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Subunits of RNA Polymerase in Function and Structure. 7. Structure of Premature Core Enzyme†

Akira Ishihama,* Hiroji Aiba,‡ Tsunao Saitoh, and Sho Takahashi§

ABSTRACT: The structure of premature core enzyme, an obligatory intermediate in both in vivo and in vitro assembly of *Escherichia coli* DNA-dependent RNA polymerase, was compared with that of native core enzyme. Though this assembled but inactive form of core enzyme harbors the gross conformation similar to that of native enzyme, minor and presumably local differences exist, which were identified by near-ultraviolet circular dichroism spectra, tritium–hydrogen exchange rate, protease sensitivity, intersubunit cross-linking rate by bifunctional reagents, sedimentation behavior, and elution profile from phosphocellulose. Taken together these results indicate that the core enzyme subunits are loosely associated in the premature core. The temperature-dependent maturation is required for the core subunits to be tightly associated, leading to the formation of structurally stable and functionally active RNA polymerase.

The DNA-dependent RNA polymerase [ribonucleoside 5′-triphosphate:RNA nucleotidyltransferase (EC 2.7.7.6)] is the key enzyme for the transcription of genetic informations in *Escherichia coli*. The RNA polymerase[1] holoenzyme is a protomer of molecular weight about 500 000 and has subunit composition of $\alpha_2\beta\beta'\sigma$ with the molecular weight of each subunit being 38 500, 155 000, 165 000, and 87 000, respectively (Burgess, 1969; Berg & Chamberlin, 1970). The formation of the complex structure has been studied extensively in in vitro reconstitution systems and it has been revealed that the core enzyme subunits are assembled in a stepwise fashion under the following sequence:

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

(Ishihama & Ito, 1972; Ishihama et al., 1973; Palm et al., 1975; Saitoh & Ishihama, 1976). Evidences have been obtained which indicate that the pathway of core enzyme assembly in vivo is identical with that found in vitro (Ito et al., 1975; Taketo & Ishihama, 1976, 1977). One of the remarkable features of the assembly mechanism is the formation of an assembled but inactive form of core enzyme ("premature core"), which indicates that proper arrangement of the subunits is necessary for the RNA polymerase activity to be exposed. In the in vitro reconstitution system, the activation of premature core ("enzyme maturation"), the rate-limiting step in the assembly sequence, can be achieved in the three different ways, i.e., self-reactivation, σ subunit-promoted reactivation, and DNA-promoted reactivation, which require respectively strict but different conditions (Saitoh & Ishihama, 1976). It remains, however, to be determined which of the three pathways operates in vivo. Systematic comparison of the structure and function of premature, reactivated, and native core enzymes is one of the ways to answer this question.

The present report deals with one of our efforts on the subject and describes the conformational differences between premature and native core enzymes.

Materials and Methods

Chemicals. Unlabeled ribonucleoside 5′-triphosphates were purchased from P-L Biochemicals and Boehringer Mannheim GmbH (West Germany), and labeled nucleotides were from Schwarz/Mann. Recrystallized products of urea, sodium dodecyl sulfate ($NaDodSO_4$), acrylamide, and *N,N′*-methylenebis(acrylamide) were obtained from Wako Chemical, Japan. Diisopropyl phosphofluoridate (Dip-F) and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) were obtained from Sigma Chemicals. Beef liver catalase (EC 1.11.1.6) and trypsin (EC 3.4.21.4) were obtained from Sigma Chemicals, while β-galactosidase is a product of Boehringer Mannheim GmbH, West Germany. T7 DNA was purified by phenol extraction from phage stocks isolated by CsCl centrifugation.

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‡ Radioisotopes Laboratory, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.
§ Department of Enzyme Chemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan.

[1] Abbreviations used: RNA polymerase, ribonucleoside 5′-triphosphate:RNA nucleotidyltransferase (DNA-dependent) (EC 2.7.7.6); $NaDodSO_4$, sodium dodecyl sulfate; Dip-F, diisopropyl phosphofluoridate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; CD, circular dichroism; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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Phosphocellulose P11 was a product of Whatman; Sephadex G-25, Sepharose 6B, and DEAE-Sephadex A-50 were products of Pharmacia, Sweden. DNA-cellulose was prepared as described previously (Fukuda et al., 1974).

Bifunctional reagents, dimethylsuberimidate and dimethyladipimidate, were synthesized essentially according to Davies & Stark (1970).

RNA Polymerase. RNA polymerase holoenzyme I was purified from *Escherichia coli* K12 W3350 and assayed essentially as described in the previous report (Fukuda et al., 1974). Frozen cells were disrupted in a RiBi cell fractionator and crude extract was prepared after centrifugation at 30000 rpm for 120 min. RNA polymerase precipitated with protamine sulfate was eluted by buffer A [10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetate (EDTA), and 0.1 mM dithiothreitol (DTT)] containing 0.2 M ammonium sulfate. Ammonium sulfate precipitate at 60% saturation was subjected to successive chromatography on low-salt Sepharose 6B, DEAE-Sephadex A-50, and high-salt Sepharose 6B columns. Holoenzyme I was isolated by DNA-cellulose column or by phosphocellulose column chromatography in the presence of 50% glycerol, while core enzyme used in this report was obtained by repeated chromatography of the high-salt Sepharose fraction on phosphocellulose columns in the presence of 5% glycerol. Residual σ subunit in the core enzyme preparations used was less than 0.1%. The σ subunit dissociated by the phosphocellulose column chromatography was used without further purification.

