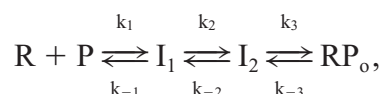


General Method of Analysis of Kinetic Equations for Multistep Reversible Mechanisms in the Single-Exponential Regime: Application to Kinetics of Open Complex Formation between $E\sigma^{70}$ RNA Polymerase and λP_R Promoter DNA

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ABSTRACT A novel analytical method based on the exact solution of equations of kinetics of unbranched first- and pseudofirst-order mechanisms is developed for application to the process of $E\sigma^{70}$ RNA polymerase (R)– λP_R promoter (P) open complex formation, which is described by the minimal three-step mechanism with two kinetically significant intermediates (I_1 , I_2),



where the final product is an open complex RP_o . The kinetics of reversible and irreversible association (pseudofirst order, $[R] \gg [P]$) to form long-lived complexes (RP_o and I_2) and the kinetics of dissociation of long-lived complexes both exhibit single exponential behavior. In this situation, the analytical method provides explicit expressions relating observed rate constants to the microscopic rate constants of mechanism steps without use of rapid equilibrium or steady-state approximations, and thereby provides a basis for interpreting the composite rate constants of association (k_a), isomerization (k_i), and dissociation (k_d) obtained from experiment for this or any other sequential mechanism of any number of steps. In subsequent papers, we apply this formalism to analyze kinetic data obtained in the reversible and irreversible binding regimes of $E\sigma^{70}$ RNA polymerase (R)– λP_R promoter (P) open complex formation.

INTRODUCTION

Many protein processes are multistep, initiated by a bimolecular association step that is often pseudofirst order (e.g., enzyme catalysis, protein–nucleic acid interactions). In vivo, the concentration of substrate and enzyme may be maintained constant by coupled reactions so an analogous situation may apply even if one reactant is not in excess. In general, the kinetics of such multistep processes are multiexponential in both relaxation to equilibrium and irreversible cases even where all steps are first (or pseudofirst) order. The maximum number of exponential terms is equal to the number of steps; in favorable cases, one can numerically decompose the kinetics into a sum of exponentials and interpret the corresponding exponential decay rate constants in terms of elementary rate constants for individual steps. In many other cases, however, including the formation of open complexes by $E\sigma^{70}$ RNA polymerase (R) at the λP_R promoter (P), the kinetics are multistep but single exponential (Roe et al., 1984, 1985; Roe and Record, 1985). What quantitative information regarding individual steps of

a multistep pseudofirst-order process can be unambiguously derived from its single-exponential kinetic behavior?

The initial steps in transcription initiation are the formation of a so-called open complex between RNA polymerase and promoter DNA. Kinetic-mechanistic studies at the λP_R promoter (Roe et al., 1984, 1985; Roe and Record, 1985; Craig et al., 1998) and *lacUV5* promoter (Buc and McClure, 1985) show that this unbranched process consists of at least three major steps: formation of the first kinetically-significant intermediate I_1 , subsequent isomerization to form the second kinetically significant intermediate I_2 , and a DNA-opening step that forms the open complex (RP_o) [designated as RP_{o2} in Mg^{2+} , conditions under which both the start site and the adjacent upstream region of DNA are open, and designated as RP_{o1} in the absence of Mg^{2+} , where the start site is closed (Suh et al., 1992; Zaychikov et al., 1997; Craig et al., 1995)]. Recent work (McQuade, 1996) shows significant reversibility of all three steps in the 7–15°C temperature range. Previous quantitative treatments of association and dissociation kinetics in the context of the three-step process (Roe et al., 1985; Roe and Record, 1985; Buc and McClure, 1985) have justified to the extent possible and used rapid equilibrium and/or steady-state approximations to analyze kinetic data. The implicit assumption in these cases has been that no exact solution of the system of differential equations of kinetics for such a complex system was available, and that general numerical fitting procedures

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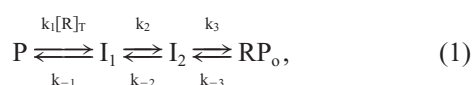
contained too many parameters to determine uniquely. In many other polymerase–promoter kinetic studies, the mechanism has been reduced to two steps (reversible initial binding and subsequent irreversible composite conformational changes), before analysis by rapid equilibria or steady-state approximations. The approximations involved in reducing the three-step mechanism to two steps and neglecting reversibility of open complex formation generally have not been considered in these studies. Here, we show that all these approximate analyses are unnecessary, and that the observation of single-exponential kinetics allows an exact analytical treatment of observed rate and equilibrium constants. This analysis avoids approximations in the mechanism and the analysis and yields an exact analytic solution for the proposed three-step mechanism and, in some cases, even for an unbranched pseudofirst order mechanism of any number of reversible steps.

RESULTS

General analytical solution to the kinetics of a reversible three-step mechanism for RNA polymerase–promoter open complex formation

The general solution of the system of linear differential equations of kinetics for pseudofirst-order reversible three-step mechanisms (Castellan, 1963) has been applied in numerous relaxation kinetics studies (e.g., Hammes and Schimmel, 1966, 1967; Hammes and Haslam, 1969; Haslam, 1972; Bernasconi, 1976). Various approximations to the general form of the solution (steady-state, rapid equilibrium, “bottleneck” step approximations) have been made prior to data analysis to relate the observed rate constants to the microscopic rate constants and initial reactant concentrations. To our knowledge, the general theory has never been specialized for the case of single-exponential kinetics, as observed in studies of the kinetics of interactions of RNA polymerase with λP_R promoter, where a minimal three-step reversible mechanism is required (e.g., Roe et al., 1984, 1985; Roe and Record, 1985; Craig et al., 1998). In what follows, we state the general results of the study by Castellan (1963) as applied to this system in the case of distinct characteristic roots of the matrix of the system of differential equations of kinetics for a three-step reversible mechanism. The basic theory leading to these results is outlined in Appendix A.

The minimal three-step pseudofirst-order mechanism for RNA polymerase–promoter open complex formation is



where $[R]_T$, the total concentration of polymerase, is typically in large excess over promoter. Craig et al. (1998) characterized I_1 and I_2 as extended complexes in which RNA polymerase contacts the promoter DNA at least from -40 to $+20$; I_1 is short-lived and I_2 is long-lived; the

conformational change in $I_1 \rightarrow I_2$ appears to involve closing of the polymerase jaws on the DNA downstream of the start site ($+1$), forming the long-lived intermediate I_2 . The DNA in the start site region opens in the subsequent step ($I_2 \rightarrow RP_o$). In excess RNA polymerase ($[R]_T \gg [P]_T$), where the initial binding step is pseudofirst order, the time-dependent vector of concentrations of reactants, intermediates, and products for the approach to equilibrium from the association direction C_a (the subscript “a” denotes reversible association in all subsequent abbreviations) of Mechanism 1 depends on time (t) as a linear combination of exponential terms (cf. Appendix A)

$$C_a = \sum_{i=1}^4 M_{ai} B_{ai} e^{-\lambda_i t}, \quad (2)$$

where vectors B_{ai} and constants M_{ai} are defined in Appendix A.

