MECHANISM OF *E. coli* RNA POLYMERASE-PROMOTER INTERACTIONS

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The regulation of gene expression in procaryotes is accomplished primarily at the level of transcription. Initiation of transcription is subject to numerous promoter-specific controls which act to ensure coordinate expression of disparate genes. The kinetics of formation of a functional ("open") complex at a promoter, prior to the catalytic steps of RNA chain initiation and elongation, is thought to play a major role in controlling the efficiency of transcription of that promoter, since the subsequent processes of nucleotide binding and phosphodiester bond formation are rapid and are not promoter-specific(Mangel and Chamberlin, 1974 Shimamoto *et al.*, 1981).

Structural, thermodynamic and kinetic studies have been performed on the interactions of *E. coli* RNA polymerase holoenzyme (RNAP)with various viral and bacterial promoters(cf. reviews by Chamberlin, 1976; Rosenberg and Court, 1979; von Hippel *et al.*, 1982, 1984). In the functional open complex, a region of approximately 10-17 base pairs in the vicinity of the interaction site is thought to be conformationally altered("open" or denatured)(Hsieh and Wang, 1978; Melnikova *et al.*, 1978; Siebenlist *et al.*, 1980; Gamper and Hearst, 1982).

Thermodynamic and kinetic studies on specific RNAP-promoter interactions have been performed using a variety of techniques including the filter binding assay(Hinkle & Chamberlin, 1972; Seeburg *et al.*, 1977; Strauss *et al.*, 1980a.b), electron microscopy(Williams & Chamberlin, 1977; Giacomoni *et al.*, 1977 a), abortive initiation(Hawley *et al.*, 1982) and other transcription assays(Stefano & Gralla, 1980; Kadesch *et al.*, 1982). Different thermodynamic properties were observed for promoter complexes formed below 15℃ and above 20℃(Strauss *et al.*, 1980a,b). Those promoter complexes formed at low temperature may be examples of a "closed" promoter complex, which is thought to be an intermediate on the pathway to formation of an open promoter complex.

Kinetics of the Formation of Open Complex

Evidence for formation of a closed complex(RP_c) as an intermediate on the pathway to formation of the functional open complex(RP_o) between RNA polymerase(R) and a promoter site(P) has been accumulated for several promoters(cf. Mulligan *et al.*, 1984; Hawley and McClure, 1980; Kadesch *et al.*, 1982). Under conditions where the formation of RP_o is irreversible, the one-intermediate mechanism(McClure, 1980)

$$R + P \underset{\text{rapid equilibrium}}{\overset{K_B}{\rightleftharpoons}} RP_c \xrightarrow[\text{slow}]{k_2^M} RP_o$$

has been used to analyze the kinetic data. (The superscript "M" is used to distinguish the rate constant K_2 of McClure's mechanism from that of the mechanism proposed in this paper.) Under pseudo-first order experimental conditions where the concentration of RNA polymerase([RNAP]) is in stoichiometric excess over

the concentration of promoter sites, a plot of the reciprocal of the observed pseudo first order rate constant ($1/k_{obs}$, denoted τ_{obs}) as a function of the reciprocal of the total polymerase concentration is linear with an intercept on the τ axis of $1/k_2^M$ and a slope of $1/k_B k_2^M$.

$$\tau_{obs} = \frac{1}{k_2^M} + \frac{1}{K_B k_2^M [RNAP]}$$

In this rapid equilibrium limit, the overall second order association rate constant k_a is given by $k_a = k_B k_2^M$. McClure and coworkers have observed non-zero intercepts in τ-plots constructed for many promoters, indicating that the first order isomerization step

$$RP_c \xrightarrow{k_2^M} RP_o$$

becomes rate limiting at sufficiently high polymerase concentrations.

Effect of Salt and Temperature on the Kinetics of the Formation of Open Complex

The kinetics of formation and dissociation of specific(open) complexes between active *E. coli* RNA polymerase holoenzyme(RNAP) and the λp_R promoter have been studied by selective nitrocellulose filter binding assays at two temperatures(25℃, 37℃) and over a range of ionic conditions(Roe *et al.*, 1984). Competition with a polyanion(heparin) or stabilization of open promoter complexes at p_R by incubation with specific combinations of nucleoside triphosphates was employed to obtain selectivity in the filter assay. This study provides a useful example of how information about mechanism may be obtained from the quantitative analysis of the effects of salt concentration and temperature on the rate constants of a protein-DNA interaction.

