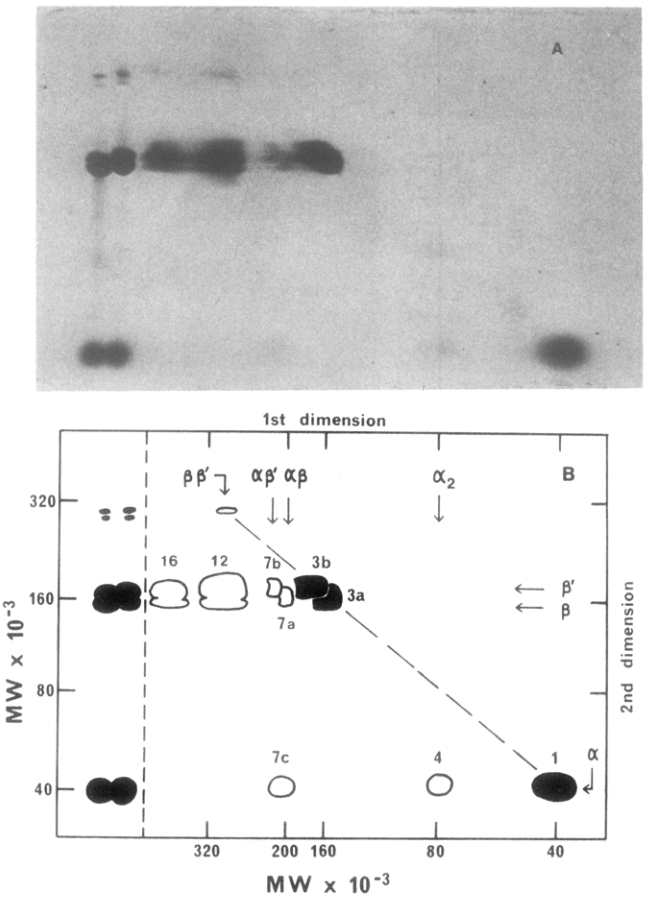


FIGURE 5: (A) Two-dimensional electrophoretic pattern of core enzyme cross-linked with methyl 4-mercaptobutyrimidate. Core enzyme was cross-linked with MMB and electrophoresis was performed in 3.7% acrylamide gels in phosphate buffer as described under Experimental Procedures. (B) Schematic representation of the gel pattern. The tracks to the left of the vertical dashed line represent duplicate controls of unmodified core enzyme subject to electrophoresis in the second dimension. The diagonal line marks un-cross-linked polypeptides. Binary cross-linked complexes are labeled along the first dimension and un-cross-linked subunits are labeled along the second dimension.

$\alpha$. The intrasubunit cross-links could give the polypeptide a more compact structure causing it to move faster in the first dimension. After cleavage of the cross-links, it would move normally in the second dimension.

Figure 4 (A and B) shows the electrophoretic pattern and schematic representation for core polymerase cross-linked with MMB. Again the un-cross-linked $\alpha$ and $\beta + \beta'$ subunits on the diagonal line (spots 1 and 3) were used as molecular weight markers. Except for the absence of spots containing $\sigma$ subunit, the pattern for core enzyme is similar to that obtained for holoenzyme. The identification of cross-linked complexes for core enzyme is given in Table II. Again, spot 7 is probably $\beta$ and $\beta'$ derived from either $\alpha\beta$ or $\alpha\beta'$.

In order to enhance the resolution of the higher molecular weight bands, we have also run two-dimensional electrophoresis in 3.7% acrylamide gels (rather than 5–15% acrylamide gradient) in phosphate buffer. This gel system has the advantage of allowing reliable estimation of mol wt of bands up to 320 000 from their electrophoretic mobility. The results obtained for core polymerase cross-linked with MMB are shown in Figure 5 (A and B). In this electrophoretic pattern, partial separation between the $\beta$ and $\beta'$ subunits can be observed for spots 3, 7, 12, and 16. The un-cross-linked $\alpha$ is seen in spot 1, whereas free $\beta$ and $\beta'$ are seen in spots 3a and 3b, respectively. Some unidentified contaminants in the core enzyme preparations are barely visible on the diagonal line. The identification of the cross-linked complexes in Figure 5 is also included in Table II. Spot 16 (mol wt $\geq$360 000) probably represents the cross-linked core enzyme complex.

