2005

Mechanisms of Telomerase Binding to Telomeres

George Czerlinski
Western Washington University

Tjalling Ypma
Western Washington University, tjalling.ypma@wwu.edu

Follow this and additional works at: https://cedar.wwu.edu/math_facpubs
Part of the Physical Sciences and Mathematics Commons

Recommended Citation
https://cedar.wwu.edu/math_facpubs/96

This Article is brought to you for free and open access by the College of Science and Engineering at Western CEDAR. It has been accepted for inclusion in Mathematics by an authorized administrator of Western CEDAR. For more information, please contact westerncedar@wwu.edu.
Mechanisms of Telomerase Binding to Telomeres

George Czerlinski\(^1\)* and Tjalling Ypma\(^2\)

\(^1\)Department of Biology
\(^2\)Department of Mathematics
Western Washington University
Bellingham, WA 98225

*Corresponding author; E-mail: george@submicron.com

Abstract: There are essentially two alternative mechanisms for the binding of telomerase to telomeres, assuming that a protective component is initially bound to the telomerase binding region on the telomeres. Either the protective (or blocking) agent first dissociates and telomerase binds thereafter, or telomerase binds first and the protective agent then dissociates from the ternary complex. In the limit, this second possibility permits the ternary complex to become a transition complex (creating another possible mechanism). Numerical simulation of both rapid mixing and chemical relaxation is used to study these alternatives. We aim to determine how the mechanisms may be distinguished experimentally and identify an appropriate experimental design. We show that rapid mixing experiments are better than chemical relaxation experiments, since the latter are more affected by the statistics of single molecule kinetics. However, hidden fast steps can only be revealed by chemical relaxation. The detection of mechanistic changes hinges on linking fluorescence reporters to the reaction components, either directly (chemically) or indirectly (via an indicator reaction). Fluorescence is excited by two-photon absorption in a small reaction volume. Various detection strategies and design issues are examined, including limitations imposed by diffusion. Constant rather than stopped flow is shown to be preferable.

KEY WORDS: telomerase, telomeres, blocking-agent, rapid mixing, chemical relaxation, two-photon absorption

Telomerase, a reverse transcriptase, was discovered by Greider and Blackburn (1985). They obtained the enzyme from tetrahymena. Morin (1989) used the same method to confirm the presence of telomerase in certain human cells. In humans telomerase is only active in germ cells, stem cells and proliferating tumor cells (Blackburn, 1991). Ideas about the functioning of the enzyme were first described by Greider and Blackburn (1989). Exactly how the initial telomere-telomerase complex is formed remains unclear.
(Baumann and Cech, 2001). There is some indication that the molecular weight of fully active human telomerase is very high (1000 kDa or more, Schnapp et al, 1998). A recent review on telomerase was given by Thomas Cech (2004).

In the binding of telomerase to the (3’-) ends of chromosomes one may distinguish the three mechanistic alternatives introduced in Figure 1. All involve a protective agent (component 3 in Figure 1) which may be a compound like Pot1 (Baumann and Cech, 2001). In Model A the protective agent first dissociates and telomerase (component 2) binds thereafter. In Model B telomerase binds first and the protective agent then dissociates from the ternary complex. A limiting case is considered in Model C, where the intermediate complex becomes a transition complex. These last two cases are shown with poten-

---

**FIGURE 1.** Two basic mechanisms of telomerase binding and a third limiting case are shown. The meaning of the circled numbers depends upon the mechanism/model as follows:

For all three models: 1 = telomere-blocker complex, 2 = free telomerase, 3 = free blocker, 4 = telomere-telomerase complex. Component 5 is free telomere-ends for model A, but telomerase-telomere-blocker complex for model B, and absent from model C. For all models component 6 is free nucleotide with fluorophor attached and component 7 is its bound form (see text for more details).
tial energy profiles in Figure 2 which also shows an intermediate case with the short-lived complex in low concentration. We aim to determine how these mechanisms may be distinguished experimentally. The third step in these models (involving component 6) represents an indicator for the active telomere-telomerase complex.