The association reaction between RNAP and λP_R was investigated under ionic conditions where the process is essentially irreversible, and under pseudo-first order conditions of excess polymerase. The pseudo-first order rate constant is directly proportional to the concentration of active polymerase over the entire range investigated(2-1.0 nM) at both 25℃ and 37℃, within experimental uncertainty(Fig.1). Second order association rate constants(k_a), calculated from these data at standard ionic conditions(0.12 M KCl, 0.01

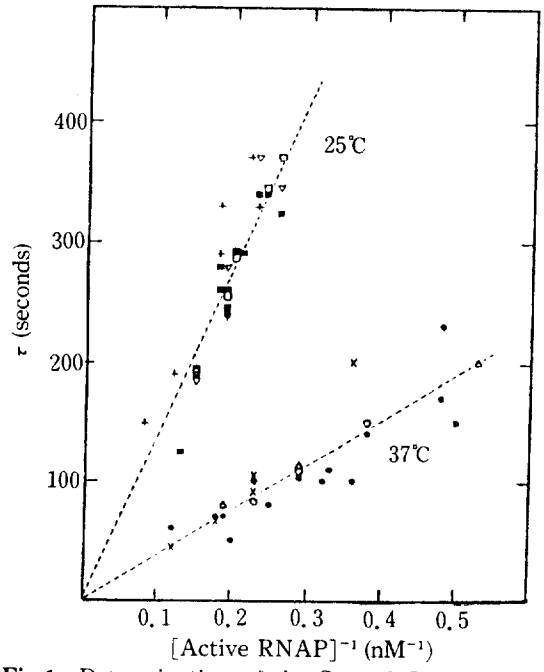

Fig.1. Determination of the Second Order Rate Constant(k_a) for Formation of Open Complexes between RNAP and the λP_R Promoter at 37℃ and 25℃.
Time constants τ_{obs}, defined as the inverse of pseudo-first order rate constants obained in RNAP excess by three different filter assays, are plotted as a function of the inverse of the active RNAP concentration for experiments with the Hae III-890 bp fragment performed in BB. The assays used were the heparin competition assay (● and ■ for 37℃ and 25℃, respectively) and the NTP-stabilization assay(using UTP, GTP, and either CpA(x,+) or ATP(△, ▽). Data obtained with the AluI-194 bp fragment by the heparin competition assay are also included(o, □). The dashed line(···) through the data was calculated by assuming that each measured τ is inversely proportional to the corresponding active RNAP concentration, and averaging the apparent second order rate constants(k_a) obtained from such an analysis. The average ka(±1 S.D), which is the reciprocal of the slope of the line, is $(2.6 \pm 0.4) \times 10^6$ M^{-1} sec^{-1} at 37℃ and $(7.2 \pm 1.4) \times 10^5$ M^{-1} sec^{-1} at 25℃.

M $MgCl_2$ 0.04 M tris(pH 8)) were strongly temperature-dependent: $k_a = (2.6 \pm 0.4) \times 10^6$ M^{-1} sec^{-1} at 37℃ and $k_a = (7.2 \pm 1.4) \times 10^5$ M^{-1} sec^{-1} at 25℃, corresponding to an activation energy of the association reaction of approximately 20 ± 5 kcal. In addition, k_a decreases strongly with increasing KCl concentration, corresponding to the net release of the thermodynamic equivalent of at least 9 monovalent ions prior to or during the rate-limiting step of the association reaction. This strong dependence of k_a on the ionic environment suggests that inorganic cations should be considered as possible regulators of *in vivo* transcription initiation.

Dissociation rate constants(k_d) were also measured under irreversible reaction conditions. At the standard ionic conditions, $k_d = (2.2 \pm 0.3) \times 10^{-5}$ sec^{-1} at 37℃ and $k_d = (4.0 = 0.4) \times 10^{-5}$ sec^{-1} at 25℃(Table 1). The increase in k_d with decreasing temperature corresponds to a *negative* activation energy of dissociation (-9 ± 4 kcal). In addition, k_d increases with increasing KCl concentration, corresponding to the net uptake of the thermodynamic equivalent of at least 6 monovalent ions in or prior to the rate limiting step of the dissociation reaction. Equilibrium constants $K_{obs}^{RP} = k_a/k_d$ for the association of RNA polymerase with the P_R promoter are also strong functions of temperature and ionic conditions(Table 1). At 37℃ in the standard buffer, $K_{obs}^{RP} = (1.2 \pm 0.3) \times 10^{11}$ M^{-1}. The van't Hoff enthalpy ΔH° of the process is estimated to be 29 ± 9 kcal. The thermodynamic equivalent of at least 15 monovalent ions is released in the overall process of association. The entropic contribution from release of these ions provides much of the thermodynamic driving force for the binding reaction under the ionic conditions investigated.