*Cross-Linking with N,N'-(1,4-Phenylene)bismaleimide (PBM).* Lack of reactive residues in the protein or improper orientation of these residues may lead to a negative result in cross-linking reaction. To overcome such potential difficulties, we have employed cross-linking reagents of differing specificity. In particular, the sulfhydryl-specific bismaleimide was used in addition to the amino-specific bisimido esters. The electrophoretic pattern of core polymerase cross-linked with PBM is shown in Figure 6. The major cross-linked bands are II and III with mol wt of approximately 80 000 (track 3) which do not appear in the gel pattern of un-cross-linked core enzyme (track 1). Track 2 shows that incubation of core enzyme with PBM in the presence of 1% sodium dodecyl sulfate failed to produce bands II and III, suggesting that the native structure of the enzyme is essential for the cross-linking. Based on the estimated molecular weights, we assigned these two bands to be two forms of $\alpha$ dimers. It seems likely that band II moves faster than band III because the former has one or more intrasubunit cross-links, similar to that observed in $\alpha$ cross-linked with MMB. In order to verify this assignment, we have cross-linked the $\alpha_2\beta$ subcomplex of the enzyme and the isolated $\alpha$ subunit with PBM. The results of these experiments are

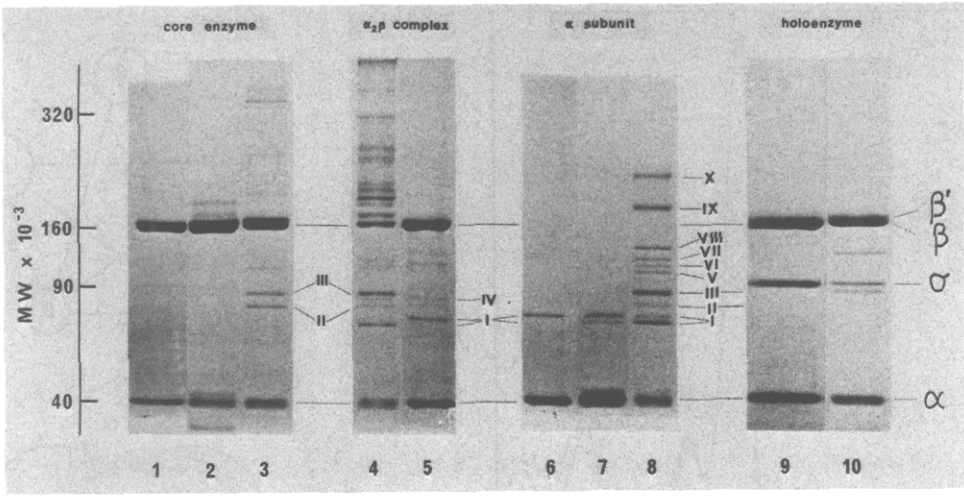


FIGURE 6: Electrophoretic patterns of core polymerase, $\alpha_2\beta$ complex, isolated $\alpha$ subunit, and holoenzyme cross-linked with *N,N'*-(1,4-phenylene)bismaleimide. Cross-linking was carried out as described under Experimental Procedures. Electrophoresis was performed in 5–15% acrylamide gradient gels with a discontinuous buffer. The un-cross-linked subunit bands are identified at the extreme right: (track 1) unmodified core enzyme; (track 2) core enzyme cross-linked after denaturation in 1% sodium dodecyl sulfate for 30 min at 50 °C; (track 3) cross-linked core enzyme; (track 4) cross-linked $\alpha_2\beta$ complex; (track 5) unmodified $\alpha_2\beta$ complex; (track 6) unmodified isolated $\alpha$ subunit; (track 7) isolated $\alpha$ subunit cross-linked after denaturation in 1% sodium dodecyl sulfate for 30 min at 50 °C; (track 8) cross-linked isolated $\alpha$ subunit; (track 9) unmodified holoenzyme; (track 10) cross-linked holoenzyme.