Preparation and Reactivation of Premature Core Enzyme. Premature core enzyme was prepared essentially as described in the preceding paper (Saitoh & Ishihama, 1976). Purified core enzyme (3 mg/mL) was dissociated by dialysis against dissociation buffer (Ishihama & Ito, 1972), which contained 10 mM Tris-HCl (pH 7.6 at 4 °C), 0.1 mM EDTA, 10 mM DTT, 0.1 M KCl, 10% glycerol, and deionized 6 M urea, for 10 h in an ice bath. The dissociated enzyme was then dialyzed for 12 h in an ice bath against reconstitution buffer (Ishihama & Ito, 1972), which was modified to achieve the best recovery of enzyme activity in the absence of promoting factors for enzyme maturation (Saitoh & Ishihama, 1976). The dialysate was used as premature core enzyme preparations. In some experiments premature core was further purified by centrifugation of the dialysate through glycerol gradients. Premature core enzyme thus prepared was reactivated by incubation at 30 °C for 60 min. The subunit concentration of premature, reactivated, and native core enzymes was determined by weighing each subunit peak of the tracings after $NaDodSO_4$–polyacrylamide gel electrophoresis.

Glycerol gradient centrifugation of premature, reactivated, and native core enzymes was performed on 5 mL of 15–30% linear gradient of glycerol containing 10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT in a Spinco SW50.1 rotor at 40000 rpm for 11 h at 4 °C.

Circular Dichroism Measurement. Premature and native core enzymes were prepared as described above and dialyzed against the modified reconstitution buffer before use. Circular dichroism (CD) spectra were determined with a Jasco J-20 spectropolarimeter. Measurements were performed at 3–4 °C in a 0.1-mm cylindrical jacketed cell for far-ultraviolet spectra or in 10-mm cell for near-ultraviolet spectra. To avoid an ambiguity caused by change in protein concentration and to make accurate comparison of molecular ellipticities, the CD spectrum of premature core was first recorded at 3–4 °C. The cell was immediately heated at 30 °C for 60 min to convert the premature core into reactivated form and its CD spectrum was measured at 3–4 °C.

Hydrogen–Tritium Exchange. Labeling of RNA polymerase proteins with tritium was accomplished by incubation of the proteins in 0.65 mL of buffer HX [10 mM Tris-HCl (pH 7.6 at 4 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.3 M KCl, and 20% glycerol] containing 5 mCi of ^3H_2O. After more than 10 days of incubation, during which virtually all the slowly exchangeable sites were equilibrated with tritium, labeled proteins were separated from tritiated water by passing the solution through a Sephadex G-25 column (1 × 10 cm) equilibrated with buffer HX [10 mM Tris-HCl (pH 7.6 at 4 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.3 M KCl, and 20% glycerol]. For measurement of slowly exchangeable sites, the isolated ^3H-labeled proteins were passed through a Sephadex column at various times after isolation and the amounts of radioactivity remained bound were determined. For measurement of rapidly exchangeable sites, aliquots of the reaction mixture for exchange-in were passed through the column in the presence of pressure addition. Filtration time was about 10 min in the absence of pressure addition.

The protein concentration of each eluate was estimated by optical density using a Zeiss spectrophotometer. Based on the protein content measured by the method of Schaffner & Weissmann (1973), the extinction coefficients used for each sample of RNA polymerase core enzymes were determined as follows: premature core enzyme, $\epsilon_{280nm} = 2.863 \times 10^5$; native core, $\epsilon_{280nm} = 2.636 \times 10^5$.

For scintillation counting, 0.5 mL of each eluant was counted with a Nuclear Chicago liquid scintillation counter, Mark II.

Protease Digestion. To RNA polymerase solution in 10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM DTT, 0.2 M KCl, and 20% glycerol, various amounts of trypsin dissolved in the same buffer were added and the mixture was incubated at 0 °C. After incubation, the mixtures were made 0.1 mM in Dip-F and 0.1 mM in $PhCH_2SO_2F$ and subjected to electrophoresis on polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$.

Cross-Linking with Bifunctional Reagents. The synthesis of bifunctional reagents was essentially as described by Davies & Stark (1970). The reaction buffer used for cross-linking was similar to that of Hillel & Wu (1977). Bifunctional reagents were dissolved in buffer Cl [0.05 M Bicine–NaOH (pH 8.5), 0.1 mM DTT, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.3 M KCl, and 20% glycerol] immediately before use and diluted to various concentrations, into which 0.1 volume of RNA polymerase core enzymes in buffer HX was added. After standing in an ice bath for 60 min, the reaction was quenched by adding ethanolamine hydrochloride (pH 8.0) at the final concentration of 0.14 M. The solution was made 1% in $NaDodSO_4$ and 0.1 M in DTT and subjected to electrophoresis in the presence of 0.1% $NaDodSO_4$. The molecular weight of cross-linked protein complexes was estimated based on the electrophoretic mobility of un-cross-linked RNA polymerase subunits as markers.