It is difficult to study the mechanism of a reaction sequence which is highly localized on the cell nucleus. Even if we could move the chromosome ends into a very small space, we would not be able to use conventional mixing techniques. Fortunately, very small volumes can be observed with two-photon absorption under a microscope. One reactant remains fixed in space and the other reactant flows over it. The reaction system may be simplified by attaching chromosome ends to the object glass under the microscope and letting the telomerase solution flow over the attached reactant (with blocker bound). In principle we may use stopped flow or constant flow. This is the basic experimental setup.
which we discuss in the method section to the extent needed for the kinetic modeling. To investigate any possible faster steps in the reaction sequence (such as the binding of component 6), we introduce chemical relaxation by temperature jump.

Our goal is to find the best experimental conditions for distinguishing between the mechanisms. To accomplish this goal we simulate the kinetics under a variety of conditions. The kinetic concentration profiles show us which components should be connected to suitable fluorophores for their actual detection.

Wenz et al (2001) conducted experiments which point to human telomerase acting as dimers. We will consider models with telomerase dimers in a later paper.

Methods

Basic Experimental Design

The basic design consists of a fluorescence microscope with a dichroic mirror to deflect the pulsed laser light onto the object carrier focused to the diffraction limit, resulting in two-photon absorption from a volume of less than 1 $\mu$m$^3$ (Denk et al, 1991). The dichroic mirror is designed to transmit the fluorescence emitted from the fluorophors within the absorption volume, which contains the target polydeoxynucleotides either on the microscope objective or as telomere ends on a cell nucleus. The quartz object holder is temperature controlled such that switching between two fluids of different temperature can be quickly accomplished to produce the desired temperature jump to observe chemical relaxation. Pulses of infrared (laser) light may have to be used to raise the temperature quickly.

Definition of Reaction Parameters

Figure 1 details the three alternative binding models and identifies the components and rate constants. Rate constants $k_1$, $k_3$ and $k_5$ are always bimolecular, $k_2$, $k_4$ and $k_6$ are monomolecular in models A and B, while $k_2$ is bimolecular and $k_6$ is monomolecular in model C. For all three models, components 1, 4, 5 and 7 refer to the various complexes of telomere ends containing the telomerase binding site; these are fixed in space (in situ).

Concentrations are defined by lower case “c” with the component number (i) as subscript. Without superscript, $c_i$ refers to the concentration as a function of time; superscript 0 (that is $c_i^0$) denotes initial concentrations; and superscript T (that is $c_i^T$) identifies total concentrations of any basic structure (time-independent). Often $c_i^T = c_i^0$.

Chemical Kinetics

All models begin with telomere ends saturated with blocker. This is accomplished in a pre-equilibrium with $c_5^0 \gg K_{2,1} = k_2/k_1$. The details are shown in Table I. The actual cylindrical volume of two-photon absorption is close to 0.5 $\mu$m$^3$ and the excitation depth close to 1 $\mu$m leading to the radius given. The value for the bimolecular rate constant $k_1$ derives from the consideration that it may be diffusion limited and require rather precise orientation, as in the binding of NADH to liver alcohol dehydrogenase (Czerlinski, 1993). The value of $k_2$ then follows from $K_{2,1} = k_2/ k_1$ with an equilibrium constant for relatively tight binding.
We simulated the chemical kinetics of each of the models A, B and C, for both constant and stopped flow, denoting the cases by Ac, Bc, Cc for constant flow and As, Bs, Cs for stopped flow respectively. We conducted experiments with a variety of different values of \( c_3^0 \) listed in Tables II through IV, that is, different initial concentrations of blocker; the respective cases are referred to as As-1, As-2, As-3 etc. for the different initial values. For Model A we further tried two alternative values of \( k_2 \) (and thus \( K_{2,1} \)), while for Model B we used two alternative values of \( k_4 \) (and thus \( K_{4,3} \)).