The four rate constants λ_i in Eq. 2 are the roots of the quartic equation

$$\lambda_i^4 - D_{a1}\lambda_i^3 + D_{a2}\lambda_i^2 - D_{a3}\lambda_i = 0. \quad (3)$$

Without approximation, the coefficients D_{ai} of this equation are functions of elementary rate constants and $[R]_T$.

$$D_{a1} \equiv k_1[R]_T + k_{-1} + k_2 + k_{-2} + k_3 + k_{-3} \quad (4)$$

$$D_{a2} \equiv k_1[R]_T(k_2 + k_{-2} + k_3 + k_{-3}) + k_{-1}(k_{-2} + k_3 + k_{-3}) + k_2(k_3 + k_{-3}) + k_{-2}k_{-3}$$

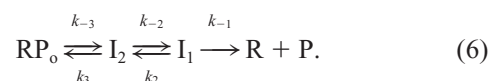
$$D_{a3} \equiv k_1[R]_Tk_{-2}k_{-3} + k_{-1}k_{-2}k_{-3} + k_1[R]_Tk_2k_3 + k_1[R]_Tk_2k_{-3}.$$

One solution of Eq. 3 is $\lambda = 0$. Therefore, one of the terms in Eq. 2 is a constant. The other λ_i are the solutions of the cubic equation

$$\lambda_i^3 - D_{a1}\lambda_i^2 + D_{a2}\lambda_i - D_{a3} = 0. \quad (5)$$

The roots λ_i of Eq. 5 are the observed relaxation rate constants (or reciprocal time constants $1/\tau_i$, commonly used in relaxation kinetics). For the mechanism considered, λ_i are positive because D_{ai} are positive (Eq. 4).

In the dissociation direction, disappearance of preformed complexes is irreversible when the dissociation reaction is performed in the presence of a large excess of a polyanionic competitor (e.g., heparin) that binds polymerase and prevents it from rebinding DNA. Therefore, in this case, we can neglect the bimolecular reassociation step,



Mechanism 6 is a special case of Mechanism 1 where $[R] = 0$ at all times.

The solution of the system of differential equations of dissociation kinetics for Mechanism 6, C_d (the subscript “d”

denotes irreversible dissociation in all subsequent abbreviations), is also a linear combination of exponentials (cf. Appendix A),

$$C_d = \sum_{i=1}^3 M_{di} B_{di} e^{-\lambda_i t}. \quad (7)$$

The three distinct λ_i for dissociation solutions of secular Eq. 5, where coefficients D_{ai} are replaced by coefficients D_{di} given by Eq. 4 at $[R]_T = 0$, i.e.,

$$\begin{aligned} D_{d1} &\equiv k_{-1} + k_2 + k_{-2} + k_3 + k_{-3}, \\ D_{d2} &\equiv k_{-1}(k_{-2} + k_3 + k_{-3}) + k_2(k_3 + k_{-3}) \\ &\quad + k_{-2}k_{-3} \\ D_{d3} &\equiv k_{-1}k_{-2}k_{-3}. \end{aligned} \quad (8)$$

The λ_i , or $1/\tau_i$ in this case, are the observed dissociation relaxation rate constants. In general, as follows from Eqs. 2 and 7, the concentrations of the experimentally observed complexes can be linear combinations of as many as three exponential terms. That is, the observed association and/or dissociation kinetics can generally exhibit single, double, or triple exponential behavior.

Single-exponential kinetics

In many cases, more than one set of theoretical parameters, or even an infinite number of them, provide statistically indistinguishable best fits to the experimental data, given their uncertainty. For example, this occurs when the number of the independent observed parameters is smaller than the number of unknown microscopic rate constants. In such cases, the explicit form of the exact solution of the differential equation of kinetics discussed above does not by itself provide any insight into the interrelationships between the elementary rate constants in Eq. 4 or 8. However, other situations exist in which the exact solution of a mechanism of a specified number of steps can be greatly simplified (without reducing the number of steps) on the basis of the experimental observations, allowing these interrelationships to be established. In this study, we focus on one such case, namely, when the observed kinetics are single-exponential.

Under the conditions examined in filter binding assays, both the association and dissociation kinetics of $E\sigma^{70}$ RNA polymerase- λP_R promoter complexes exhibit single exponential behavior within experimental uncertainty (Roe et al., 1984, 1985; Roe and Record, 1985; Schlax, 1995). In this assay, the observable is the concentration of long-lived (heparin-resistant) complexes, which include RP_o and I_2 . Complex I_1 is a short-lived (heparin-sensitive) complex that is present at equilibrium at temperatures below 15°C (Craig et al., 1998). From the single exponential character of the kinetics in the association direction, it follows that the solutions of secular Eq. 5 satisfy the relationships $\lambda_3 \ll \lambda_2$, λ_1 , where λ_3 , the observed rate constant, is a function of the

microscopic rate constants and $[R]_T$ (Appendix B). Therefore, given Eq. 5 and the derivation given in Appendix B, it follows that

$$\lambda_3 \cong \frac{D_3}{D_2}, \quad (9)$$

where appropriate expressions for D_3 and D_2 for association are given by Eq. 4.

Because the kinetics of dissociation of long-lived complexes at the λP_R promoter are also single exponential, the rate constant in the dissociation direction is also calculated from Eq. 9, where the appropriate expressions for D_3 and D_2 for dissociation are given by Eq. 8. Hence, Eq. 9 describes the consequence of single-exponential character of both association and dissociation kinetics.

Application to $E\sigma^{70}$ RNA polymerase- λP_R promoter DNA open complex formation kinetics

Filter binding and DNase I footprinting assays used to study kinetics of RNA polymerase-promoter open complex formation typically detect long-lived (LL) complexes. The observed fractional extent of conversion of unbound promoter DNA to long-lived complexes (RP_{LL} ; $I_2 + RP_o$) at equilibrium in the excess of RNA polymerase is

$$\theta_{LL}^{eq} = \frac{[I_2]_{eq} + [RP_o]_{eq}}{[P]_T} = \frac{K_{eq}^{LL}[R]_T}{1 + (K_1 + K_1K_2 + K_1K_2K_3)[R]_T}, \quad (10)$$

where

$$K_{eq}^{LL} = K_1K_2 + K_1K_2K_3 \quad (11)$$

and $K_1 = k_1/k_{-1}$, $K_2 = k_2/k_{-2}$, $K_3 = k_3/k_{-3}$ (cf. Eq. 1).