Polyacrylamide Gel Electrophoresis. Proteins were electrophoresed in phosphate buffer containing $NaDodSO_4$ as described by Shapiro et al. (1967). In some experiments, the electrophoresis system of Laemmli (1970) was also employed. Gels were stained with Coomassie brilliant blue and scanned with a Joyce-Loebl microdensitometer MKIII.

Results

Subunit Composition. Chromatography of *E. coli* RNA

polymerase on the phosphocellulose column in the presence of elevated glycerol concentrations leads to highly purified preparations of holoenzyme and core enzyme (Gonzalez et al., 1977), while in the absence of glycerol holoenzyme is dissociated into core enzyme and σ subunit (Burgess, 1969; Burgess et al., 1969). The protomeric forms of holoenzyme and core enzyme thus purified have the subunit composition $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$, respectively. In addition, a low molecular weight polypeptide called ω (Burgess, 1969) was found in all the enzyme preparations used in the present study. Though virtually all the σ subunit can be displaced from enzyme by phosphocellulose column chromatography in the absence of glycerol, the polypeptide ω is stably associated with core enzyme lacking σ subunit (Ishihama and Ito, unpublished results) raising the possibility that the polypeptide ω may play a functional role in some aspect of RNA polymerase action. When premature core enzyme was constructed from dissociated core enzyme, it contained polypeptide ω associated with $\alpha_2\beta\beta'$ complex. The subunit composition of premature core enzyme was indistinguishable from native core enzyme used. Polypeptide ω was recovered as bound form when premature core enzyme was isolated by centrifugation through glycerol gradients.

Absorbance Spectrum. When an absorbance spectrum was measured for purified native and premature core enzymes in buffer HX, no significant difference was found in the shape of spectra between the two enzymes but the degree of absorbance after calibration of protein concentration was much higher for premature core enzyme than native core enzyme, e.g., 8.6% higher at 280 nm. The molecular extinction at 280 nm was 2.863×10^5 and 2.636×10^5 for premature and native core enzymes, respectively. The observation indicated that the conformation of premature core enzyme was different from that of native core enzyme, though the subunit constitution was identical for the two polymerases.

Circular Dichroism Spectrum. In order to analyze the structural difference(s) between premature and native core enzymes, circular dichroism (CD) spectra were examined for two core enzymes and one of the typical results is shown in Figure 1, which in addition includes the CD spectrum of mature core enzyme reactivated in the absence of maturation-enhancing factors. Premature, reconstituted, and native core enzymes exhibited similar far-ultraviolet CD spectra with minimum at 208–209 nm and maximum at 193 nm. Less prominent minimum was also observed at near 220–222 nm for all three core enzymes. The molecular ellipticities at 222 nm of premature and reconstituted core enzymes were 83.6% and 85.6% of that of native core enzyme and the ellipticities at 209 nm were 79.8 and 88.4%, respectively. Since the denatured core enzyme in 6 M urea buffer exhibited a completely different spectrum, essentially similar to that of guanidine-treated RNA polymerase (Harding & Beychok, 1974), these results indicate that most of the core enzyme structure is established in premature core and the minor change in structure accompanied with the activation of premature core (enzyme maturation) occurs on local regions and/or subunit arrangement.

On the contrary, the near-ultraviolet CD spectra, which are indicative of the optical activity of aromatic chromophores, indicated that the magnitude of the Cotton effect was 1.5-fold higher for native core enzyme than premature core enzyme. In addition, the ratio of $[\theta]_{269nm}/[\theta]_{278nm}$ was lower for native (0.85) than premature core (0.95). Taken together the apparent difference in the shape of CD spectra at near-ultraviolet region implies that the enzyme maturation is accompanied by