The values for the equilibrium constants in Tables II to IV were selected relative to \( K_{2,1} \) in Table I as follows: \( K_{4,3} \) (Table II) = 10 \( K_{2,1} \) (Table I), with an alternative value for \( K_{2,1} \) (Table II) = 10 \( K_{4,3} \) (Table II) to cover a wide range of possibilities. These equilibrium values are reversed in Tables III and IV. The value for \( K_{6,5} \) (identical in all Tables) is consistent with currently available literature; the value for \( k_5 \) was chosen somewhat arbitrarily, but should be in the right range (Stryer, 1988). Values for the bimolecular rate constants in these tables were chosen such that \( k_1 \) (Table II) = \( k_1 \) (Table I), and \( k_3 \) (Table II) = 10 \( k_1 \) (Table I). These values are reversed in Tables III and IV. Values for the monomolecular rate constants in Tables II and III follow from the values for the equilibrium constants and bimolecular rate constants.

The applicable differential equations and the mass conservation equations depend on the individual model and whether we use constant or stopped flow. For each of the models A, B and C, and for both constant and stopped flow, several differential equations and conservation equations hold; we selected and list only enough of these relationships to provide the unique, well-defined solution for each case. A complete set of differential equations can be derived by direct reference to Figure 1, but some of those equations will be redundant.

**TABLE I. Parameter values for pre-equilibration**

<table>
<thead>
<tr>
<th>PARAMETER, UNITS</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avogadro’s Number, molecules/mole</td>
<td>6.022 x 10^{23}</td>
</tr>
<tr>
<td>Radius of 2-photon abs. cylin., µm</td>
<td>0.4</td>
</tr>
<tr>
<td>Depth of 2-photon abs. cylin., µm</td>
<td>1.00</td>
</tr>
<tr>
<td>Volume at chromosome ends, µm³</td>
<td>0.500</td>
</tr>
<tr>
<td># of DNA-ends in volume, molecules</td>
<td>50</td>
</tr>
<tr>
<td>( c_1^0 ), µM</td>
<td>0</td>
</tr>
<tr>
<td>( c_3^0 ), µM</td>
<td>5.0</td>
</tr>
<tr>
<td>( c_5^0 ), µM</td>
<td>0.166</td>
</tr>
<tr>
<td>( K_{2,1} ), µM</td>
<td>0.01</td>
</tr>
<tr>
<td>( k_1 ), µM⁻¹ s⁻¹</td>
<td>0.1</td>
</tr>
<tr>
<td>( k_2 ), s⁻¹</td>
<td>0.001</td>
</tr>
<tr>
<td>( c_{one} ), µM</td>
<td>0.0033</td>
</tr>
</tbody>
</table>

(concentration of one molecule in microvolume)
Constant Flow

For constant flow, in which the concentrations of components 2, 3 and 6 are held constant, the relevant constants, initial conditions (including alternative values of $c_3^0$), differential equations and conservation equations are displayed in Tables II through IV corresponding to models A through C respectively. These are open systems with only one conservation equation (for the components immobilized on the cell nucleus or by attaching them to the objective). Since the concentrations of components 2, 3 and 6 are held constant, the system of differential equations is linear in the remaining variables, greatly facilitating the computation.

Stopped Flow

For stopped flow $c_2$, $c_3$ and $c_6$ are no longer held constant, requiring the introduction of additional equations to supplement those listed in Tables II to IV. These are closed systems governed by conservation equations; the conditions $c_i = c_i^0$ (for $i = 2, 3, 6$ and all $t$) of Tables II through IV are replaced by the relationships listed in Tables V through VII respectively. Again, two alternate values are used for the dissociation constant of component 3. To what extent stopped flow can actually be implemented will be discussed later.

In each case the differential equations form a stiff system which was solved numerically by the Matlab (Mathworks, 2004) routine ode23s (Shampine and Reichelt, 1997).