For application to thermodynamic and kinetic data obtained from assays that monitor only the amount of open complex (RP_o) but not I_2 , such as abortive initiation technique (Buc and McClure, 1985) or $KMnO_4$ footprinting (Craig et al., 1998), θ_{LL}^{eq} is replaced by $\theta_{RP_o}^{eq} = [RP_o]/[P]_T$ and, therefore, in Eq. 10, the equilibrium constant K_{eq}^{LL} is replaced by $K_{eq}^{RP_o} = K_1K_2K_3$.

At λP_R promoter, the fractional occupancy θ_{LL} is found experimentally to exhibit single exponential kinetic behavior under all conditions (reversible or irreversible association and irreversible dissociation) and follows the rate law (Schlax et al., 1995; Record et al., 1996)

$$-\frac{d \ln |\Delta \theta_{LL}|}{dt} = \beta, \quad (12)$$

where β is the relaxation rate constant. For single exponential relaxation kinetics, the above analysis shows that $\beta = \lambda_3$ in Eq. 9. To apply Eq. 12 in the association direction, $|\Delta \theta_{LL}| = \theta_{LL}^{eq} - \theta_{LL}$; in the dissociation direction $|\Delta \theta_{LL}| = \theta_{LL} - \theta_{LL}^{eq}$. Alternatively, one can rewrite the rate law in the

association direction from Eqs. 10 and 12:

$$\frac{d[\text{RP}_{\text{LL}}]}{dt} = \alpha[\text{P}]_{\text{T}} - \beta[\text{RP}_{\text{LL}}], \quad (13)$$

where $\alpha \equiv \beta\theta_{\text{LL}}^{\text{eq}} = \lambda_3\theta_{\text{LL}}^{\text{eq}}$. If association is irreversible, $\theta_{\text{LL}}^{\text{eq}} = 1$ and $\lambda_3 = \beta = \alpha$. For irreversible dissociation (in the presence of competitor) $\theta_{\text{LL}}^{\text{eq}} = 0$; therefore $\alpha = 0$ and $\beta = \lambda_3|_{[\text{R}]=0} = k_{\text{d}}$, the experimentally observed first-order dissociation rate constant.

For RNA polymerase–promoter association kinetics to form either long-lived, open or abortively-initiating complexes, the reciprocal of α (designated as τ_{obs} in earlier studies) is found to be a linear function of the reciprocal of the concentration of RNA polymerase (McClure, 1980; Schlax et al., 1995),

$$\frac{1}{\alpha} = \frac{1}{k_{\text{a}}[\text{R}]_{\text{T}}} + \frac{1}{k_{\text{i}}}, \quad (14)$$

where k_{a} is a composite second order association rate constant and k_{i} is a composite first-order isomerization rate constant. The general analytical theory presented in the previous section yields Eq. 14 for the single-exponential case without any other approximations. For the formation of long-lived complexes, calculation of α from its definition (Eq. 13) using Eqs. 4, 9, and 10 yields a result of the same functional form as Eq. 14, and predicts k_{a} and k_{i} :

$$\frac{1}{k_{\text{a}}} = \frac{1}{K_1k_2} + \frac{1}{K_{\text{eq}}^{\text{LL}}k_{-3}} + \frac{K_1 + K_1K_2 + K_1K_2K_3}{k_1K_{\text{eq}}^{\text{LL}}} \quad (15)$$

$$\frac{1}{k_{\text{i}}} = \frac{1}{k_2} + \frac{1}{k_{-3}(1 + K_3)} + \frac{K_1}{K_{\text{eq}}^{\text{LL}}k_{-3}}. \quad (16)$$

If one measures θ_{RP_0} instead of θ_{LL} and observes single exponential association, then modified forms of Eqs. 15 and 16 apply with $K_{\text{eq}}^{\text{LL}}$ replaced by $K_{\text{eq}}^{\text{RP}_0}$. Eqs. 15 and 16 are general (not steady state!) results, subject only to the requirement of single-exponential kinetics. If, experimentally, the kinetics are shown to be single-exponential at some $[\text{R}]_{\text{T}}$, then, by continuity, they are single-exponential over a range of $[\text{R}]_{\text{T}}$ in some neighborhood of this $[\text{R}]_{\text{T}}$, so Eqs. 14–16 must be applicable over this range, in which the slope, $1/k_{\text{a}}$, and the intercept, $1/k_{\text{i}}$, exist and, generally, can be experimentally determined.

The relaxation rate constant for irreversible dissociation of either long-lived or open complexes in the single exponential regime is determined from Eqs. 8 and 9,

$$\frac{1}{k_{\text{d}}} = \frac{1}{k_{-3}} + \frac{1 + K_3}{k_{-2}} + \frac{K_{\text{eq}}^{\text{LL}}}{K_1k_{-1}} + \frac{1}{k_{-1}}. \quad (17)$$

Where long-lived complexes are monitored, comparison of Eqs. 15 and 17 then yields

$$\frac{k_{\text{a}}}{k_{\text{d}}} = K_1K_2 + K_1K_2K_3 = K_{\text{eq}}^{\text{LL}}. \quad (18)$$

Where open complexes are monitored,

$$\frac{k_{\text{a}}}{k_{\text{d}}} = K_1K_2K_3 = K_{\text{eq}}^{\text{RP}_0}. \quad (18')$$

Eqs. 18 and 18' are not trivial results because such relationships between rate constants and an equilibrium constant are generally valid only for reversible single-step (elementary) reactions. The validity of these relationships for a sequential pseudofirst-order three-step mechanism is a result of single-exponential kinetics. This is a general result for a mechanism showing single-exponential kinetics in that it does not involve the steady-state or rapid equilibrium assumptions. One does not need to have any information about fast/slow steps in the mechanism a priori to obtain Eq. 18. The derivations of Eqs. 18 and 18' are valid regardless of the number of steps in the mechanism.

Relationships between observed and microscopic rate constants

In this section, we present five important inequalities, each of which follows solely from the single-exponential character of the kinetics. These relationships are used in subsequent parts of this study to relate observed relaxation rate constants to the microscopic rate constants of individual steps in the mechanism. (Details of the derivations are given in the Appendices C and D.) These relationships are derived for specific application to the kinetics of formation and dissociation of long-lived complexes (I_2 , RP_0) between RNA polymerase and promoter DNA. For this case, the systems of differential rate equations of Mechanisms 1 and 6 are rewritten to incorporate the observation of single-exponential kinetics.