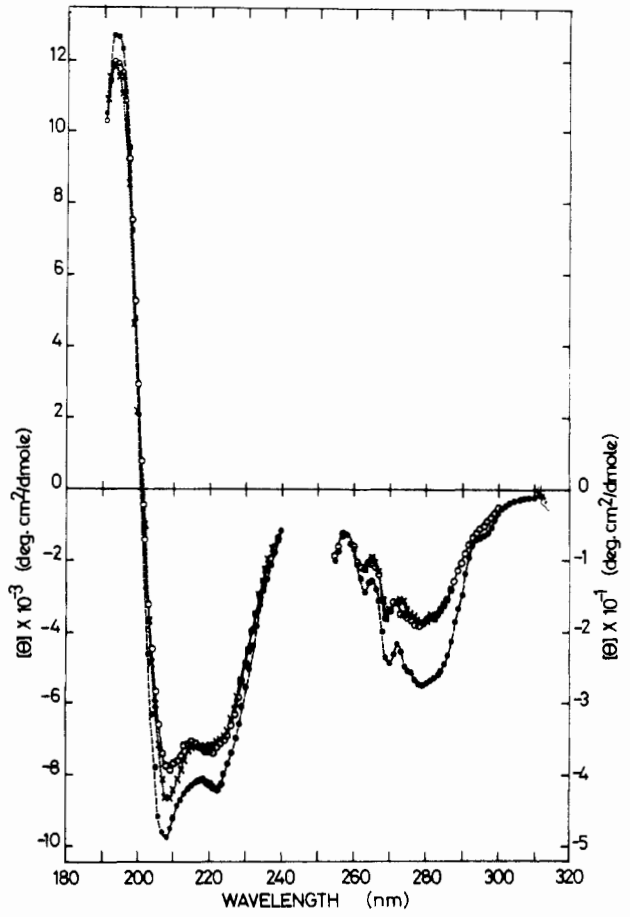

FIGURE 1: Circular dichroism spectra of RNA polymerase core enzymes. CD spectra of premature, reconstituted, and native core enzymes were measured as described in Materials and Methods. (O—O) Premature core enzyme; (X---X) reconstituted core enzyme; (●---●) native core enzyme. The intensity of spectra above 250 nm is enlarged 200-fold.

structural alteration at least in the environment of aromatic amino acid residues in core enzyme subunits. The near-ultraviolet CD spectrum for self-reactivated core enzyme was considerably different from that for native core but rather close to that for premature core. The present observation supports the notion that the core enzyme identical with the native enzyme in the structure and function is reconstituted only in the presence of σ subunit (Saitoh & Ishihama, 1976, 1979).

Hydrogen–Tritium Exchange Rate. The gross structural difference between premature and native core enzymes was also examined by measuring the rate of hydrogen–tritium exchange, which is known to be influenced by states of exchangeable hydrogens in macromolecules. For the purpose, exchangeable hydrogens in RNA polymerase proteins were fully labeled with tritium and the exchange-out rates of bound tritium were compared. When native core enzyme was exposed to preequilibration in the presence of tritium water, the amount of tritium remaining bound after passing through a Sephadex column reached almost a plateau after 7-days incubation under the conditions employed and at 4 °C (Figure 2). Since the tritium in the rapidly exchangeable hydrogen sites, which are known to be on the side chains or surface, was exchanged out within the time (approximately 10 min) required for separation of tritiated protein from tritium water by Sephadex columns, the level of labeling thus estimated represents stably bound tritium on slowly exchangeable hydrogen sites in buried portions of highly structured core enzyme. Based on the amino acid composition of core enzyme subunits, the level was es-

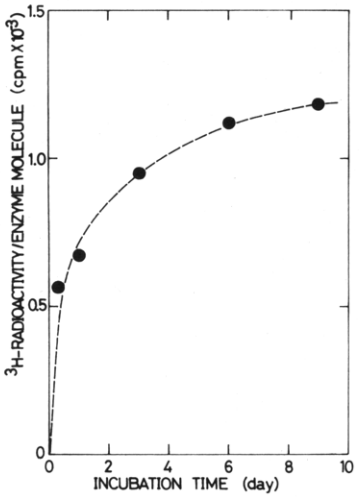

FIGURE 2: Time course of ^{3}H exchange-in for native core enzyme. Purified RNA polymerase core enzyme was dialyzed against buffer HX. To 1.81 mL of the dialyzed enzyme containing 1.92 mg of protein, 0.01 mL of 100 mCi/mL ^3H_2O was added and the mixture was stored at 4 °C in a sealed tube. At the times indicated, 0.4-mL aliquots were passed through Sephadex G-25 columns (1 × 10 cm). Fractions of 0.585 mL were collected and determined for ^{3}H radioactivity and A_{280nm}. The ratio of radioactivity (cpm per 0.5 mL of filtrate) to A_{280nm} of the enzyme peak is plotted.

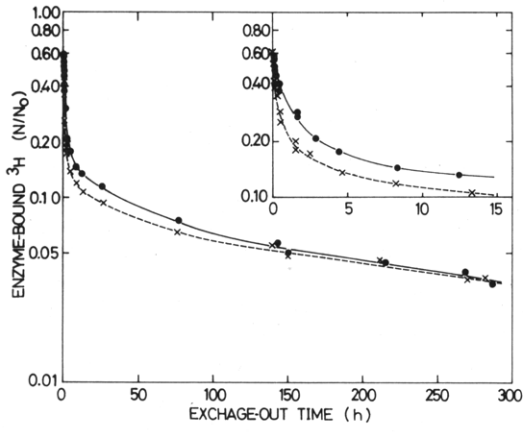

FIGURE 3: Time course of ^{3}H exchange-out for native and premature core enzymes. Solutions of native (10.45 mg/0.65 mL) or premature core enzyme (10.45 mg/0.61 mL) in buffer HX containing 5 mCi of ^3H_2O were stored at 4 °C for 11 days. ^{3}H-Labeled core enzymes were isolated by passing through Sephadex G-25 columns (1 × 10 cm). Enzyme protein was eluted within 30 min for both core enzymes. At the times indicated, aliquots of the enzyme peak were passed through Sephadex columns for measurement of radioactivity remaining bound to enzyme. The time of the start for isolation of labeled proteins was taken as the time zero for this experiment. The amount of enzyme-bound ^{3}H is represented as the ratio of the number of exchanged (N) to that of exchangeable hydrogen sites (N_0), which was calculated based on the amino acid composition of core enzyme (Fujiki & Zurek, 1975). The exchange-out curves for the first 15 h are enlarged (see insert).

timated to be about 20% of the total exchangeable hydrogen in core enzyme.

