Conformational Transition of *Escherichia coli* RNA Polymerase Induced by the Interaction of σ Subunit with Core Enzyme†

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ABSTRACT: The isolated σ subunit of *Escherichia coli* RNA polymerase has been labeled covalently with a fluorescent probe, *N*-(1-pyrene)maleimide. The labeled σ subunit (PM-σ) still retained its biological activity in stimulating transcription of T7 DNA by core enzyme. When a stoichiometric amount of core enzyme was added to a solution of PM-σ, there was a decrease in fluorescence intensity without shifts in emission maxima of PM-σ. The kinetics of the interaction between the σ subunit and core enzyme was investigated with the stopped-flow technique by monitoring the fluorescence quenching. A biphasic change of fluorescence intensity with respect to time was observed when PM-σ was rapidly mixed with an excess of core enzyme. The kinetic data can be analyzed in terms of a mechanism in which a fast bimolecular binding of σ to core enzyme is followed by a relatively slow isomerization of the holoenzyme formed. From the best-fit kinetic parameters, an overall binding constant of $\leq 3 \times 10^{-10}$ M was estimated for the PM-σ-core complex, in agreement with that obtained by the fluorimetric titration. In addition, we have studied the effect of temperature on the rate constant associated with the conformational change of the holoenzyme, which shows a temperature transition around 20 °C. The nonlinear Arrhenius plot obtained implies that the conformational transition is complex and may be composed of several processes. The activation energy for the "overall" conformational change was estimated to be 6.7 kcal/mol. The kinetic evidence for the conformational transition of holoenzyme induced by the interactions of σ subunit with core enzyme presented here further supports the proposition that the σ subunit acts on core enzyme to trap a unique conformation of RNA polymerase which recognizes the proper promoters and initiates the synthesis of specific RNA chains.

The DNA-dependent RNA polymerase of *Escherichia coli* is a multisubunit enzyme, with a subunit composition of two α (mol wt 40 000), one β (mol wt 155 000), one β' (mol wt 165 000), and one σ (mol wt 95 000) (Burgess, 1969; Zillig et al., 1970). The σ subunit can easily be isolated by passing the holoenzyme through a phosphocellulose column, which also yields the core enzyme ($\alpha_2\beta\beta'$) (Burgess et al., 1969). Core polymerase retains catalytic activity, but the resulting RNA product is transcribed from both DNA strands and apparently the initiation is nonspecific. Addition of σ, which by itself has no synthetic function, leads to the promotion of specific initiations that yield asymmetric transcripts resembling the in vivo RNA products (Burgess et al., 1969; Bautz et al., 1969). Furthermore, σ also stimulates the overall RNA synthesis by increasing the rate of initiation through "the σ cycle": after RNA synthesis has begun, σ is released and can be reused by added core enzyme (Travers and Burgess, 1969; Wu et al., 1975).

The specific initiation exerted by σ could be due to the following two possibilities. First, σ by itself recognizes the specific promotor on the template and serves as a signal for core polymerase. This is less likely due to the observation that the isolated σ does not bind DNA (Zillig et al., 1970). The second possibility is that σ acts on core enzyme to induce a specific conformation of the holoenzyme, which then favors the proper sites on the template over the wrong ones. Indeed σ has been shown to facilitate the dissociation of core polymerase from nonspecific binding sites on DNA (Hinkle and Chamberlin, 1972). Evidence from our laboratory by chemical modification of RNA polymerase (Yarbrough and Wu, 1974a) also suggests that the second possibility may, in fact, be the case, namely, the σ-core interaction plays an important role in template recognition and specific initiation in RNA synthesis. Nevertheless, a prerequisite of this hypothesis is that there must be a conformational transition of RNA polymerase induced by the σ-core interaction. Thus far, such a conformational transition has not been demonstrated.

By covalently labeling the isolated σ subunit with a fluorescent probe, *N*-(1-pyrene)maleimide, we have developed a spectroscopic method to study the σ-core interaction (Yarbrough and Wu, 1974b). Taking advantage of this spectroscopic signal, we have carried out equilibrium and kinetic studies to delineate the molecular mechanism of the σ-core interaction and to determine its kinetic and thermodynamic parameters. Our results indicate that a conformational transition of RNA polymerase is indeed induced upon binding of σ to core enzyme. The physicochemical basis and physiological significance of this conformational change are discussed.