1. Applying the single-exponential character to the kinetics in the association direction yields the following inequality (cf. Appendix C, Eq. C6):

$$\beta \equiv \lambda_3 \ll k_3 + k_{-3}. \quad (19)$$

Inequality 19 means that equilibration between I_2 and RP_0 occurs rapidly on the time scale of their accumulation. It is a relationship between the relaxation rate constant and an elementary constant in the reverse direction.

2. A relationship that is stronger than inequality 19 can be obtained using results in Appendix C and vector $\mathbf{B}_{\text{a}3}$ (Eqs. A5 and A6). The full derivation is given in Appendix D. It yields

$$\beta \ll k_{-3}. \quad (20)$$

Therefore, the accumulation of long-lived complexes is much slower than conversion from RP_0 to I_2 .

3. The derivation in Appendix C also yields

$$\begin{aligned} \left(\frac{1}{k_{\text{a}}[\text{R}]_{\text{T}}} + \frac{1}{k_{\text{i}}} \right)^{-1} &= \alpha \equiv \lambda_3 \theta_{\text{LL}}^{\text{eq}} \leq \lambda_3 \\ &\equiv \beta \ll k_1[\text{R}]_{\text{T}} + k_{-1}. \end{aligned} \quad (21)$$

These inequalities relate irreversible or reversible association relaxation rate constants α and β to the microscopic rate constants of the first step and demonstrate that both α and β are much smaller than the relaxation rate constant for equilibration of the initial binding step.

4. and 5. Two other important inequalities are derived by applying to the dissociation direction considerations analogous to those applied to the kinetics in the association direction in Sections 1 and 3. The final results (consequences of single-exponential character of dissociation kinetics) are

$$k_d = \lambda_3(\text{at } [R] = 0) \ll k_3 + k_{-3}, \quad (22)$$

indicating that dissociation is much slower than the equilibration between I_2 and RP_o , and

$$k_d = \lambda_3(\text{at } [R] = 0) \ll k_{-1}. \quad (23)$$

Therefore, dissociation of heparin-resistant complexes is much slower than the dissociation of I_1 .

Rapid equilibria and rate-limiting steps in $E\sigma^{70}$ RNA polymerase- λP_R promoter open complex formation

By considering the ranges of polymerase concentrations in which single-exponential kinetics are experimentally observed, one can draw conclusions about which steps of its mechanism must equilibrate rapidly and which steps occur irreversibly. For the case in which the kinetics of forming long-lived complexes ($I_2 + RP_o$) are single-exponential at $[R]_T \geq 0.3k_i/k_a$, one can simplify the expressions for observed association, isomerization, and dissociation rate constants (k_a , k_i , k_d) given by Eqs. 15–17. These simplified expressions are given in Table 1, and their derivation is given below.

Interpretation of isomerization rate constant k_i

When single-exponentiality is observed at RNA polymerase concentrations $[R]_T$ on the order of or greater than k_i/k_a (specifically $[R]_T \geq 0.3k_i/k_a$; or $[R]_T \geq 3$ nM for $E\sigma^{70}$ - λP_R kinetics at 7–15°C (McQuade, 1996)), the second term on the right hand side in Eq. 16 for k_i can be neglected on the

basis of Eq. 14 and Inequality 19. The third term in Eq. 16 is also negligible for the analysis of $E\sigma^{70}$ - λP_R kinetics, on the basis of the following argument. From Eq. 10 it follows that

$$\frac{K_1}{K_{eq}^{LL}} = \frac{1 - \theta_{LL}^{eq,max}}{\theta_{LL}^{eq,max}}, \quad (24)$$

where $\theta_{LL}^{eq,max}$ is the fraction of promoters in the form of long-lived complexes at infinitely large $[R]_T$. Because experimental values of $\theta_{LL}^{eq,max}$ increase monotonically from $\theta_{LL}^{eq,max} = 0.4$ at 7°C to $\theta_{LL}^{eq,max} = 1$ above 15°C (McQuade, 1996; cf. Craig et al., 1998), therefore $K_1/K_{eq}^{LL} \leq 1$ for $T > 7^\circ\text{C}$. This result and Inequality 20 allow us to neglect the third term on the right hand side of Eq. 16 in the temperature range accessible to association kinetic experiments for λP_R ($T > 7^\circ\text{C}$), yielding

$$k_i \cong k_2. \quad (25)$$

Eq. 14 and Inequality 20 then yield

$$k_2 \ll k_{-3}, \quad (26)$$

that is, conversion from I_1 to I_2 occurs much more slowly than conversion from RP_o to I_2 . Where there is appreciable isomerization of product (RP_o) from I_2 (so a three-step mechanism is required), then $k_3 \geq k_{-3}$ and, therefore, from Eq. 26,

$$k_2 \ll k_3. \quad (27)$$

For $E\sigma^{70}$ - λP_R case, Inequality 27 demonstrates that conversion of I_1 to I_2 is much slower than conversion from I_2 to RP_o at $T > 7^\circ\text{C}$.

Interpretation of observed association rate constant k_a

We rewrite the expression for k_a (Eq. 15) in the form

$$\frac{1}{k_a} = \frac{1}{K_1} \left(\frac{1}{k_2} + \frac{K_1}{K_{eq}^{LL} k_{-3}} \right) + \frac{K_1 + K_{eq}^{LL}}{k_1 K_{eq}^{LL}}. \quad (28)$$

As shown in the derivation of Eq. 25, $K_1/K_{eq}^{LL} \leq 1$ for open complex formation at the λP_R promoter at 7–37°C. Therefore, because of Inequality 26, we can neglect the second

TABLE 1 Relationships between relaxation and microscopic rate constants for different types of mechanisms and their analyses

Mechanism	Analysis	k_a	k_i	k_d
$R + P \xrightleftharpoons[k_{-1}]{k_1} I_1 \xrightleftharpoons[k_{-2}]{k_2} I_2 \xrightleftharpoons[k_{-3}]{k_3} RP_o$	Single-exponential over an arbitrary range of $[R]_T$	Eq. 15	Eq. 16	Eq. 17
	Single-exponential at $[R]_T \geq 0.3k_i/k_a$	$K_1 k_2$	k_2	$k_{-2}/(1 + K_3)$
	Rapid Equilibria/Steady-State Approximations (Roe et al., 1985)	$K_1 k_2$	$(k_2^{-1} + k_3^{-1})^{-1}$	k_{-2}/K_3
	Rapid Equilibria, Two-Step Approximations (Buc and McClure, 1985)	$K_1 k_2$	k_2	$k_{-2}/(1 + K_3)$
$R + P \xrightleftharpoons[k_r]{K_B} I \xrightleftharpoons[k_r]{K_B} RP_o$	Rapid Equilibrium (McClure, 1980)	$K_B k_f$	k_f	k_r