We argue on the basis of the magnitude of k_a and the dependences of k_a and k_d on temperature and salt concentration that a *minimum* reaction mechanism for the interaction of RNA polymerase(R) with the λP_R promoter(P) to form an open complex(RP_0) must be

$$R + P \rightleftharpoons I_1 \rightleftharpoons I_2 \rightleftharpoons RP_0$$

where I_1 and I_2 are intermediates which do not accumulate under the reaction conditions investigated and where the rate-limiting steps, under the conditions investigated, are the interconversions of the transient intermediates I_1 and I_2, which presumably correspond to conformationally distinct forms of an intermediate(closed) promoter complex. The kinetic constants for the individual steps in the association reaction differ significantly from

Table 1. Dependencies of the Rate and Equilibrium Constants for λP_R-RNAP Interaction on Temperature and [KCl][a].

T.℃	[KCl] [M]	$k_a \times 10^{-5}$[b] (M^{-1} sec^{-1})	number of association experiments	$k_d \times 10^5$[c] (sec^{-1})	$K_P (= \frac{k_a}{k_d}) \times 10^{-10}$[b] ($M^{-1}$)
25	0.12	7.2−1.4	30	4.0±0.4	1.8±0.5
37	0.10	31±3	2	···	···
37	0.12	26±4	25	2.2±0.3	12±3
37	0.16	4.9±0.5	2	3.6±1.5	1.4±0.9
37	0.18	3.1±0.3	6	8.0±2.5	0.4±0.2
37	0.20	···	···	11±2.0	···

(a) Buffer: 40 mM Tris(pH 8), 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/m/BSA
(b) Calculated on the basis of 100% active enzyme
(c) Single experiments with duplicate sampling; error was estimated from the slope of semi-log plot.

those previously obtained for P_R from the abortive initiation assay(Hawley and McClure, 1980), although the overall association rate constants(k_a) measured by the two assays are comparable.

Characterization of the Steps Involved in the 3-Step Mechanism

The kinetics of formation and of dissociation of open complexes(RP_o) between *E. coli* RNA polymerase(R) and the λP_R promoter(P) have been studied as a function of temperature in the physiological range using the nitrocellulose filter binding assay(Roe *et al*., 1985). Kinetic data were analyzed according to the mechanism $R+P \rightleftharpoons I_1 \rightleftharpoons I_2 \rightleftharpoons RP_o$, where I_1 and I_2 are kinetically distinguishable intermediate complexes at this promoter which do not accumulate under the reaction conditions investigated(Roe *et al.*, 1984). The overall second order association rate constant increases dramatically with increasing temperature, yielding a temperature-dependent activation energy in the range 20 kcal(near 37℃) to 40 kcal(near 13℃)(Fig.2). Both isomerization steps($I_1 \rightarrow I_2$ and $I_2 \rightarrow RP_o$) appear to be highly temperature dependent; except at low temperatures(<13℃) the step $I_1 \rightarrow I_2$, which we attribute to a conformational change in the polymerase with a large negative ΔC_P°, is rate-limiting at the reactant concentrations investigated and hence makes the dominant contribution to the apparent activation energy of the pseudo first order association reaction(Fig.2, Table 2). The subsequent step $I_2 \rightarrow RP_o$, which we attribute to DNA melting, has a higher activation energy(in excess of 100 kcal) but only becomes rate-limiting at low temperature(<13℃). The initial binding step $R+P \rightleftharpoons I_1$ appears to be in equilibrium on the time scale of the isomerization reactions under all conditions investigated; the equilibrium constant for this step is not a strong function of temperature and is approximately $10^7 M^{-1}$ under the standard ionic conditions of the assay(40 mM Tris, 10 mM $MgCl_2$, 0.12 M KCl).