Materials and Methods

Chemicals. *N*-(1-Pyrene)maleimide was synthesized by the method of Weltman et al. (1973). All other chemicals were of the highest reagent grade available.

RNA Polymerase and σ Subunit. RNA polymerase holoenzyme was isolated from *Escherichia coli* B as described previously (Wu and Wu, 1973). Core enzyme and σ subunit were purified as described by Berg et al. (1971). The preparation of σ was at least 95% pure when examined by acrylamide gel electrophoresis in sodium dodecyl sulfate.

N-(1-Pyrene)maleimide-Labeled σ (PM-σ). Since we have

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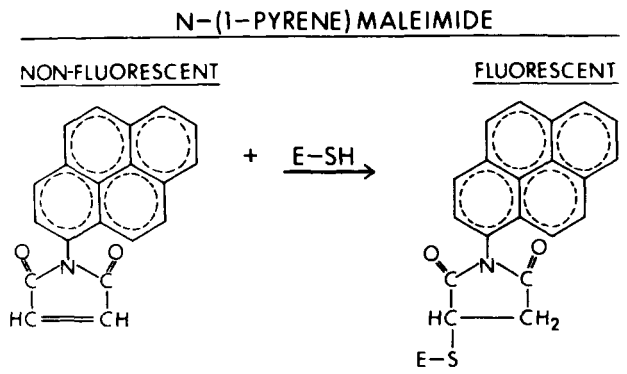

FIGURE 1: Structures of *N*-(1-pyrene)maleimide and its adduct.

found that *N*-(1-pyrene)maleimide adducts are not stable in Tris[1] or other buffers containing primary amino groups (Wu et al., 1976a), either phosphate or Bicine buffer was used in all the experiments described below. Purified σ (0.5–1 mg) was dialyzed against a N_2-flushed phosphate buffer, pH 7.5 (0.05 M potassium phosphate, 0.05 M KCl, 10^{-4} M EDTA, and 5% glycerol), for 4 h at 3 °C (four changes). A threefold molar excess of *N*-(1-pyrene)maleimide from a freshly prepared solution in 50% ethanol–acetone was added to the dialyzed σ. The reaction mixture was incubated for 1–2 h at 3 °C and the unreacted *N*-(1-pyrene)maleimide was removed by passing through a Sephadex G-75 column equilibrated with Bicine buffer, pH 7.5 (0.02 M Bicine, 0.05 M KCl, 10^{-4} M EDTA, 5% glycerol). The labeled σ was stored at 3 °C and used within 2 days. The average number of dyes bound was determined by $A_{340\,nm}$ of PM-σ using a molar absorptivity of 4.1×10^4 M^{-1} cm^{-1} for the pyrene chromophore (Rawitch et al., 1969), and the protein concentration was measured by the method of Bücher (1947). Assays for the stimulation of core enzyme transcription on T7 DNA template by unlabeled and PM-σ were performed as described previously (Yarbrough and Wu, 1974a).

Absorption and Fluorescence Spectra. Absorption spectra were obtained with a Cary 118C recording spectrophotometer. Corrected fluorescence emission spectra were recorded on a Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with a corrected spectra attachment. The absorbance of solutions used was always less than 0.05 at the excitation wavelength to prevent inner filter effects. For all spectral measurements, the temperature of the sample was maintained at 22 ± 0.1 °C.

Stopped-Flow Measurements. The stopped-flow apparatus with fluorescence detection was constructed in this laboratory and has been described before (Wu and Wu, 1974). The dead time of the apparatus was about 2 ms. The light source was a Hanovia 200-W Xe–Hg arc lamp. The excitation wavelength was selected at 340 nm by a Bausch & Lomb, high-intensity uv monochromator. The fluorescence emission at right angle was passed through Kodak Wratten 2C and Corning 7-59 filters to remove exciting light. The kinetic information was recorded on a Tektronix 543 storage oscilloscope and transferred on line to a PDP-11 digital computer through a Biomation 802 transient recorder. Relaxation times were calculated by a nonlinear, least-squares analysis of the data. Unless noted otherwise, each relaxation time represents the mean value obtained from at least five experiments. In some cases the oscilloscope traces were photographed and analyzed graphically. The results were in good agreement with the computer analysis. The experimental uncertainty in the relaxation times was estimated to be about 10%.