term in the parenthesis in Eq. 28. We simplify Eq. 28 further by using, again, the single-exponentiality at $[R]_T$ on the order of or greater than k_i/k_a . After substituting k_i/k_a for $[R]_T$ in Inequality 21 and using the fact that $k_1 k_i/k_a$ is on the order of $k_{-1} + k_2$, which is a consequence of Eqs. 25 and 28, neglecting the second term in parenthesis ($K_1/(K_{eq} k_3) \ll 1/k_2$), we obtain

$$k_2 \ll k_{-1}. \quad (29)$$

Inequality 29 remains valid at temperatures below 7°C based on the extrapolation of the values of k_2 and k_{-1} reported by McQuade (1996). For $E\sigma^{70}-\lambda P_R$ case, Inequality 29 shows that conversion of I_1 to I_2 is much slower than dissociation of I_1 to free P and R. This allows us to simplify further Eq. 28, in which the first term is proved now to be much greater than the other two, and we obtain

$$k_a = K_1 k_2. \quad (30)$$

This expression is valid at all temperatures at which association kinetic experiments have been performed for λP_R .

Interpretation of observed dissociation rate constant k_d

To simplify the general equation for k_d (Eq. 17) and obtain a result applicable to $E\sigma^{70}-\lambda P_R$ promoter kinetic data, we rewrite Eq. 17 as

$$\frac{1}{k_d} = \frac{1}{k_{-3}} + \frac{1 + K_3}{k_{-2}} + \frac{1}{k_{-1}}(1 + K_2 + K_2 K_3). \quad (31)$$

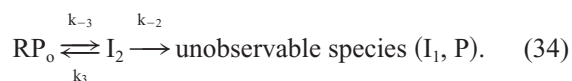
For RP_o complexes at λP_R promoter, Craig et al. (1998) reported $K_3 = 0.3$ at 0°C. This result together with Inequality 22 (derived based only on the observation of single-exponential dissociation kinetics) yield $k_d \ll k_{-3}$ at 0°C. Because k_{-3} increases with increasing temperature (it is postulated to behave like an elementary rate constant) and k_d decreases with increasing temperature (Roe et al., 1985; Roe and Record, 1985; McQuade, 1996), we can neglect the first term in Eq. 31 above 0°C. In addition, $1/k_{-1}$ is much smaller than $1/k_d$, as follows from Inequality 23. These two simplifications yield

$$\frac{1}{k_d} = \left(1 + \frac{k_2}{k_{-1}}\right) \left(\frac{1 + K_3}{k_{-2}}\right). \quad (32)$$

For $E\sigma^{70}-\lambda P_R$ case, Inequality 29 (proved at $T > 7^\circ\text{C}$ and extended to lower temperatures (cf. derivation of Inequality 29 above) then yields

$$\frac{1}{k_d} = \frac{1 + K_3}{k_{-2}}. \quad (33)$$

To examine possible rapid equilibria in the dissociation direction, we rewrite Mechanism 6 using rapid equilibrium 29,



The treatment of this mechanism is given in Appendix E. Comparison of Eq. E5 and Eq. 33 yields

$$\frac{k_{-2}}{k_{-3}} \ll K_3 + 1. \quad (35)$$

As noted above, the equilibrium constant of the third step, K_3 , is on the order of unity or greater above 0°C. Therefore, to a good approximation, we can drop the unity in Inequality 35, which yields

$$k_{-2} \ll k_3. \quad (36)$$

For $E\sigma^{70}-\lambda P_R$ case, Inequality 36 means that conversion of I_2 to I_1 is much slower than conversion from I_2 to RP_o at all temperatures.

DISCUSSION

Analytical solution to the reversible three-step mechanism under pseudofirst-order binding conditions

The novel kinetic analysis reported here should provide a general approach to the quantitative treatment of association and dissociation kinetic data for RNA polymerase–promoter DNA long-lived or open complexes, if the association and/or dissociation kinetics are single-exponential and conditions are pseudofirst order. Open complex formation, the key initial process in transcription initiation, involves at least two kinetically significant intermediates and three mechanistic steps for the *lacUV5* and λP_R promoters, the only ones for which detailed quantitative kinetic data over a wide temperature range are available. The multistep nature of the mechanism of open complex formation poses a problem in the analysis of data, even of experiments performed under pseudofirst-order conditions in a large excess of polymerase over promoter DNA. Although the exact solution of the system of differential equations of kinetics can be obtained for a pseudofirst-order mechanism of any number of steps without any simplifying assumptions, its use for data analysis is limited. The number of observed parameters is often smaller than the number of unknown rate constants, such that direct fitting does not give a unique result. Furthermore, it does not provide any insight as to what the relative rates of the steps in the mechanism are. The question one tries to answer in this case is what information about the mechanism still can be extracted and what fitted parameters are correlated. To do this, one commonly makes some simplifying assumptions. Rapid equilibrium and/or steady-state approximations are examples. Efforts were made to justify the rapid equilibrium approximation for polymerase–promoter kinetics experimentally (Roe et al., 1984, 1985; McClure, 1980; Buc and McClure, 1985) and this approximation has been used with success in the kinetic studies of the mechanism of RNA polymerase–promoter open complex formation. A potentially more serious approximation is to reduce the number of steps in the mech-

anism. This may allow one to solve the problem without making steady-state or other approximations, but rate or equilibrium constants obtained in this way are composite functions of the parameters of the original larger mechanism and not readily interpretable. (A similar situation exists in the use of the over-simplified two-step mechanism of enzyme kinetics.) It is very important to preserve the number of kinetically significant intermediates in the analysis.

This study shows that the single-exponential character of the kinetics of association and dissociation provides a way to find the observed rate constants as a function of microscopic rate and equilibrium constants for any unbranched pseudofirst-order mechanism without using steady-state approximations or reducing the number of mechanistic steps below the minimum required by the data. The idea of this approach lies in the fact that the observed rate constant is much smaller in magnitude than the magnitudes of the other relaxation rate constants λ_i for a sequential (unbranched) mechanism. The mathematical side of this statement is detailed in Appendix B. This approach *derives* the conditions (such as rapid equilibrium) that are necessary for the mechanism to exhibit the observed single-exponential character. This study shows that rapid equilibria in the first step in the forward direction and in the last step in the reverse direction guarantee the single-exponential behavior in the three-step mechanism of $E\sigma^{70}$ RNA polymerase- λP_R promoter DNA open complex formation. Another important consequence of the single-exponential character is the derivation without approximations of the expression $K_{eq} = k_a/k_d$ (Eq. 18). The essential features of this analysis remain the same for any unbranched pseudofirst mechanism of any number of steps. We expect that the minimal three-step mechanism of open complex formation will be general, independent of promoter sequence, and, therefore, that this approach will be generally useful in analysis of kinetic and thermodynamic data for other promoters. One must note, though, that rapid equilibrium/rate limiting steps can be distributed differently for different polymerases and/or promoters. In this sense, the kinetic and/or thermodynamic significance of different intermediates may vary.