shown in tracks 4 to 8. The $\alpha_2\beta$ subcomplex yielded cross-linked bands II and III in addition to multiple bands of mol wt >160 000 (track 4) which are assumed to contain cross-linked aggregates of $\alpha\beta$ and $\alpha_2\beta$ complexes. Bands I and IV are impurities which can also be seen in the gel pattern of un-cross-linked $\alpha_2\beta$ (track 5). The introduction of intrasubunit cross-links is probably responsible for the shift to a lower apparent molecular weight of band I upon cross-linking. Bands II and III observed in the gel pattern of the cross-linked isolated $\alpha$ subunit (track 8) are identified as $\alpha$ dimers based on their apparent mol wt (~80 000) and the fact that these two bands are also seen in the cross-linked $\alpha_2\beta$ complex (track 4) and core enzyme (track 3) which contain only two $\alpha$ subunits. Additional bands of apparent mol wt higher than 80 000 (bands V, VI, VIII, IX, and X in track 8) represent oligomers of $\alpha$ larger than dimer. Bands I and VII are impurities since they also appear in the gel pattern of un-cross-linked $\alpha$ (track 6). When the isolated $\alpha$ subunit cross-linked with PBM was subjected to glycerol gradient centrifugation, essentially two broad peaks were obtained: the lighter peak which consisted of more than 60% of the material in these two peaks had the same mobility as un-cross-linked $\alpha$ while the heavier one sedimented slightly slower than the $\alpha_2\beta$ complex. Electrophoresis of these separated peaks (data not shown) revealed that the slower sedimenting material contained gel bands I, II, and III whereas the faster sedimenting material contained bands V through X (cf. Figure 6, track 8). These results suggest that the isolated $\alpha$ subunit exists largely as dimer and can form higher-order aggregates. Incubation of isolated $\alpha$ subunit with 1% sodium dodecyl sulfate disrupts the aggregation and thereby prevents the formation of cross-linked complexes (track 7).

Cross-linking of holoenzyme with PBM produced, in addition to the two $\alpha$ dimers (bands II and III), a band having mobility intermediate to that of $\beta$ and $\sigma$ (Figure 6, track 10). The apparent mol wt of 130 000 suggests that it has the composition of $\alpha\sigma$.

*Cross-Linking of the Reconstituted Enzyme Complexes.* When RNA polymerase was denatured in 7 M urea and subsequently reconstituted at 0 °C, an inactive "premature" enzyme complex was formed, which sedimented as a discrete band considerably slower than native enzyme (Fukuda and Ishihama, 1974; Palm et al., 1975). We have isolated both the cold reconstituted core and holoenzyme complexes and found that they had the same subunit composition as native enzyme. At an ionic strength of 0.2 both these preparations had an $s_{20,w}$ value of about 10, while the corresponding values for core and holoenzyme were 13 and 15, respectively. Upon warming at 31 °C for 90 min, the enzymatic activities of the cold reconstituted enzyme complexes were markedly enhanced: a five-fold increase for the holoenzyme and a 50-fold increase for the core enzyme preparations (the specific activity of warm reconstituted enzyme complex was about 50% of the original native enzyme). Furthermore, the warm reconstituted enzyme complex showed sedimentation properties similar to those of native RNA polymerase.

The cold and warm reconstituted core enzyme preparations were cross-linked with DS at 0 °C; both produced $\alpha\beta$ and $\beta\beta'$ cross-linked bands as analyzed on dodecyl sulfate–polyacrylamide gels in phosphate buffer (Figure 7). However, comparison between the cold reconstituted and warm reconstituted complexes revealed that the extent of $\beta\beta'$ cross-linking in the latter was approximately twice that in the former (after correction for the small amount of $\beta\beta'$ complex seen on the gel of unmodified core enzyme; for example, see Figure 2, track C), while the extent of $\alpha\beta$ cross-linking appeared to be the same. Gels of DS cross-linked cold and warm reconstituted holoenzyme exhibited cross-linked $\alpha\beta$, $\sigma\beta$, $\sigma\beta'$, and $\beta\beta'$ bands. Again, the warm reconstituted holoenzyme showed about twice the amount of $\beta\beta'$ complex when compared to the cold reconstituted preparation. No significant differences in the $\alpha\beta$, $\sigma\beta$, and $\sigma\beta'$ bands were observed.