### Table II. Model A, constant flow (Ac)

<table>
<thead>
<tr>
<th>PARAMETER, UNITS</th>
<th>VALUE</th>
<th>ALT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_1^0$, µM</td>
<td>0.166</td>
<td></td>
</tr>
<tr>
<td>$c_2^0$, µM</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$c_3^0$, µM (Ac-1, Ac-2)</td>
<td>0, 0.2</td>
<td></td>
</tr>
<tr>
<td>$c_6^0$, µM</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$c_i^0 = 0$ for $i = 4, 5, 7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{6,5}$, µM</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>$k_5$, µM$^{-1}$ s$^{-1}$</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>$k_6$, s$^{-1}$</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>$K_{4,3}$, µM</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$k_3$, µM$^{-1}$ s$^{-1}$</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$k_4$, s$^{-1}$</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$K_{2,1}$, µM</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>$k_1$, µM$^{-1}$ s$^{-1}$</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>$k_2$, s$^{-1}$</td>
<td>0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>$dc_1/dt = k_1 c_3 c_5 - k_2 c_1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dc_5/dt = k_2 c_1 - k_1 c_3 c_5 + k_4 c_4 - k_3 c_2 c_5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dc_7/dt = k_5 c_4 c_6 - k_6 c_7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c_i^0$ (i = 0) = $c_i + c_4 + c_5 + c_7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>for all $t$: $c_i = c_i^0$ for $i = 2, 3, 6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At equilibrium: $K_{2,1} = c_3 c_5 /c_1$, $K_{4,3} = c_2 c_5 /c_4$, $K_{6,5} = c_4 c_6 /c_7$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE III. Model B, constant flow (Bc)

<table>
<thead>
<tr>
<th>PARAMETER, UNITS</th>
<th>VALUE</th>
<th>ALT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_1^0$, µM</td>
<td>0.166</td>
<td></td>
</tr>
<tr>
<td>$c_2^0$, µM</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$c_3^0$, µM</td>
<td>0, 0.2, 2</td>
<td></td>
</tr>
<tr>
<td>$c_6^0$, µM</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$c_i^0 = 0$ for $i = 4, 5, 7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{6,5}$, µM</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>$k_5$, µM$^{-1}$s$^{-1}$</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>$k_6$, s$^{-1}$</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>$K_{2,1}$, µM</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$k_1$, µM$^{-1}$s$^{-1}$</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$k_2$, s$^{-1}$</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$K_{4,3}$, µM</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>$k_3$, µM$^{-1}$s$^{-1}$</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>$k_4$, s$^{-1}$</td>
<td>0.1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
dc_1/dt &= k_2 c_3 c_4 - k_1 c_1 c_2 \\
dc_5/dt &= k_1 c_1 c_2 - k_2 c_5 + k_3 c_3 c_4 - k_4 c_5 \\
dc_7/dt &= k_3 c_4 c_6 - k_6 c_7 \\
c_1^i (t = c_i^0) &= c_1 + c_4 + c_7 \\
\text{for all } t: c_i = c_i^0 \text{ for } i = 2, 3, 6 \\
\text{At equilibrium: } K_{2,1} = c_2 c_1 /c_5, K_{4,3} = c_3 c_4 /c_5, K_{6,5} = c_4 c_6/c_7
\end{align*}

TABLE IV. Model C, constant flow (Cc)