All solutions used in steady-state fluorescence measurements and stopped-flow experiments contained 0.2 M KCl, 0.02 M Bicine (pH 7.5), 0.1 mM dithiothreitol, and 0.1 mM EDTA. The temperature of solutions was held constant with a Lauda K-2/RD refrigerated circulator.

[1] Abbreviations used are: PM, *N*-(1-pyrene)maleimide; PM-σ, the pyrenemaleimide-labeled σ subunit of RNA polymerase; EDTA, ethylenediaminetetraacetic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Tris, tris(hydroxymethyl)aminomethane.

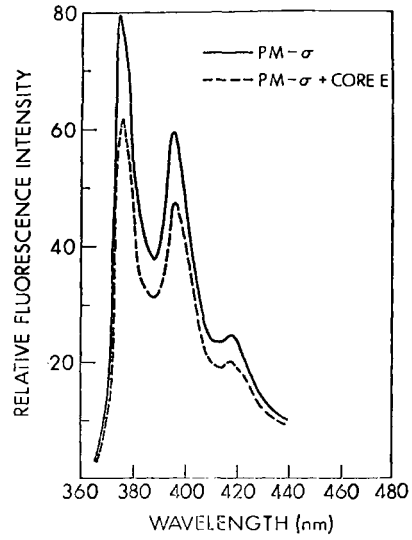

FIGURE 2: Corrected fluorescence emission spectra of PM-σ in the absence and presence of core polymerase. The concentrations of both PM-σ and core enzyme were 4×10^{-8} M in 0.02 M Bicine buffer (pH 7.5), 0.2 M KCl, 10^{-4} M dithiothreitol, and 10^{-4} M EDTA. The excitation wavelength was 340 nm. (—) In the absence of core enzyme; (- - -) in the presence of core enzyme.

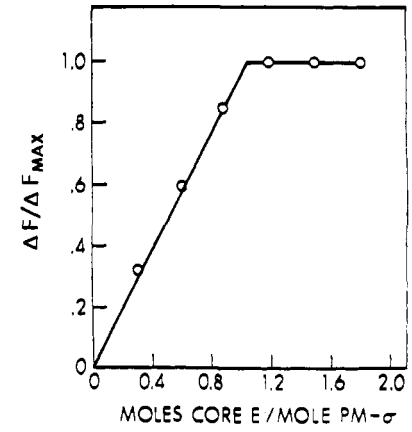

FIGURE 3: Fluorimetric titration of PM-σ with core enzyme. The indicated amount of core enzyme was added to a solution (0.4 ml) containing 4×10^{-8} M PM-σ in Bicine buffer as described in the legend to Figure 2. $\Delta F/\Delta F_{max}$ represents the fractional quenching of fluorescence intensity of PM-σ at 376 nm. The excitation wavelength was 340 nm.

Results

Fluorescent Labeling of σ Subunit with N-(1-Pyrene)-maleimide. *N*-(1-Pyrene)maleimide (Figure 1) reacts with sulfhydryl groups in proteins or other sulfhydryl compounds to form highly fluorescent derivatives. When purified σ subunit of RNA polymerase was reacted with a threefold molar excess of *N*-(1-pyrene)maleimide, about 2 mol of the dye was incorporated per mol of σ. The labeled σ (PM-σ) retained about 80%

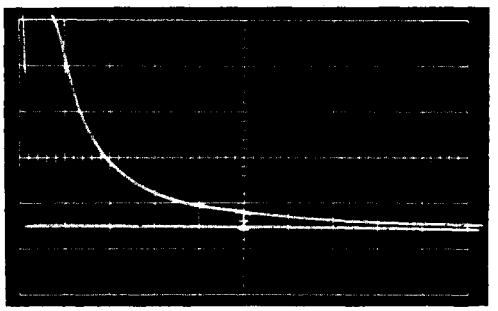

FIGURE 4: Oscilloscope trace of the kinetics of PM-σ binding to core enzyme. The concentrations of PM-σ and core enzyme were 3×10^{-8} and 4×10^{-7} M, respectively. Measurements were made at 22 °C. Other conditions were as described in Materials and Methods. The ordinate represents the fluorescence change at 376 nm (0.1 V/division, with total signal setting at 3 V) and the abscissa is time (2 s/division).

of its activity in stimulating transcription of T7 DNA by core enzyme as compared with unlabeled σ. The corrected fluorescence emission spectrum of PM-σ is shown in Figure 2. Emission maxima were observed at 376, 396, and 417 nm. The quantum yield of PM-σ was determined to be 0.13 using a standard quantum yield of 0.55 for quinine sulfate in 1 N H_2SO_4 (Melhuish, 1964).