## Discussion

Previous attempts to obtain structural information about *E. coli* RNA polymerase using the cross-linking technique have yielded an incomplete picture. One report on cross-linking of core polymerase with dimethyl suberimidate indicated the formation of a single large aggregate (Young and Blumenthal, 1975). This was probably the result of over-reaction. A different study (King et al., 1974) using the same reagent noted

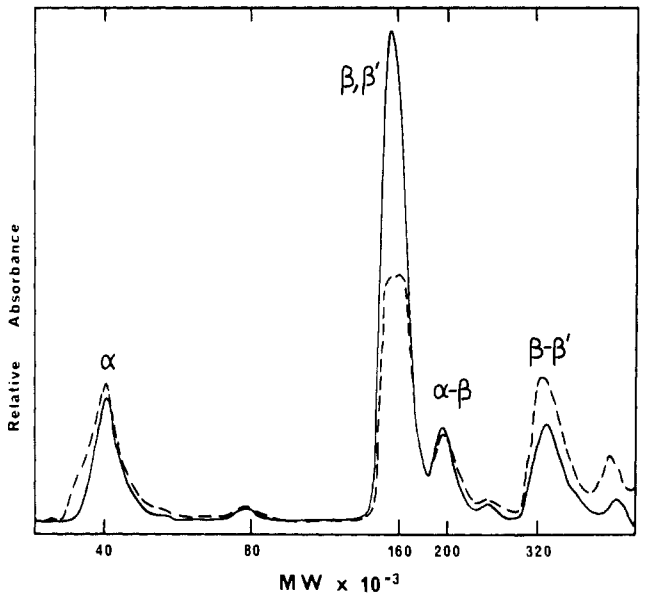


FIGURE 7: Spectrophotometric gel scans of reconstituted core enzyme cross-linked with dimethyl suberimidate. Cross-linking and analysis by dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described under Experimental Procedures. Electrophoresis was in the direction of decreasing molecular weight: (—) cross-linked cold reconstituted core enzyme complex; (- - -) cross-linked warm reconstituted core enzyme complex. The amounts of protein used in the electrophoresis, and thus the total areas under all peaks, are approximately the same for these two cases.

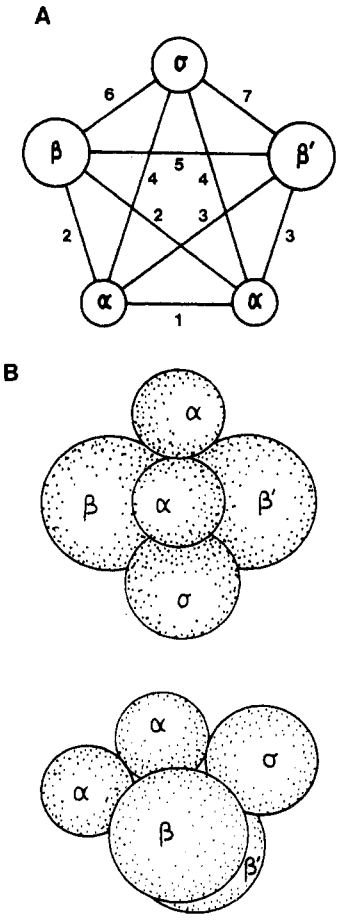


FIGURE 8: Schematic illustration of the spatial arrangement of subunits in RNA polymerase holoenzyme: (A) possible subunit neighbors in the oligomeric complex $\alpha_2\beta\beta'\sigma$: 1, $\alpha\alpha$; 2, $\alpha\beta$; 3, $\alpha\beta'$; 4, $\alpha\sigma$; 5, $\beta\beta'$; 6, $\beta\sigma$; 7, $\beta'\sigma$. (B) The proposed model based on our cross-linking results. For simplicity all the subunits were assumed to be spherical in shape with relative volumes corresponding to their respective molecular weights. Two different views of the same subunit arrangement are shown.

the formation of an intense $\beta\beta'$ complex and a $\beta\beta'\sigma$ complex for holoenzyme. It was pointed out that the $\alpha$ subunit failed to cross-link with any other subunit in both core and holoenzyme.