Comparison with approximate solutions to the three-step mechanism and the approximate two-step mechanism of RNA polymerase-promoter open complex formation

Table 1 compares the expressions for the composite rate constants, k_a , k_i , and k_d , derived in this work with those used previously in the studies of the kinetics of transcription initiation.

Roe et al. (1985) analyzed association and dissociation kinetics of long-lived polymerase-promoter complexes. They used an approach based on approximations of rapid equilibria in the first step in the association direction and in the third step in the dissociation direction that are rigorously proved to be valid in the present study. These rapid equi-

librium approximations were tested using salt effect on k_a (originating in K_1) and negative activation energy (E_{act}) of k_d (originating in K_3). The steady-state assumption on I_2 in the association direction and on I_1 in the dissociation direction are less satisfactory. Table 1 shows that the expression of Roe et al. (1985) for k_a is correct but that their steady-state result for k_i is only accurate if $k_2 \ll k_3$. The latter inequality is valid at λP_R at $T > 15^\circ\text{C}$ (Inequality 27). The expression of Roe et al. for k_d is correct in the temperature range of their experiments ($T > 10^\circ\text{C}$), where $K_3 \gg 1$. Tsodikov et al. (1998) demonstrated $K_3 \gg 1$ at 37°C for λP_R but $K_3 = 0.3$ at 0°C (Craig et al., 1998) and, therefore, the assumption that I_2 does not accumulate is no longer accurate at temperatures below 10°C .

Buc and McClure (1985) used the abortive initiation assay to monitor open complexes (RP_o). They analyzed the data by using the general solution of the equations of kinetics applied to individual two-step mechanisms of association and dissociation obtained from the minimal three-step mechanism by assuming rapid equilibrium of the first step and irreversibility of the second step in each direction. These assumptions regarding rapid equilibria and slow steps, which we deduce in the present analysis from the observation of single exponential kinetics at $[R]_T \geq 0.3k_i/k_a$, yield correct expressions for k_a , k_i , and k_d in terms of elementary rate and equilibrium constants (Table 1). However, Buc and McClure (1985) reported values of k_a and k_i calculated using analysis of β (the relaxation rate constant) and not α ; because open complex formation is reversible even in the presence of nucleoside triphosphates at the *lacUV5* promoter under at least some of conditions investigated, $\beta \neq \alpha$ and systematic errors in K_1 and k_2 may have been introduced (Eq. 14; see also Schlax et al., 1995).

Despite the complexity of the three-step mechanism and difficulty of determining the six rate constants of its steps and their dependences on temperature, [salt], and other solution variables, it is a *minimal* kinetic mechanism of open complex formation and both intermediates are kinetically significant. One inevitably introduces the possibility for misinterpretation when simplifying a three-step mechanism to a two-step mechanism. For example, the second and third steps of open complex formation are often collapsed into one step. Because the rate-limiting step in the association direction is usually the second (and the last!) step in this two-step mechanism with the open complex RP_o as the final product, one draws an apparent, but unjustified, conclusion for this mechanism that the DNA opening step is rate limiting. However, in the case of the λP_R promoter, such a conclusion would be erroneous because the interconversion of the two intermediates is rate limiting in both directions at this promoter under the conditions of interest (Craig et al., 1998; Tsodikov et al., 1998).

In conclusion, for the situation in which an unbranched pseudofirst-order multistep process exhibits single exponential kinetics, we derive algebraic expressions for composite association, isomerization, and dissociation rate constants (k_a , k_i , and k_d) in terms of elementary rate constants and

locate rapid equilibrium and rate-limiting steps. This generality is important in analyzing not only the kinetics of open complex formation in transcription initiation, but also in analysis of other unbranched enzymatic mechanisms exhibiting similar experimental behavior. This approach should be especially valuable in cases in which no information exists regarding the validity of the rapid equilibrium or steady-state assumptions for the intermediates, rapid equilibria, or regarding rate-limiting steps.

APPENDIX A

Association

In excess of RNA polymerase ($[R]_T \gg [P]_T$), the concentrations of P, I_1 , I_2 , RP_o as functions of time in Mechanism 1 are the solutions of the following system of differential equations

$$\frac{d[P]}{dt} = -k_1[R]_T[P] + k_{-1}[I_1], \quad (A1)$$

$$\frac{d[I_1]}{dt} = k_1[R]_T[P] - (k_{-1} + k_2)[I_1] + k_{-2}[I_2],$$

$$\frac{d[I_2]}{dt} = k_2[I_1] - (k_{-2} + k_3)[I_2] + k_{-3}[RP_o],$$

$$\frac{d[RP_o]}{dt} = k_3[I_2] - k_{-3}[RP_o].$$

This system can be rewritten in the matrix form

$$\frac{d\mathbf{C}_a}{dt} = \hat{\mathbf{A}}_a \mathbf{C}_a, \quad (A2)$$

where

$$\mathbf{C}_a = \begin{pmatrix} [P] \\ [I_1] \\ [I_2] \\ [RP_o] \end{pmatrix} \quad (A3)$$

and

$$\hat{\mathbf{A}}_a = \begin{pmatrix} -k_1[R]_T & k_{-1} & 0 & 0 \\ k_1[R]_T & -(k_{-1} + k_2) & k_{-2} & 0 \\ 0 & k_2 & -(k_{-2} + k_3) & k_{-3} \\ 0 & 0 & k_3 & -k_{-3} \end{pmatrix}$$

(The subscript “a” denotes (reversible) association starting with R and P.)