Equilibrium Binding of PM-σ with Core Polymerase. σ subunit binds stoichiometrically to the core enzyme of RNA polymerase to form the holoenzyme (Burgess, 1969). When a saturating amount of core enzyme was added to PM-σ, there was a 25% decrease in fluorescence intensity without shifts in emission maxima (Figure 2). Monitoring the change in fluorescence intensity at 376 nm, a solution of PM-σ (4×10^{-8} M) was titrated with core enzyme. As shown in Figure 3, the change in fluorescence intensity increased linearly until a 1 to 1 mol ratio of core enzyme was added. Further addition of core enzyme did not produce any more fluorescence change. The titration curve indicates that the binding of PM-σ to the core polymerase was essentially stoichiometric under the experimental conditions. Although the dissociation constant of the PM-σ–core-enzyme complex could not be accurately determined, an upper limit of the dissociation constant on the order of 10^{-9} M was estimated based on the concentrations of both PM-σ and core enzyme used in the experiments.

Kinetics of the σ–Core Interaction. Again using the fluorescence change at 376 nm as a signal, the kinetics of interaction of PM-σ with core polymerase was studied by means of stopped-flow technique. When PM-σ was mixed rapidly with a large excess of core enzyme, the observed decrease in fluorescence intensity at 376 nm with respect to time was biphasic (Figure 4) and could be fitted within the experimental error to two exponential decays by a nonlinear, least-squares analysis:

$$\Delta F = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \qquad (1)$$

where a_1 and a_2 are amplitude factors and τ_1 and τ_2 are relaxation times. The dependence of $1/\tau_1$ and $1/\tau_2$ on the concentration of core polymerase is shown in Figures 5a and 5b, respectively. As can be seen, $1/\tau_1$ increases linearly with increasing core enzyme concentrations. In contrast, the concentration dependence of $1/\tau_2$ levels off at higher core enzyme concentrations.

Since two clearly resolvable exponential terms are observed in the kinetics of the binding reaction, at least two sequential steps must be involved in the binding of PM-σ to core enzyme. A simple two-step mechanism consistent with the observed kinetics is a rapid bimolecular binding of σ to core polymerase