Kinetics of the exchange-out of stably bound tritium were then compared for premature and native core enzymes. As shown in Figure 3, the rate of exchange-out is more than 10% higher at 1 h after the start of exchange-out for premature core than for native core enzyme. The result suggests that native core is more highly structured than premature core enzyme. However, the amount of most tightly bound tritium, which was not exchanged out even after 5 h, was virtually the same between the two enzyme proteins (approximately 3–4% of total exchangeable tritium) within the range of experimental

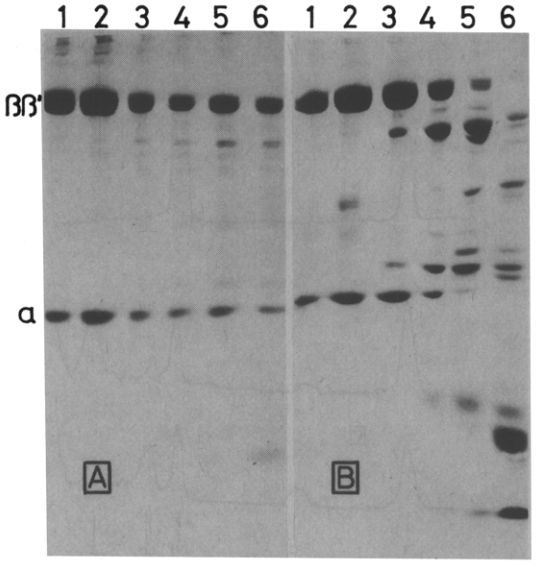

FIGURE 4: Trypsin digestion of native and premature core enzymes. Native (16.4 μg/10 μL) and premature core enzymes (15.6 μg/10 μL) in buffer HX were incubated for 30 min at 30 °C in the presence of following amounts of trypsin: (1) 0; (2) 1 ng; (3) 10 ng; (4) 100 ng; (5) 1 μg; (6) 10 μg. After addition of Dif-P and $PhCH_2SO_2F$, the mixtures were heated for 5 min at 70 °C in the presence of 0.5% $NaDodSO_4$ and 10 mM DDT and subjected to electrophoresis on polyacrylamide slab gel according to Laemmli (1970). (A) Native core enzyme; (B) premature core enzyme.

errors. Since tritium-labeled premature core enzyme was reactivated nearly to the same extent as that prior to preincubation, incubation of RNA polymerase for 10 days at 4 °C in HX buffer seems not to alter the structure and activity of RNA polymerase. The observation supported the notion that the backbone structure of RNA polymerase is established until premature core is assembled, and temperature-dependent maturation of premature core enzyme is accompanied by minor alterations in the structure. In good agreement with these observations, the rate and extent of tritium exchange-out for isolated individual subunits are higher and more than those for both premature and native core enzymes (Aiba & Ishihama, unpublished observation).

Protease Sensitivity. Since isolated RNA polymerase subunits are more sensitive to proteases than native holo- and core enzymes (Ishihama, unpublished observation), the structure of the various forms of RNA polymerase core enzyme may be compared by testing the sensitivity to protease digestion. Figure 4 shows one of the experiments, in which native and premature core enzymes were exposed to various concentrations of trypsin and analyzed for the cleavage products by polyacrylamide gel electrophoresis under dissociating conditions. Several fragments of different intensity were found for premature core, which exhibited apparent molecular weights of 125 000 and 47 000 for the two major fragments, identifiable even with low concentrations of trypsin, and of 135 000, 115 000, 75 000, and 52 000 for the minor fragments. The size of these fragments indicated that they were generated from the two large subunits, β and β', of core enzyme. In addition, at least three polypeptides smaller than 20 000 daltons were found in the presence of high concentrations of trypsin. The origin of each polypeptide mentioned above was identified by testing cross-reaction to antisera against each subunit and will be described elsewhere (Ishihama & Ito, submitted for publication).