We have been able to identify binary cross-linked complexes containing each of the four subunits of RNA polymerase. With the two bisimido esters used in this study the predominant cross-linking product of both core and holoenzyme is the $\beta\beta'$ complex. These imido esters react quite specifically with the $\epsilon$-amino group of lysine residues, as indicated by the observation that the rate of cross-linking increases with increasing pH from 8 to 10. The relative number of lysine residues in each subunit of RNA polymerase varies little, from 4.8 mol % in $\alpha$ to 6.1 mol % in $\beta'$ (Fujiki and Zurek, 1975). Thus, the high reactivity of $\beta$ and $\beta'$ relative to $\sigma$ and $\alpha$ subunits is probably due to their larger surface area and hence larger number of potential sites for cross-linking. In agreement with a previous report (King et al., 1974), we found that the reactivity of DS toward $\alpha$ subunit was low. However, by employing the cleavable MMB reagent, we have demonstrated the cross-linking of $\alpha$ dimers in both holo- and core enzyme (Figures 3 and 4). From the length of the MMB molecule (Traut et al., 1973), the maximum separation of the cross-linked lysine residues in the two $\alpha$ subunits is 15 Å. Further evidence for the existence of $\alpha$-$\alpha$ neighbors was provided by using the sulfhydryl reagent PBM which produced two cross-linked bands of mol wt ~80 000 with holoenzyme, core enzyme, $\alpha_2\beta$ subcomplex, and isolated $\alpha$ subunit (bands II and III, Figure 6). The two $\alpha$ subunits in these molecular species are expected to be within 12 Å based on the length of PBM molecule (Chang and Flacks, 1972). The direct identification of $\beta$ and $\beta'$ as $\alpha$ subunit neighbors was obtained with MMB (Figure 5). Cross-linking results with DS (Figures 1 and 2) agreed with these assignments which indicate that the $\alpha$-$\beta$ and $\alpha$-$\beta'$ separations are $\leq$11.5 Å. As shown in Figure 1, the $\sigma$ subunit is more reactive with DS than the $\alpha$ subunit and forms the $\beta\sigma$ and $\beta'\sigma$ cross-linked complexes. These two complexes were also identified from the products obtained with MMB (Figure 3). Furthermore, both MMB and PBM yielded a cross-linked $\alpha\sigma$ band, indicating that in holoenzyme at least one $\alpha$ subunit is adjacent to $\sigma$. In summary, we have identified directly seven pairs of subunit neighbors, $\alpha_2$, $\alpha\beta$, $\alpha\beta'$, $\alpha\sigma$, $\beta\sigma$, $\beta'\sigma$, and $\beta\beta'$, in holoenzyme from the cleaved cross-linked complexes generated by MMB (Table II). These assignments were further verified by the estimated molecular weights of the complexes cross-linked with both cleavable and noncleavable reagents (see Tables I and II).

It can be shown that the maximum number of subunit neighbors (or contacts) attainable in an oligomer composed of $n$ different subunits is $n(n-1)/2$. Furthermore, for an oligomer composed of $n$ single-copy subunits and $m$ different subunits each present in multiple copies, the corresponding expression is $[(m+n)^2+m-n]/2$ independent of the multiplicity. Thus, the maximum number of different subunit neighbors that can exist for RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) is seven ($n=3$, $m=1$). This is schematically represented in Figure 8A. All seven different subunit neighbors shown in this diagram have been experimentally observed. However, these seven neighbor relations are not sufficient to assign an unequivocal subunit arrangement in space. From

simple topological considerations, such an assignment for a pentameric protein requires ten different neighbor relations (for an oligomer with $n$ subunits, this number is $4n - 10$; $n \geq 4$). The additional ambiguity in subunit topography involves the disposition of the subunits present in multiple copies.