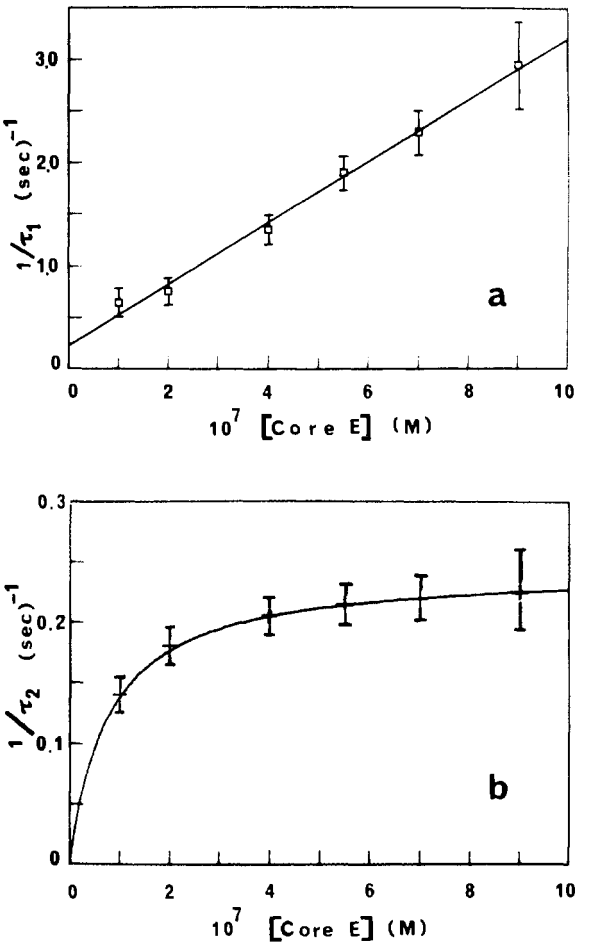

FIGURE 5: Concentration dependence of $1/\tau_1$ (a) and $1/\tau_2$ (b) for PM-σ binding to core enzyme. Data were obtained and analyzed as described in Materials and Methods. The solutions were the same as for Figure 4, except that the concentration of core enzyme was varied. The solid lines in a and b are the theoretical curves calculated according to eq 3 and 4, respectively, using the kinetic parameters in Table I.

followed by a relatively slow conformational transition (isomerization) of the holoenzyme formed:

$$E + \sigma \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\sigma \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\sigma' \qquad (2)$$

where E represents the core enzyme, $E\sigma$ and $E\sigma'$ are two different conformational forms of the holoenzyme, and k_i's are rate constants for the corresponding steps. When the concentration of core polymerase is in excess of the concentration of σ ($[E] \gg [\sigma]$), the bimolecular step becomes pseudo-first-order with respect to core polymerase and thus may be characterized by a single exponential decay, τ_1, for which

$$1/\tau_1 = k_{-1} + k_1[E] \qquad (3)$$

Thus $1/\tau_1$ has a linear dependence on the core enzyme concentration as shown in Figure 5a. By a linear, least-squares analysis of the data in Figure 5a, the values $k_1 = 3 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 0.23$ s^{-1} were obtained. This leads to a value of the intrinsic dissociation constant for the bimolecular step, K_1 ($=k_{-1}/k_1$), of 7.8×10^{-8} M.

The relaxation time for the unimolecular isomerization step is related to the equilibrium concentration and the rate constants as follows (Amdur and Hammes, 1966):

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_2}{1 + ((k_{-1}/k_1)/[E])} \qquad (4)$$

It can be seen from Figure 5b that k_{-2} is very small and $1/\tau_2$ reaches a plateau at high concentrations of core enzyme. In this situation (i.e., $1/\tau_2 \gg k_{-2}$), eq 4 can be rearranged to give

$$\tau_2 \simeq \frac{1}{k_2} + \frac{K_1}{k_2}\frac{1}{[E]} \qquad (5)$$

By a linear, least-squares analysis of the plot of τ_2 vs. $1/[E]$ (not shown), values of $k_2 \simeq 0.24\ s^{-1}$ and $K_1 \simeq 7 \times 10^{-8}$ M were estimated. Using these approximate values as the first trial parameters in a nonlinear, least-squares analysis of the data (Figure 5b) according to eq 4, the best fit values of $k_2 = 0.26\ s^{-1}$, $k_{-2} \leq 1 \times 10^{-3}\ s^{-1}$, and $K_1 = 7.8 \times 10^{-8}$ M were obtained.

Effect of Temperature on the Rate of Conformational Transition. Studies on the temperature dependence of rate constants can provide thermodynamic information about the elementary steps involved in molecular interactions. For this purpose, it is usually necessary to carry out kinetic analysis varying concentrations of reactants at several different temperatures. Such studies required large quantities of both σ subunit and core enzyme that we could not afford. However, according to eq 5, k_2 is linearly proportional to $1/\tau_2$ at a constant concentration of the core enzyme. Therefore, we have investigated the effect of temperature on the forward rate constant, k_2, associated with the conformational transition by examining the temperature dependence of $1/\tau_2$ at constant concentrations of PM-σ (3×10^{-8} M) and core enzyme (4×10^{-7} M). Under these conditions, $1/\tau_2 \simeq 0.83\ k_2$. Figure 6 shows the results obtained by varying the temperature from 10 to 34 °C. Within the experimental error, the plot is sigmoidal with an inflection point near 20 °C. The Arrhenius plot of the data shown in Figure 6 is also nonlinear.