The theory of ordinary differential equations allows one to find a general solution of this system by first finding the roots λ_i of the secular equation

$$\det(\hat{\mathbf{A}}_a + \lambda_i \hat{\mathbf{I}}) = 0, \quad (A4)$$

where $\hat{\mathbf{I}}$ is the identity matrix. In the case of four distinct λ_i ($\lambda_1 \neq \lambda_2 \neq \lambda_3 \neq \lambda_4$; their equality, which is usually defined to a very high computational precision, would be a very unlikely coincidence) one solves 4 linear algebraic systems

$$(\hat{\mathbf{A}}_a + \lambda_i \hat{\mathbf{I}}) \mathbf{B}_{ai} = 0, \quad i = 1, \dots, 4 \quad (A5)$$

for the characteristic vectors \mathbf{B}_{ai} . Then, the solution of Eq. A2 is

$$\mathbf{C}_a = \sum_{i=1}^4 M_{ai} \mathbf{B}_{ai} e^{-\lambda_i t}, \quad (A6)$$

where M_{ai} are arbitrary constants. M_{ai} can be obtained from the initial conditions, which, in the association direction, often are

$$\mathbf{C}_a(0) = \begin{pmatrix} [P]_T \\ 0 \\ 0 \\ 0 \end{pmatrix},$$

which yields a system of linear algebraic equations

$$\sum_{i=1}^4 M_{ai} \mathbf{B}_{ai} = \begin{pmatrix} [P]_T \\ 0 \\ 0 \\ 0 \end{pmatrix}. \quad (A7)$$

By solving Eq. A4 we obtain

$$\begin{aligned} & (k_1 - \lambda_i)(k_{-1} + k_2 + \lambda_i)(k_{-2} + k_3 + \lambda_i)(k_{-3} + \lambda_i) \\ & - (k_1 + \lambda_i)(k_{-1} + k_2 + \lambda_i)k_3k_{-3} - (k_1 + \lambda_i)(k_{-3} + \lambda_i)k_2k_{-2} \\ & - k_1k_{-1}(k_{-2} + k_3 + \lambda_i)(k_{-3} + \lambda_i) + k_1k_{-1}k_3k_{-3} = 0. \end{aligned} \quad (A8)$$

After simplification, Eq. A8 yields Eq. 3 of the text. Without approximation, the coefficients D_{ai} in Eq. 3 are related to the microscopic rate constants and the (excess) concentration of RNA polymerase by Eqs. 4 in the text.

Dissociation

For the mechanism of Eq. 6, the system of differential equation of kinetics is

$$\frac{d\mathbf{C}_d}{dt} = \hat{\mathbf{A}}_d \mathbf{C}_d \quad (A9)$$

$$\mathbf{C}_d = \begin{pmatrix} [I_1] \\ [I_2] \\ [RP_o] \end{pmatrix} \quad (A10)$$

$$\hat{\mathbf{A}}_d = \begin{pmatrix} -(k_{-1} + k_2) & k_{-2} & 0 \\ k_2 & -(k_{-2} + k_3) & k_{-3} \\ 0 & k_3 & -k_{-3} \end{pmatrix},$$

where the subscript “d” denotes (irreversible) dissociation starting from the equilibrium mixture of I_2 and RP_o . By analogy to the association case,

$$\det(\hat{\mathbf{A}}_d + \lambda_i \hat{\mathbf{I}}) = 0. \quad (A11)$$

In the general case of three distinct characteristic roots,

$$(\hat{\mathbf{A}}_d + \lambda_i \hat{\mathbf{I}}) \mathbf{B}_{di} = 0. \quad (A12)$$

The solution is given again by the linear combination of the corresponding exponential terms,

$$\mathbf{C}_d = \sum_{i=1}^3 M_{di} \mathbf{B}_{di} e^{-\lambda_i t}. \quad (A13)$$

The vector of initial concentrations is found from the initial conditions,

$$\mathbf{C}_d(0) = \begin{pmatrix} [I_1]_0 \\ [I_2]_0 \\ [RP_o]_0 \end{pmatrix} = \sum_{i=1}^3 \mathbf{M}_{di} \mathbf{B}_{di}, \quad (\text{A14})$$

where $[I_1]_0$, $[I_2]_0$, and $[RP_o]_0$ are related by the equilibrium between these complexes that is established prior to the addition of heparin:

$$[I_2]_0/[I_1]_0 = K_2 \quad \text{and} \quad [RP_o]_0/[I_2]_0 = K_3. \quad (\text{A15})$$

Solution of secular Eq. A11 in the dissociation direction yields Eq. 5 in the text, where D_i are defined by Eq. 8.

APPENDIX B

In the approach of the reversible sequential three-step mechanism Eq. 1 to equilibrium in the association direction, the complexes I_2 and RP_o reach equilibrium last. The observed relaxation rate constant should therefore correspond to the smallest (in magnitude) solution of Eq. 5. The magnitudes of the other two nonzero λ_i values should be much larger than the observed rate because the kinetics are single-exponential, i.e., there is no detectable lag phase within experimental uncertainty. Then, the smallest characteristic root of Eq. 5 is determined as follows.

Consider the cubic equation,

$$\lambda^3 - D_1\lambda^2 + D_2\lambda - D_3 = 0, \quad D_{1,2,3} > 0, \quad (\text{B1})$$

and let the roots $\lambda_1, \lambda_2, \lambda_3$ be such that

$$\lambda_3 \ll \lambda_1, \lambda_2. \quad (\text{B2})$$

Eq. B1 can be rewritten as

$$(\lambda - \lambda_1)(\lambda - \lambda_2)(\lambda - \lambda_3) = 0. \quad (\text{B3})$$

Eqs. B3 and B1 yield

$$\begin{aligned} D_1 &= \lambda_1 + \lambda_2 + \lambda_3; & D_2 &= \lambda_1\lambda_2 + \lambda_2\lambda_3 + \lambda_1\lambda_3; \\ D_3 &= \lambda_1\lambda_2\lambda_3. \end{aligned} \quad (\text{B4})$$

Conditions B2 then yield

$$D_1 \approx \lambda_1 + \lambda_2; \quad D_2 \approx \lambda_1\lambda_2; \quad D_3 = \lambda_1\lambda_2\lambda_3. \quad (\text{B5})$$

After solving B5 for $\lambda_1, \lambda_2, \lambda_3$ we obtain

$$\lambda_3 \approx D_3/D_2 \quad (\text{B6})$$

$$\lambda_{1,2} = \frac{D_1 \pm \sqrt{D_1^2 - 4D_2}}{2}. \quad (\text{B7})$$

The $\lambda_{1,2}$ can be obtained explicitly for a four-step mechanism as well (as solutions of a cubic equation), but their determination for a larger mechanism requires additional approximations. Eq. B6 for λ_3 has the same form for a mechanism of any number of steps.