Figure 8B illustrates a model we propose for the spatial arrangement of subunits in RNA polymerase holoenzyme based on our cross-linking results. As stated above, this model is not unique. In fact, other models are also consistent with the results but differ from the one presented in Figure 8B only in the disposition of the $\alpha$ subunits. We have chosen the present model on the basis of maximum subunit contacts (nine contacts) and symmetrical disposition of the two $\alpha$ subunits relative to $\beta$ and $\beta'$ subunits. Such a structure would be thermodynamically more stable than other models; hence, it is most likely to represent the real quaternary structure of the enzyme. In this model, the backbone of holoenzyme is the $\beta\beta'$ complex. Around the contact domain between $\beta$ and $\beta'$ subunits, $\sigma$ and two $\alpha$ subunits are strung in sequence. The relative similarity of the cross-linking patterns obtained for core and holoenzyme suggests that the binding of $\sigma$ to core polymerase does not significantly alter the subunit arrangement in core enzyme. The structure of core enzyme would therefore be the structure shown in Figure 8B with the $\sigma$ subunit eliminated. The structure of the $\alpha_2\beta$ complex would in turn be that of core enzyme lacking the $\beta'$ subunit; such a structure would have a "mickey-mouse" like shape. From studies of RNA polymerase by digestion with matrix-bound proteases, Lill and Hartmann (1975) have concluded that the $\sigma$ subunit interacts with both $\beta$ and $\beta'$ subunits. Based on this and other evidence, they proposed a tentative model of the quaternary structure of RNA polymerase similar to that shown in Figure 8B.

A question may be raised as to whether the proximity relations of subunits determined by cross-linking experiments in fact reflect the native quaternary structure of the protein. We have examined a number of our cross-linked samples by sedimentation analysis in order to detect alterations in structure due to the chemical modification. A finite extent of aggregation was apparent in some samples. Nevertheless, the majority of the cross-linked proteins co-sedimented with unmodified controls and formed well-defined bands. These results indicate that, in most cases, the cross-linked proteins had undergone no gross-structural change as shown by velocity sedimentation. Electrophoresis of the cross-linked RNA polymerase which co-sedimented with the unmodified enzyme gave the same gel patterns as the unpurified material except for the absence of several bands with mol wt >320 000. This observation supports our notion that the subunit topography shown in Figure 8B, which was deduced from the cross-linked gel bands of mol wt $\leq$320 000, represents the structure of the RNA polymerase protomer rather than its aggregate. To further test the dependence of the observed cross-linked bands on the native structure of the enzyme, we have attempted to cross-link RNA polymerase and its isolated subunits in the presence of the denaturing reagent sodium dodecyl sulfate. From tracks 2 and 7 in Figure 6, it is evident that little cross-linking takes place when the proteins have been denatured. Although two bands are seen above the $\beta'$ subunit in track 2, these could be due to some nonspecific interaction of the polypeptide complexes in the presence of sodium dodecyl sulfate. Such interactions would not occur in the cross-linking reactions of native enzyme since these reactions were halted by mercaptoethanol or ethanolamine (see Experimental Procedures) before addition of sodium dodecyl sulfate. The essential point is that bands II and III seen with the native enzyme are not observed when cross-linking occurs in the presence of the denaturing reagent.

Another potential approach to the determination of proximity relationships between macromolecules is fluorescence energy transfer (Gennis and Cantor, 1972; Hillel and Wu, 1976b). By labeling the isolated subunits, pair by pair, with fluorescent donors and acceptors, the detailed spatial organization of a multisubunit enzyme can be revealed by energy transfer measurements of the reconstituted enzyme. Using this approach we have found that the two $\alpha$ subunits in the $\alpha_2\beta$ complex are within contact distance (Z. Hillel and C.-W. Wu, unpublished results). The quaternary structure of RNA polymerase shown in Figure 8B will be confirmed by further energy transfer experiments.