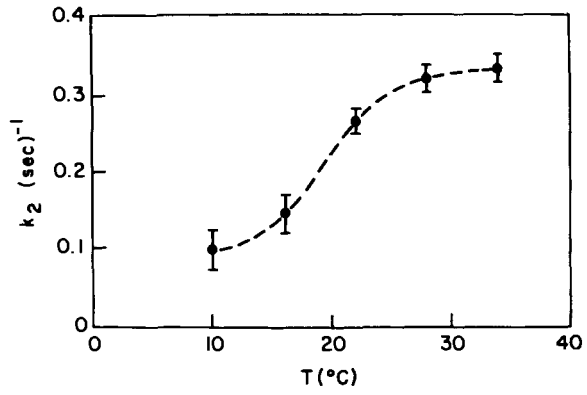

FIGURE 6: Effect of temperature on the forward rate constant, k_2, associated with the conformational transition of holoenzyme. The concentrations of PM-σ and core enzyme were 3×10^{-8} and 4×10^{-7} M, respectively. Other conditions were as described in the legend to Figure 4, except that the temperature was varied from 10 to 34 °C. The values of k_2 were calculated from $1/\tau_2$ as described in the text.

TABLE I: Kinetic Parameters for the σ-Core Interaction.

$k_1 = 3 \times 10^6\ M^{-1}\ s^{-1}$	$K_1 = k_{-1}/k_1 = 7.8 \times 10^{-8}$ M
$k_{-1} = 0.23\ s^{-1}$	$K_2 = k_{-2}/k_2 \leq 4 \times 10^{-3}$
$k_2 = 0.24\ s^{-1}$	$K_0 = K_1K_2/(1 + K_2)$
$k_{-2} \leq 1 \times 10^{-3}\ s^{-1}$	$\leq 3.2 \times 10^{-10}$ M

Discussion

On the basis of amino acid analysis, the σ subunit of *Escherichia coli* RNA polymerase contains eight half-cysteine residues per protein molecule (Fujiki and Zurek, 1975). We have shown that PM-σ, in which two cysteine residues have been labeled with *N*-(1-pyrene)maleimide, still retains 80% activity in stimulating the transcription of T7 DNA by core polymerase. In earlier studies, we also found that, in the σ subunit, two cysteine residues could be modified by tetrathionate (Yarbrough and Wu, 1974a), while a single cysteine residue was labeled with a fluorescent derivative of iodoacetamide, 1,5-I-AENS (Wu et al., 1976b). These modified σ subunits were biologically as active as unmodified σ. Thus at least two reactive cysteine residues in the σ subunit are not essential for biological activity.

The kinetic data obtained for the interaction of PM-σ with core polymerase are consistent with a simple two-step mechanism (eq 2) in which a rapid bimolecular reaction is followed by a relatively slow unimolecular isomerization (conformational change) of the holoenzyme formed. The best-fit kinetic parameters are listed in Table I. The overall binding constant, K_0, calculated from the kinetic parameters ($\leq 3.2 \times 10^{-10}$ M) is in reasonable agreement with that estimated from the fluorimetric titration ($<10^{-9}$ M; Figure 3). RNA polymerase isolated by several procedures has been shown to contain amounts of the σ subunit ranging from 60 to 80% (Berg et al., 1971). This is consistent with the very low value of overall binding constant that we obtained for the σ-core complex. Comparison of the values of K_0 and K_1 reveals that the stability of σ-core complex is enhanced by two orders of magnitude due to the practically irreversible conformational transition of the bimolecular encounter complex, Eσ.

A value of $3 \times 10^6\ M^{-1}\ s^{-1}$ was obtained for the bimolecular rate constant (k_1) associated with the σ-core interaction. Relatively few data are available in the literature concerning the kinetics of protein-protein interactions. Most of the bimolecular rate constants reported for protein-protein interactions fall in the range 10^5 to $10^7\ M^{-1}\ s^{-1}$ (Finlayson et al., 1969; Noble et al., 1969; Kellett and Gutfreund, 1970; Anderson et al., 1971; Schweitz et al., 1973; Quast et al., 1974), the highest value being $2.1 \times 10^7\ M^{-1}\ s^{-1}$ for the association of 30S and 50S ribosomal subunits to form the 70S complex in the presence of 8 mM Mg^{2+} (Wishnia et al., 1975). These reactions are not diffusion controlled, in the sense that every encounter is not productive. The bimolecular rate constant for an ideal diffusion-controlled reaction between two spherical molecules having molecular weights equivalent to that of the σ subunit and core polymerase can be calculated to be about $7 \times 10^9\ M^{-1}\ s^{-1}$ in aqueous solution at 23 °C (Caldin, 1964). This value is more than three orders of magnitude higher than the value of k_1 reported in Table I. Such difference may be due to the fact that only a specific fraction of the surface area of each molecule is engaged in binding and that the binding requires correct orientation of the molecule. If this combined steric and orientation factor is of the order of 10^{-2} for each protein molecule (e.g., the target area for binding covers 10% of total surface area of a molecule and the orientation factor is assumed to be 0.1 for each molecule), then the calculated bimolecular rate constant would approach the observed value.