<table>
<thead>
<tr>
<th>PARAMETER, UNITS</th>
<th>VALUE</th>
<th>ALT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_1^0$, µM</td>
<td>0.166</td>
<td></td>
</tr>
<tr>
<td>$c_2^0$, µM</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$c_3^0$, µM</td>
<td>0, 0.2, 2</td>
<td></td>
</tr>
<tr>
<td>$c_6^0$, µM</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$c_i^0 = 0$ for $i = 4, 7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{6,5}$, µM</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>$k_5$, µM$^{-1}$s$^{-1}$</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>$k_6$, s$^{-1}$</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>$K_{2,1}$, µM</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$k_1$, µM$^{-1}$s$^{-1}$</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$k_2$, s$^{-1}$</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

\[
\begin{align*}
dc_1/dt &= k_2 c_3 c_4 - k_1 c_1 c_2 \\
dc_5/dt &= k_5 c_4 c_6 - k_6 c_7 \\
c_1^i (t = c_i^0) &= c_1 + c_4 + c_7 \\
\text{for all } t: c_i = c_i^0 \text{ for } i = 2, 3, 6 \\
\text{At equilibrium: } K_{2,1} = c_2 c_1 /c_5, K_{6,5} = c_4 c_6/c_7
\end{align*}

This is an implementation of a variable-steplength second order modified Rosenbrock method. Explicit expressions for the Jacobian matrix were provided. To verify the numerical results we repeated selected experiments using ode15s, and replacing the analytical Jacobian with a numerical approximation. In all cases the results obtained by the different methods were essentially identical. As an additional check the final values were verified by independently computing the equilibrium concentrations.

To obtain results for chemical relaxation initiated during the course of the flow at specified times, we assumed a sufficiently fast temperature perturbation (referred to as a “temperature jump”) which we simulate by instantaneously increasing all bimolecular rate constants (except $k_2$ in model C) by 30%. The numerical computation was then repeated and the data with unaltered rate constants subtracted from those with increased rate constants, resulting in the difference curves shown. Instead of temperature jump perturbation, one could also consider “concentration jumps” as introduced by Czerlinski (1966, p. 269). While the latter perturbation is straightforward for constant flow, the effects of diffusion have to be carefully considered for stopped flow, as we show below.

We use a logarithmic timescale for our graphs in order to be able to observe fast processes as well as slower ones. As a result, sideways shifts in the graphs correspond to changes in the order of magnitude of the corresponding reaction rates. To show how close the obtained curves are to single-molecule kinetics (Lu et al, 1998) corresponding to one molecule in the observation volume ($c_{\text{one}}$ in Table I; see also below), horizontal lines at 3 $c_{\text{one}}$ and 10 $c_{\text{one}}$ were added to the graphs.

### TABLE V. Model A, stopped flow (As)

- $c_2^T = c_2 + c_4 + c_7$
- $c_3^T = c_1 + c_3$
- $c_6^T = c_6 + c_7$

### TABLE VI. Model B, stopped flow (Bs)

- $c_2^T = c_2 + c_4 + c_5 + c_7$
- $c_3^T = c_1 + c_5 + c_3$
- $c_6^T = c_6 + c_7$

### TABLE VII. Model C, stopped flow (Cs)

- $c_2^T = c_2 + c_4 + c_7$
- $c_3^T = c_1 + c_3$
- $c_6^T = c_6 + c_7$
Results and Discussion

Constant Flow

In the context of Model A (Figure 1A; Table II) it is not known whether \( K_{2,1} (= k_2/k_1) \) is smaller or larger than \( K_{4,3} (= k_4/k_3) \). Thus, Table II shows two alternative possibilities for the values of \( K_{2,1} \). Figure 3 shows for Model A (with \( c_3^0 = 0.2 \mu M \); model Ac-2) that in constant flow the curves for large \( K_{2,1} \) differ from those for small \( K_{2,1} \); the concentrations of components 4 and 7 are smaller for small \( K_{2,1} \), as the latter prevents all of component 1 from dissociating. The rightward shift is due to the change in the value of \( k_2 \) from 0.1 s\(^{-1}\) to 0.001 s\(^{-1}\) (Table II). The concentration peak for component 5 disappears, but a small level of component 5 appears simultaneously with component 4.