APPENDIX C

To derive Inequalities 19 and 21, we explicitly incorporate the single-exponential accumulation of the observable (long-lived) products I_2 and RP_o (whose total concentration is designated by $[RP_{LL}]$) into the initial system of differential Eqs. A1 of association kinetics. Integration of the single-exponential rate law yields

$$[RP_{LL}] = \theta_{LL}^{\text{eq}}[P]_T(1 - e^{-\lambda_3 t}). \quad (\text{C1})$$

Substitution of $[I_2] = [RP_{LL}] - [RP_o]$ from Eq. C1 into the last equation in system A1 yields

$$\frac{d[RP_o]}{dt} = k_3(\theta_{LL}^{\text{eq}}[P]_T(1 - e^{-\lambda_3 t}) - [RP_o]) - k_{-3}[RP_o]. \quad (\text{C2})$$

The solution of Eq. C2 at the initial condition $[RP_o] = 0$ at $t = 0$ is

$$[RP_o] = A_1 + A_2 e^{-(k_3 + k_{-3})t} + A_3 e^{-\lambda_3 t}, \quad (\text{C3})$$

where

$$\begin{aligned} A_1 &= \frac{k_3 \theta_{LL}^{\text{eq}}[P]_T}{k_3 + k_{-3}}; \\ A_2 &= -(A_1 + A_3); \\ A_3 &= \frac{k_3 \theta_{LL}^{\text{eq}}[P]_T}{\lambda_3 - (k_3 + k_{-3})}. \end{aligned} \quad (\text{C4})$$

The concentration of I_2 as a function of time can be obtained after substituting $[RP_o]$ from Eq. C3 into Eq. C1,

$$\begin{aligned} [I_2] &= \theta_{LL}^{\text{eq}}[P]_T - A_1 - A_2 e^{-(k_3 + k_{-3})t} \\ &\quad - (A_3 + \theta_{LL}^{\text{eq}}[P]_T) e^{-\lambda_3 t}. \end{aligned} \quad (\text{C5})$$

Therefore, the concentrations of RP_o and I_2 are a sum of two exponential terms and a constant (Eqs. C3 and C5). One rate constant is λ_3 and the other is equal to $k_3 + k_{-3}$ (Eq. C3). (Note that the third exponent present in the general solution does not appear in Eqs. C3 and C5 as a result of applying the observed single-exponential rate law (Eq. C1) at the starting point of the derivation. Mathematically, this is a consequence of the fact that the preexponential factor of this exponent is negligibly small.) From the single-exponential character of kinetics we know that λ_3 is much smaller than any other rate constant in the mechanism, which yields

$$\beta \equiv \lambda_3 \ll k_3 + k_{-3}. \quad (\text{C6})$$

To derive Inequality 21, we use the fact that the rate of conversion to RP_{LL} is equal in magnitude to the rate of decay of short-lived species P and I_1) and has the opposite sign. Then, by analogy to the above approach, we obtain

$$\frac{d[P]}{dt} = -(k_1[R]_T + k_{-1})[P] - k_{-1}[I_1] - \lambda_3 \theta_{LL}^{\text{eq}}[P]_T e^{-\lambda_3 t}. \quad (\text{C7})$$

Integration of Eq. C7 with the initial condition $[P] = [P]_0$ extrapolated to $t = 0$ ($[P]_0$ generally can differ from $[P]_T$ because of very fast accumulation of I_1), yields $[P]$ as a function of time,

$$[P] = H_1 + H_2 \exp[-(k_1[R]_T + k_{-1})t] + H_3 \exp(-\lambda_3 t), \quad (\text{C8})$$

where

$$\begin{aligned} H_1 &= \frac{k_{-1} \theta_{LL}^{\text{eq}}[P]_T + [P]_T}{k_1[R]_T + k_{-1}}; & H_2 &= [P]_0 - H_1 - H_3; \\ H_3 &= \frac{k_{-1} \theta_{LL}^{\text{eq}}[P]_T}{-\lambda_3 + k_1[R]_T + k_{-1}}. \end{aligned} \quad (\text{C9})$$

Promoter concentration ($[P]$) is also a sum of two exponents, one with the rate constant of λ_3 and the other with $k_1[R]_T + k_{-1}$ (Eq. C8). By single-exponential character of kinetics, we know that λ_3 is much smaller than any other rate constant in the mechanism, and, therefore, that the first step in

the association direction equilibrates rapidly with respect to the formation of the observed complexes. From Eq. 14 and the fact that the occupancy θ_{LL}^{eq} is smaller or equal than one, we obtain

$$\left(\frac{1}{k_a[R]_T} + \frac{1}{k_i} \right)^{-1} = \alpha \equiv \lambda_3 \theta_{LL}^{eq} \leq \lambda_3 \equiv \beta \ll k_1[R]_T + k_{-1}. \quad (C10)$$

APPENDIX D

Vector \mathbf{B}_{a3} is obtained from Eq. A5,

$$\mathbf{B}_{a3} = \begin{pmatrix} 1 \\ \frac{k_1[R]_T - \lambda_3}{k_{-1}} \\ \frac{1}{k_{-2}} \left[\left(\frac{k_1[R]_T - \lambda_3}{k_{-1}} \right) (k_{-1} + k_2 - \lambda_3) - k_1[R]_T \right] \\ \frac{k_3}{(k_{-3} - \lambda_3)k_{-2}} \left[\left(\frac{k_1[R]_T - \lambda_3}{k_{-1}} \right) (k_{-1} + k_2 - \lambda_3) - k_1[R]_T \right] \end{pmatrix}. \quad (D1)$$

The ratio of the fourth and third components of this vector is equal to $k_3/(k_{-3} - \lambda_3)$. In contrast, it is equal to the ratio of the corresponding preexponential coefficients A_3 and $-(A_3 + \theta_{LL}^{eq}[P]_T)$ from Eqs. C3 and C5, respectively, which, after substituting the definition of A_3 in Eq. C4 and using Inequality 22 yields

$$\frac{A_3}{-(A_3 + \theta_{LL}^{eq}[P]_T)} = K_3. \quad (D2)$$

The approximate equality $k_3/(k_{-3} - \lambda_3) \approx K_3$ yields Inequality 20 directly.

APPENDIX E

The system of differential equations of kinetics for Mechanism 33 is

$$\begin{aligned} \frac{d[I_2]}{dt} &= -(k_{-2} + k_3)[I_2] + k_{-3}[RP_o] \\ \frac{d[RP_o]}{dt} &= k_3[I_2] - k_{-3}[RP_o], \end{aligned} \quad (E1)$$

or, in the matrix form,

$$\frac{d\mathbf{C}}{dt} = \hat{\mathbf{A}}\mathbf{C}, \quad \text{where} \quad \mathbf{C} = \begin{pmatrix} [I_2] \\ [RP_o] \end{pmatrix} \quad (E2)$$

and

$$\hat{\mathbf{A}} = \begin{pmatrix} -(k_{-2} + k_3) & k_{-3} \\ k_3 & -k_{-3} \end{pmatrix}. \quad (E3)$$

The secular Eq. A4 for matrix $\hat{\mathbf{A}}$ is then

$$\lambda^2 - \lambda(k_{-2} + k_3 + k_{-3}) + k_{-2}k_{-3} = 0. \quad (E4)$$

By following the method described in Appendix B, we obtain the magnitude of the smaller λ , which is the observed dissociation rate constant

$$k_d = \frac{k_{-2}k_{-3}}{k_{-2} + k_3 + k_{-3}}. \quad (E5)$$

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