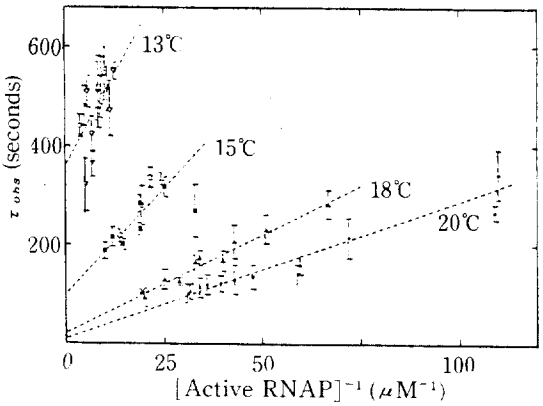

Fig.2. τ-analysis for the Formation of Open Complexes at P_R at Different Temperatures.
Values of τ_{obs} were measured by either heparin competition assay(filled symbols) or NTP-stabilization assay(open symbols) (See text for details). Dotted lines are the weighted least squares fit through the data. Kinetic parameters obtained from this analysis are listed in Table 2.

The activation energy of the dissociation reaction becomes increasingly negative at low temperatures, ranging from approximately-9 kcal near 37℃ to-30 kcal near 10℃. Thermodynamic(van't Hoff) enthalpies ΔH° of open complex formation consequently are large and temperature-dependent, increasing from approximately 29 to 70 kcal as the temperature is reduced from 37℃ to 13℃ (Table 2, Fig.3). The corresponding ΔCp°

Table 2. The Temperature Dependence of Kinetic Parameters Obtained from τ-analysis.

Tem. ℃	k_a ($M^{-1}s^{-1}$)	τ_{int} (s)	k_1^a (s^{-1})	K_1, predicted (M^{-1})
37[b]	$(2.7\pm0.3)\times10^6$	0.6 ± 9	>0.1	$\leq2.7\times10^7$
25[b]	$(7.0\pm0.5)\times10^5$	-6 ± 21	>0.07	$\leq1.0\times10^7$
20	$(3.6\pm0.3)\times10^5$	10 ± 11	0.1	$\leq7.6\times10^6$
18	$(2.5\pm0.2)\times10^5$	21 ± 10	0.05	$\leq7.8\times10^6$
15	$(1.2\pm0.2)\times10^5$	97 ± 33	0.01	$\leq1.6\times10^7$
13	$(6.8\pm2.4)\times10^4$	360 ± 45	0.003	$\leq2.7\times10^7$

a: $k_1 = \tau_{int}^{-1} = (1/k_2 + 1/k_3)^{-1}$
b: data from Roe *et al.*(1984)

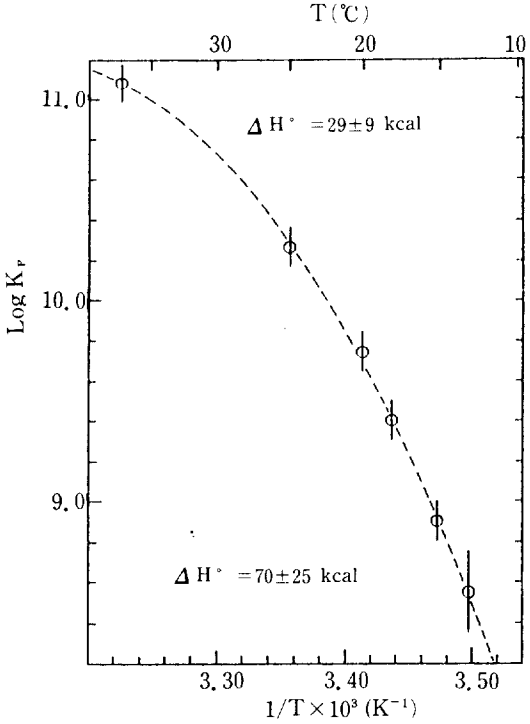

Fig.3. The van't Hoff plot of the Temperature Dependence of K_P.
The dotted line is a theoretical fit assuming that the standard heat capacity difference $\Delta C_P^\circ (\equiv \partial \Delta H^\circ / \partial T)$ is −2.4 kcal/deg, thereby yielding the integrated form $\ln Kp = 8.1832 \times 10^3 - 3.8084 \times 10^5/T - 1.2079 \times 10^3 \ln T$.($K_P$ exhibits a maximum at 42℃.) ΔH°, which is obtained from the slope of the curve, is(29±9) kcal betwen 25 and 37℃, and increases to (70±25) kcal between 13 and 15℃.

is approximately-2.4 kcal/deg. We propose that this large negative ΔC_P° arises primarily from the burial of hydrophobic surface in the conformational change(I_1 I_2) in RNA polymerase in the key second step of the mechanism.

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