It has been proposed (Fukuda and Ishihama, 1974; Palm et al., 1975) that in vitro reconstitution of RNA polymerase takes place in the following sequence:

$$2\alpha \xrightarrow{\beta} \alpha_2\beta \xrightarrow{\beta'} \alpha_2\beta\beta' \text{ (premature)} \rightarrow \alpha_2\beta\beta' \text{ (active)}$$

The activation of "premature" core complex is facilitated by the presence of $\sigma$ subunit or DNA. Our observation that isolated $\alpha$ subunit exists largely as dimer suggests that the first step in the in vitro assembly of RNA polymerase is the formation of $\alpha$ dimer. There is evidence indicating this sequence of subunit assembly ($\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$) may also be operative for the in vivo formation of the enzyme (Ito et al., 1975). The results of our cross-linking studies of RNA polymerase and its subcomplexes have shown that the stepwise formation of holoenzyme could occur without gross alteration of the subunit arrangement in the following intermediates: $\alpha_2$, $\alpha_2\beta$, and core enzyme. An interesting point here concerns the subunit arrangement of the cold reconstituted or "premature" enzyme complex. Since this complex is a highly structured intermediate in the reconstitution of active enzyme, the elucidation of its structure will be useful for studying the mechanism of assembly of RNA polymerase. Our experimental results show that for both core and holoenzyme, the extent of cross-linking between $\beta$ and $\beta'$ subunits is significantly lower in the cold reconstituted enzyme than the warm reconstituted enzyme preparations. One interpretation of these results is that the inactive complex has a structure similar to native enzyme except that the separation between $\beta$ and $\beta'$ subunits is larger. If this is the case, it would be difficult to explain the observed relatively low sedimentation coefficient for the "premature" enzyme complex.[2] Another interpretation of these cross-linking results assumes that the cold reconstituted enzyme complex is a mixture of individual subunits and/or subcomplexes of the enzyme which upon warming at 31 °C transforms into an active enzyme molecule. The gel patterns of the cross-linked "premature" enzyme complex indicate that it is composed, at least in part, of the $\alpha\beta$, $\beta\sigma$, and $\beta'\sigma$ subcomplexes. There is some $\beta\beta'$ interaction in the cold reconstituted enzyme, which can be promoted by raising the temperature. Thus, our cross-linking results are in accord with the idea that assembly of RNA polymerase subunits into active enzyme molecule proceeds with reconstitution of intermediates, such as individual

[2] The value of $s_{20,w}$ for native holoenzyme is 15 while that of the "premature" holoenzyme is 10. Assuming that the partial specific volumes of these two enzyme species are the same, a 1.5-fold increase in $s_{20,w}$ indicates a 1.5-fold increase in the Stokes radius. If the Stokes radius is taken to be representative of the size of the molecule, a 1.5-fold increase in radius means a 3.4-fold increase in volume, which is physically rather unlikely.

subunits and/or subcomplexes, followed by temperature-dependent interactions between these intermediates.

By means of chemical cross-linking with bifunctional reagents, we have obtained a spatial organization for the subunits in *E. coli* RNA polymerase. The quaternary structure of the enzyme may be important for the enzyme-promoter recognition. The promoter regions of several DNAs have been sequenced and an interesting feature of these primary structures is the existence of symmetries in analogous regions of promoter sequences (Schaller et al., 1975; Maniatis et al., 1974; Sekiya and Khorana, 1974) which are implicated in the formation of tight complexes with RNA polymerase (Pribnow, 1975). The proposed quaternary structure of RNA polymerase shown in Figure 8B has no symmetry axis. Thus if symmetry plays a role in the recognition of promoters by RNA polymerase it must arise at the secondary or tertiary rather than quaternary level of structure either within a subunit or from two or more subunits of the enzyme. Chemical cross-linking studies of RNA polymerase and DNA at various stages of the transcription process may shed some light on how RNA polymerase recognizes specific promoters and how translocation of the enzymes takes place on the DNA template.

## References


Bautz, E. K. F., Bautz, F. A., and Dunn, J. J. (1969), *Nature (London) 223,* 1022-1024.

Berg, D., Barrett, K., and Chamberlin, M. J. (1972), *Methods Enzymol. 21D,* 506-519.

Berg, D., and Chamberlin, M. J. (1970), *Biochemistry 9,* 5055-5064.

Burgess, R. R. (1969), *J. Biol. Chem. 244,* 6168-6176.