In contrast, native core enzyme was hardly digested by trypsin up to the concentrations tested, indicating that premature core is less structured than native core enzyme. Higher

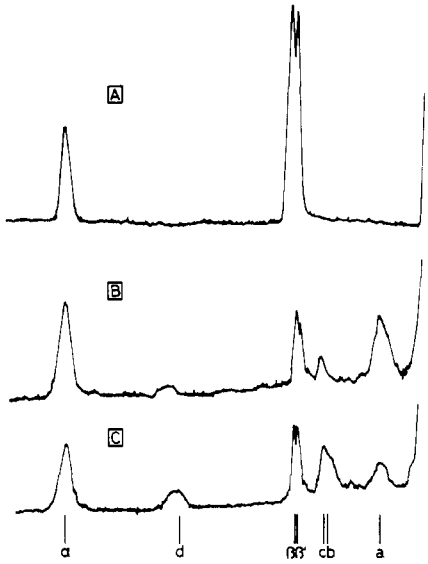

FIGURE 5: Cross-linking of native and premature core enzymes. Native (35 μg) and premature core (33 μg) enzymes in 0.02 mL of Bicine buffer were treated with 0.1 M dimethylsuberimidate for 15 min at 37 °C. After termination of the reaction by adding monoethanolamine, the mixtures were subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. Stained gels were scanned with a Joyce-Loebl microdensitometer using wedge F. Electrophoresis is from right to left. The positions of enzyme subunits are indicated by α, β, and β′, while cross-linked products are shown by a, b, c, and d, each having molecular weight of 320 000, 210 000, 190 000, and 80 000, respectively. (A) Untreated native core enzyme; (B) cross-linked native core enzyme; (C) cross-linked premature core enzyme.

sensitivity of premature core was also found when the kinetics of degradation was followed in the presence of a constant amount of trypsin, and moreover α-chymotrypsin was used in place of trypsin (data not shown).

Cross-Linking with Bifunctional Reagents. The quaternary structures of native and premature core enzymes have been investigated by chemical cross-linking with bifunctional reagents, dimethylsuberimidate and dimethyladipimidate. The time course of the appearance of cross-linked products analyzed by electrophoresis under dissociating conditions indicated that three cross-linked bands were formed for native core enzyme, each having the molecular weight of 320 000 (a), 210 000 (b), and 190 000 (c) estimated based on the mobility of un-cross-linked subunits as markers (Figure 5). These might represent ββ′, αβ′, and αβ complexes, respectively, as identified by Hillel & Wu (1977). The most extensive cross-linking had involved the β and β′ subunits for native core enzyme. Prolonged exposure to bifunctional reagents resulted in the formation of a small amount of α dimer with the molecular weight of 80 000 [band d (Figure 5)].

The cross-linked products from premature core enzyme were essentially the same for those from native core enzyme. However, the rate and extent of cross-linking appear different: the formation of ββ′ complex was slower and less for premature core, while that of αβ and αβ′ complex was faster and more for premature core enzyme than native enzyme (Figure 5). Difference in the relative yields of the cross-linked subunit complexes was observed when the mixtures of RNA polymerase core enzymes and various concentrations of bifunctional reagents were incubated for the same time (data not shown). These observations imply that the distance between β and β′ subunits is larger in premature core than in native core enzyme.

Sedimentation Behavior. The sedimentation coefficients of enzymes reactivated by the three different ways were essentially the same (12.6–13.4 S) with those of native holo- and core enzymes if the centrifugation was performed in the presence of 0.2 M KCl (Saitoh & Ishihama, 1976). In the absence of salt, however, both native and reconstituted core enzymes sedimented faster forming a peak around 20 S, which indicates that the reconstituted core enzyme as well as native core enzyme is capable of associating to form a dimer or multimers. No significant differences were also found in the sedimentation behavior among native holoenzyme and native or reconstituted core enzymes supplemented with σ subunit prior to centrifugation.

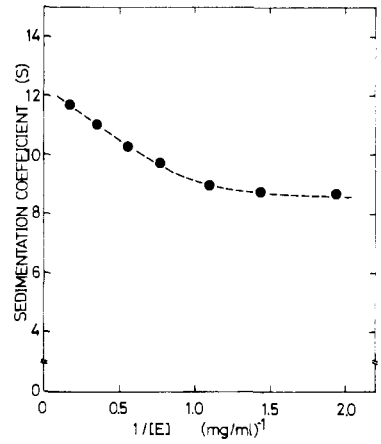

FIGURE 6: Sedimentation coefficient of native and premature core enzymes. Both native and premature core enzymes were dialyzed against 10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.2 M KCl. Aliquots of 0.2 mL containing indicated concentrations of enzymes were layered on top of a 5-mL linear gradient of glycerol (15–35%) in the same buffer and centrifuged for 15 h at 2 °C and 50 000 rpm in a Spinco SW50.1 rotor. The peak position was determined by the enzyme activity. For the assay of premature core enzyme, glycerol fractions were preincubated for 60 min at 30 °C in the presence of σ subunit prior to RNA synthesis. The apparent sedimentation coefficients of premature core enzyme were estimated after correction for protein concentration based on those of native core enzyme.

In contrast, as shown in Figure 6, the sedimentation velocity of premature core enzyme was remarkably influenced by the protein concentration at the time of centrifugation. The sedimentation coefficient at the lowest protein concentration examined was as low as that of $\alpha_2\beta$ complex and that at the highest concentration was as high as that of native and reconstituted core enzyme. Under the conditions employed, however, neither $\alpha_2\beta$ complex nor free β′ subunit peak was identified through glycerol gradients of premature core enzyme. Moreover, when the premature core enzyme peak of the lowest protein concentration was treated with antibodies against individual subunits, at least half of all the core subunits were coprecipitated, indicating that major portions of the core subunits in slowly sedimenting fractions are assembled. Taken together these observations might be best explained by saying that premature core enzyme is in rapid equilibrium with a mixture of $\alpha_2\beta$ complex and free β′ subunit, forming a single sedimentation peak in glycerol gradients, whereas matured core enzyme is apparently no more dissociated into subassemblies under the same conditions.