Figure 4 shows that the sideways shift in the flow curves also occurs in the chemical relaxation curves for \( c_4, c_5 \) and \( c_7 \) when relaxation is initiated at 100 s for Ac-2; this shift also occurs for Ac-1 and when relaxation is initiated at \( 10^4 \) s. The shift in Figure 4 arises from the fact that the reaction between components 4 and 7 is not accessible until 4 is actually formed. The dashed curve for component 1 in Figure 3 (\( K_{2,1} = 0.01 \mu M \)) shows that at 100 s only a small amount of this component has been consumed, leading to a

![Figure 3](image-url)

**FIGURE 3.** Model A, constant flow (Table II), \( c_3^0 = 0.2 \text{ mM (case Ac-2), with } K_{2,1} = 1 \text{ as solid curve and } K_{2,1} = 0.01 \text{ as dashed curve. The curves with constant } c_3^0 = 0 \text{ mM (case Ac-1, not shown) are identical to the solid curve.}**
pronounced change in the dashed curve for component 1 in Figure 4. For $K_{2,1} = 0.01 \mu M$, all reactants are participating, showing that components 4 and 7 are in fast equilibrium at this time period. That is also why components 4 and 7 move nearly synchronously in Figure 4. The time period for visible presence of component 5 increases — as does the size of constant flow — as the separation between $K_{2,1}$ and $K_{4,3}$ increases beyond the selected factor 10 shown here.

We deduce from the location of the horizontal lines in Figures 3 and 4 that single molecule kinetics may be largely neglected in flow experiments, but not in chemical relaxation experiments (where only the lines for 3 component are shown). This holds for all described simulations. Thus, many chemical relaxation curves would have to be measured for one set of data to obtain averaged curves which correspond to bulk concentrations.

Figures 5 to 7 are concerned with Model B (Figure 1B). Figure 5 shows that in constant flow there is very little difference between cases Bc-1 and Bc-2 for $K_{4,3} = 1$ (in both cases $c_3^0$ is small compared to $K_{4,3}$). Therefore, a third value for $c_3^0 (= 2 \mu M)$ was chosen, although this high concentration is rather unlikely in nature. In all cases, component 5 reaches a pronounced concentration maximum after about 1 s. The difference between Models A and B lies in the fact that component 5 is free ssDNA in Model A and ternary complex in Model B.

Figure 6 shows the corresponding curves for Model B and $K_{4,3} = 0.01 \mu M$. While case Bc-1 shows the expected longer presence of component 5 and the expected shift of the rising curves for $c_4$ and $c_7$ relative to Figure 5, case Bc-2 is very different in the final time

FIGURE 4. Model A, constant flow, as per Figure 3, with start of chemical relaxation (initiated for instance by temperature jump) at 100 s; curves show the deviations from the curves without perturbation. Please note that the line for component coincides with the abscissa axis.
FIGURE 5. Model B, constant flow (Table III), constant $c_3^0 = 0$ (solid curves), 0.2 (dotted curves), 2.0 (dashed curves) µM with $K_{4,3} = 1$.

FIGURE 6. Model B, constant flow (Table III), constant $c_3^0 = 0$ (solid curves), 0.2 (dotted curves), 2.0 (dashed curves) µM, with $K_{4,3} = 0.01$. 
period. Case Bc-3 produces almost no components 4 and 7. Because of the high concentration of the catalytically inactive ternary complex 5 over most of the time range, this alternate case is most likely not present in nature. The shape of the curves is clearly highly dependent upon the initial concentration of component 3: high $c_3^0$/$K_{4,3}$ essentially stabilizes component 5.

Figure 7 shows the chemical relaxation results for $K_{4,3} = 1 \mu$M and cases Bc-1, Bc-2 and Bc-3 with temperature jump at 100 s. The curves for Bc-1 and Bc-2 are almost identical, while those for Bc-3 are clearly different. A temperature jump at 1 s (not shown) reveals a transient appearance of component 5. The behavior is similar to that shown in Figure 4, except for the visible dependence upon the initial concentration of component 3. The value $K_{2,1} = 0.01 \mu$M leads to a shift similar to that in Figure 4 (not shown).