Burgess, R. R., and Jendrisack, J. J. (1975), *Biochemistry 14,* 4634-4638.

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature (London) 221,* 43-46.

Chamberlin, M. J. (1974), *Annu. Rev. Biochem. 43,* 721-775.

Chamberlin, M. J., McGrath, J., and Waskell, J. (1970), *Nature (London) 228,* 227-231.

Chang, F. N., and Flacks, J. G. (1972), *J. Mol. Biol. 68,* 177-180.

Colvill, A. J. E., van Bruggen, E. F. J., and Fernandez-Moran, H. (1966), *J. Mol. Biol. 17,* 302-304.

Davies, G. E., and Stark, G. R. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 651-656.

Dunn, J. J., Bautz, F. A., and Bautz, E. K. F. (1972), *Nature (London), New Biol. 230,* 94-96.

Fujiki, H., and Zurek, G. (1975), *FEBS Lett. 55,* 242-244.

Fukuda, R., and Ishihama, A., (1974), *J. Mol. Biol. 87,* 523-540.

Gennis, R. B., and Cantor, C. R. (1972), *Biochemistry 11,* 2509-2517.

Harding, J. D., and Beychok, S. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3395-3399.

Heil, A., and Zillig, W. (1970), *FEBS Lett. 11,* 165-168.

Hillel, Z., and Wu, C.-W. (1976a), *Biophys. J. 16,* 15a.

Hillel, Z., and Wu, C.-W. (1976b), *Biochemistry 15,* 2105-2113.

Ishihama, A. (1972), *Biochemistry 11,* 1250-1258.

Ishihama, A., and Ito, K. (1972), *J. Mol. Biol. 72,* 111-123.

Ito, K., Iwakura, Y., and Ishihama, A. (1975), *J. Mol. Biol. 96,* 257-271.

King, A. M. Q., Lowe, P. A., and Nicholson, B. H. (1974), *Biochem. Soc. Trans. 2,* 76-78.

Kitano, Y., and Kameyama, T. (1969), *J. Biochem. (Tokyo) 65,* 1-16.

Laemmli, U. K. (1970), *Nature (London) 227,* 680-685.

Lill, H. R., and Hartmann, G. R. (1975), *Eur. J. Biochem. 54,* 45-53.

Lubin, M. (1969), *J. Mol. Biol. 39,* 219-233.

Maitra, U. (1971), *Biochem. Biophys. Res. Commun. 43,* 443-450.

Maniatis, T., Ptashne, M., Barell, B. G., and Donelson, J. E. (1974), *Nature (London) 250,* 394-397.

Manjula, B. N., and Richards, F. F. (1975), *Anal. Biochem. 66,* 412-422.

Martin, R. G., and Ames, B. N. (1960), *J. Biol. Chem. 236,* 1372-1379.

Palm, P., Heil, A., Boyd, D., Grampp, B., and Zillig, W. (1975), *Eur. J. Biochem. 53,* 283-291.

Pribnow, D. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 784-788.

Schaller, H., Gray, C., and Herrmann, K. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 737-741.

Sekiya, T., and Khorana, G. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2978-2982.

Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815-820.

Slayter, H. S., and Hall, C. E. (1966), *J. Mol. Biol. 21,* 113-114.

Traut, R. R., Bollen, A., Sun, T.-T., Hershey, J. W. B., Sudberg, J., and Pierce, L. R. (1973), *Biochemistry 12,* 3266-3273.

Wang, K., and Richards, F. M. (1974), *Israel J. Chem. 12,* 375-387.

Wu, C.-W., Yarbrough, L. R., Hillel, Z., and Wu, F. Y.-H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3019-3022.

Wu, F. Y.-H., and Wu, C.-W. (1973), *Biochemistry 12,* 4343-4348.

Yarbrough, L. R., and Hurwitz, J. (1974), *J. Biol. Chem. 249,* 5400-5404.

Young, R. A., and Blumenthal, T. (1975), *J. Biol. Chem. 250,* 1829-1832.

Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schwieger, M., Rahmsdorf, H.-J., Ponta, H., and Hirsch-Kauffmann, M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2506-2510.