Phosphocellulose Column Chromatography. By passing through the phosphocellulose column, RNA polymerase holoenzyme is dissociated into core enzyme and σ subunit (Burgess et al., 1969); in addition, some portions of core enzyme are dissociated into $\alpha_2\beta$ complex and free β′ subunit (Fukuda & Ishihama, 1974), in particular in buffers lacking glycerol which is widely used as an enzyme stabilizer. As an attempt to distinguish the two core enzymes, native and

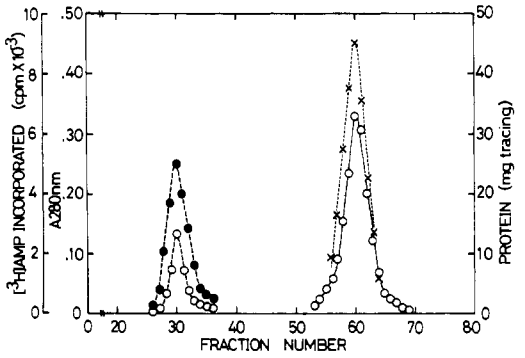

FIGURE 7: Phosphocellulose column chromatography of native and premature core enzymes. Native (5.5 mg/mL) and premature core (6.8 mg/mL) enzymes were dialyzed against 50 mM Tris-HCl (pH 7.6 at 4 °C), 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol and separately subjected to chromatography on phosphocellulose columns (0.5 × 10 cm). Elution was performed by a linear gradient of KCl (0–1.0 M) in 150 mL of the same buffer and fractions of 1.15 mL were collected. RNA polymerase activity was determined with 0.01-mL aliquots using poly[d(A-T)]·poly[d(A-T)] as template, and subunit distribution was determined by electrophoresis of 0.02-mL aliquots on polyacrylamide gels under dissociating conditions. Two separate patterns are shown in the same figure. (O—O) A_{260nm} for native core enzyme; (X---X) RNA polymerase activity for native core enzyme; (●---●) β subunit for premature core enzyme; (O---O) α subunit for premature core enzyme. β' subunit of premature core enzyme was tightly bound to phosphocellulose and eluted after native enzyme peak forming a broad peak.

premature core, in their stability of assembled state, both enzymes were passed through the phosphocellulose column. As shown in Figure 7, most of premature core enzyme was dissociated into $\alpha_2\beta$ complex and β' subunit, while the majority of native core enzyme was recovered as assembled core enzyme form. Since β' subunit alone is a basic protein and tightly bound to DNA (Fukuda & Ishihama, 1974), it is reasonable that polyanions such as phosphocellulose promote the dissociation of premature core by removal of released β' into polyanion-bound form. In accordance with this notion, $\alpha_2\beta$ complex was recovered in supernatant when mixtures of premature core and DNA were centrifuged, though in the absence of DNA all core enzyme subunits were recovered in supernatant as a complex. This polyanion-induced dissociation of premature core enzyme was not observed concomitantly upon reactivation of premature core enzyme by exposure to high temperature. By passing premature core enzyme preparations through the DEAE-Sephadex A-50 column, however, most of the core subunits were recovered in a single peak.

Discussion

The mechanism of multisubunit enzyme formation has been studied extensively with the RNA polymerase from *Escherichia coli* and it has been established that the core enzyme subunits are assembled both in vitro and in vivo stepwisely under the following sequence: $2\alpha \rightarrow \alpha_2 \xrightarrow{\beta} \alpha_2\beta \xrightarrow{\beta'} \alpha_2\beta\beta'$ (premature core) → E (active core) (Ishihama & Ito, 1972; Fukuda & Ishihama, 1974; Palm et al., 1975; Ito et al., 1975; Saitoh & Ishihama, 1976; Taketo & Ishihama, 1976, 1977). One of the unique features of the assembly mechanism is the formation of most of the secondary, tertiary, and quaternary structures before enzyme activity is expressed, i.e., the formation of premature core enzyme. The presence of highly structured but inactive core enzyme has been confirmed by several other investigators who performed the reconstitution utilizing different procedures (Harding & Beychok, 1974; Yarbrough & Hurwitz, 1974; Palm et al., 1975), and the premature core is believed to be an obligatory intermediate in the pathway of RNA polymerase formation.

The rate-limiting step in in vitro reconstitution is the activation (maturation) of this premature core enzyme. Although premature core enzyme is formed under varieties of conditions and even at low temperature, strict conditions are required for premature core to be activated such that the reaction is temperature dependent and is enhanced by either DNA or σ subunit, the fourth subunit of RNA polymerase or the initiation factor for transcription (Burgess et al., 1969), especially at salt concentrations as low as that in vivo (Ishihama et al., 1973; Fukuda & Ishihama, 1974; Saitoh & Ishihama, 1976). The in vivo process of enzyme maturation, however, remains to be determined.