It has been suggested that a necessary step in the initiation of RNA chains is the formation of a "melted-in" template-enzyme complex (Florentiev and Ivanov, 1970). Zillig et al. (1970) showed that a plot of the rate of RNA synthesis vs. temperature gives a sharp transition centered at 17.5 °C. This phenomenon was attributed to the local melting of the DNA helix. Although there is evidence for the local unwinding of DNA by RNA polymerase (Saucier and Wang, 1972), the anomalous temperature dependence of an enzymatic reaction

could also be affected by structural changes in the enzyme (Talsky, 1971). The results from our studies of the temperature effect on the rate of conformational transition of holoenzyme, which shows a transition temperature around 20 °C (Figure 6), imply that the temperature transition observed for the RNA polymerase reaction is possibly also related to the temperature-dependent conformational transition of the enzyme.

The nature of this conformational transition is unknown. In general, the Arrhenius plot is linear for a rate constant associated with a simple molecular process (e.g., unimolecular isomerization) and the slope of the plot represents the activation energy of this process. The nonlinear Arrhenius plot obtained for k_2 suggests that the conformational transition of the holoenzyme is rather complex and may consist of several molecular processes which have different activation energies. This is not surprising in view of the fact that the conformational transition of a protein may involve various processes, e.g., displacements and/or rotation of a bond (or bonds) in the backbone or side chains. Alternatively, there may be more than one kinetically distinct pathway for the conformational transition which differ in their temperature dependence. Nevertheless, detailed analysis of these processes is not simple. We have estimated the activation energy ($\Delta E^{\ddagger}$) of the "overall" conformational transition to be 6.7 kcal/mol, simply by a linear regression of the Arrhenius plot of k_2. Other activation parameters at 22 °C were also calculated: $\Delta G^{\ddagger} = 18.1$ kcal/mol, $\Delta H^{\ddagger} = 6.1$ kcal/mol, and $\Delta S^{\ddagger} = -40$ eu. The large negative value of entropy of activation ($\Delta S^{\ddagger}$) implies that the activation complex is highly ordered or there may be some solvent effects during the process of complex formation (Laidler and Bunting, 1973). Finally, from the kinetic parameters in Table I, the free energies of reaction for the bimolecular binding, the conformational transition, and the overall reaction of the σ-core interaction at 22 °C were calculated to be $\Delta G^{\circ}_1 = -9.6$ kcal/mol, $\Delta G^{\circ}_2 \leq -3.2$ kcal/mol, and $\Delta G^{\circ}_{\text{overall}} \leq -12.9$ kcal/mol.

It has been proposed that the σ subunit of RNA polymerase is responsible for the promotor selection and the subsequent specific initiation (Burgess et al., 1969; Travers and Burgess, 1969; Hinkle and Chamberlin, 1972). Previously (Yarbrough and Wu, 1974a), we showed that a complex of intact σ and chemically modified core enzyme (at sulfhydryl groups) can transcribe single-stranded DNA but is almost inactive with native double-stranded DNA template. This observation indicates that, although σ subunit may be needed for transcribing native double-stranded DNA, the intact structure of core enzyme (in particular, those regions which are involved in binding σ) is also essential. In this paper, we further demonstrated kinetically that a specific conformational transition of RNA polymerase is induced by binding of the σ subunit to core polymerase. Thus, we have provided both structural and kinetic evidence to support the idea that template recognition and specific initiation in RNA synthesis are regulated at the molecular level through the σ-core interaction of RNA polymerase.

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