The present study demonstrated that, although the gross structure is similar for premature and native core enzymes, minor but considerable differences exist, which could be observed by circular dichroism spectra, hydrogen–tritium exchange rates, sedimentation behaviors, protease sensitivities, reactions to bifunctional cross-linking reagents, and elution profiles from phosphocellulose columns. The shape of the CD spectrum for premature core was essentially the same with that of native core enzyme at the range of far-ultraviolet, as already described by Harding & Beychok (1974). The residue ellipticities at 222 nm ($[\theta]_{222nm}$), indicative of the α helix content, of premature and reactivated core enzymes were 83–86% of that of native core enzyme. On the basis of the values at 222 nm and according to the method of Greenfield & Fasman (1969), the amount of α helix was estimated to be about 20% for both premature and reactivated core and about 23% for native core enzyme. The amounts of α helix, β sheet, and other structures, determined from the CD spectra between 190 and 239 nm according to the method of Chen et al. (1974), were, however, virtually equal among the three enzymes within experimental errors and the helix contents were more than those estimated from the ellipticities only at 222 nm: 44.4–46.0% for α helix; 18.3–21.2% for β sheet; and 4.5–5.3 for peptide units per segment. Thus, premature core enzyme has the secondary structure identical with or very similar to that of native core enzyme. However, intensity of the near-ultraviolet CD spectra was considerably smaller (approximately two-thirds) for premature core enzyme. The difference in the near-ultraviolet CD spectra is best explained such that microenvironments of the aromatic chromophores supposedly on the exposed region of premature core enzyme are not identical with those of native core enzyme. Since the intensity did not increase to the native enzyme level if premature core was reactivated in the absence of maturation factors, e.g., DNA or σ subunit, the notion was further supported that the maturation of RNA polymerase proceeds either on DNA or σ subunit (Saitoh & Ishihama, 1976, 1979).

Proper analysis of tritium–hydrogen exchange rates is capable of delivering information on the structural change during the enzyme maturation. The results indeed demonstrated some changes in the enzyme structure coupled with the maturation, which produced labeled tritium shielded from contact with solvent or prevented water molecules from penetrating into some of the labeled sites buried due to the enzyme maturation. Since the rates for rapidly and slowly exchangeable sites are essentially equal for the two core enzymes but the difference lies in the exchange rate of intermediate classes, the structural difference was again indicated to be limited on local regions on premature core.

The structural difference was also demonstrated by testing protease sensitivity. Though the cleavage products obtained

by trypsin digestion of premature core enzyme were essentially the same as those from native core enzyme, for example, those described in the literatures (Hermier et al., 1971; Lill & Hartmann, 1975; Ishihama and Ito, unpublished results), the time course of their appearance was significantly different for the two core enzymes such that premature core was digested faster than native enzyme. It has already been demonstrated that assembly intermediates in vivo including premature core, accumulated in the temperature-sensitive assembly-defective *E. coli* mutants, are rapidly and preferentially degraded (Taketo et al., 1976), though assembled RNA polymerase is one of the metabolically stable constituents in exponentially growing cells without detectable degradation (Iwakura et al., 1974; Kawakami et al., 1979).

A number of evidences have accumulated which indicate that all core subunits are assembled together in premature core complex, including coprecipitation of all three subunits, α, β, and β', by specific antisera against each subunit, cross-linking among all possible subunit combinations and simultaneous elution of all subunits from the DEAE-Sephadex column. Analysis of the sedimentation behavior, however, suggested that premature core enzyme was in rapid equilibrium with the mixture of $\alpha_2\beta$ and β', in general agreement with the sedimentation behavior of complexes in instantaneous equilibration (Kegeles & Cann, 1978). Supporting the interpretation is the finding that premature core enzyme is dissociated by passing through the phosphocellulose column into respective constituents, namely, $\alpha_2\beta$ complex and dissociated β' subunit.

Previous estimations of the amount of RNA polymerase in *E. coli* growing at various rates (for example, see Ishihama et al., 1976) indicated that, though it is maintained at certain levels characteristic of the rate of cell growth, there is a surplus of nonfunctioning polymerase, which may be grouped into two classes: active (functional) free RNA polymerase and inactive polymerase (Bremer & Dalbow, 1975; Nielrich, 1978). It is indeed known that a considerable amount of RNA polymerase with virtually no enzyme activity exists in minicells free from chromosomes (Rogerson, 1975). Moreover, we have obtained evidences indicating that partition control of RNA polymerase operates in *E. coli*, by which the amount of functioning RNA polymerase on nucleoids is regulated depending upon the phase of cell growth (Kawakami et al., 1979). A considerable population of RNA polymerase is degraded concomitantly with the increase of unused fraction in the stationary-phase cells (Kawakami et al., 1979). Comparison in the structure between premature core and unused inactive polymerase remains to be made.

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