Figure 8 refers to Model C. Compared to Model B there is no component 5 present. This would also be the limiting case for Model B at very low concentrations of component 5 (and thus sufficiently fast decomposition in both directions). As the two lower values for $c_3^0$ led to identical curves, we also used $c_3^0 = 2 \mu$M (the values given for $c_2^0$ and $K_{2,1}$ dictate $c_3^0 >> c_2^0$ for a significant change in final concentrations of components 4 and 7, as $c_3^0$ is low).

Figure 9 shows chemical relaxation with perturbation initiated at 100 s, under the conditions of Figure 8. Again, the curves for the two lower values of $c_3^0$ overlap, while the curves for $c_3^0 = 2 \mu$M are clearly different. The difference for $c_1$ is substantial. One should note that high $c_3^0$ produced a visible relaxation curve for component 1, delayed from the initial change for components 4 and 7. At high $c_3^0$ there is a secondary change in 7 coinciding with the timing for the change in $c_1$. A similar change in $c_4$ is barely visible.

---

**FIGURE 7.** Chemical relaxation behavior for conditions of Figure 5, temperature jump initiated at 100 s.
FIGURE 8. Model C, constant flow (Table IV), constant $c_3^0 = 0$ (solid curves), 2.0 (dashed curves) μM.

FIGURE 9. Temperature jump at 100 s for Model C, constant flow, conditions of Figure 8 with same notation of curves. Please note that the line for $3 \text{cone}$ coincides with the abscissa axis.
Stopped Flow

Figure 10 refers to Model A and should be compared to Figure 3 involving constant flow under otherwise identical conditions. For $K_{2,1} = 1 \mu M$ there is very little difference between constant and stopped flow. But for $K_{2,1} = 0.01 \mu M$ the equilibrium values for components 1 and 7 are clearly different.

Chemical relaxation curves are shown in Figure 11 which should be compared to Figure 4. Structure and diversity are increased in Figure 11 over Figure 4. As indicated for Figure 4, the curves for $K_{2,1} = 0.01$ would be quite different for perturbation initiated at $10^4$ s (curves for components 4 and 7 shifted back to the left). For $K_{2,1} = 0.01 \mu M$, in both stopped and constant flow most of component 1 is converted to other components for $c_3^0 = 0 \mu M$ (not shown), less for $c_3^0 = 0.2 \mu M$ (Figure 10) and only about 20% for $c_3^0 = 2 \mu M$ (not shown).

Figure 12 refers to Model B for $c_3^0 = 0 \mu M$. The difference between constant and stopped flow is rather small, but increases for larger $c_3^0$ (not shown).

Figure 13 refers to Model C for $c_3^0 = 0.2 \mu M$. The difference between constant flow and stopped flow is again rather small and negligible for practical purposes. Chemical relaxation data for this model are not shown, as there is again little difference between constant and stopped flow.

Figure 14 shows the behavior of Models A and B in stopped flow with $c_3^0 = 0 \mu M$ and $c_2^0 = 0.2 \mu M$ (reduced from the 1 $\mu M$ used elsewhere in order to show the behavior of these curves together with the others; the concentration curves for component 2 have a similar shape for $c_2^0 = 1 \mu M$ but are vertically offset). The strongest difference between

FIGURE 10. Model A, stopped flow (Table V) with $K_{2,1} = 1 \mu M$ (solid curves) and $K_{2,1} = 0.01 \mu M$ (dotted curves) for case As-2 only. Data not shown in Table V are in Table II.
FIGURE 11. Difference curves after perturbation at 100 s, Model A, stopped flow (Table V) with $K_{2,1} = 1 \mu M$ (full curves) and $K_{2,1} = 0.01 \mu M$ (dotted curves) for case As-2 only. Please note that the line for $3_{\text{con}}$ coincides with the abscissa axis.

FIGURE 12. Model B, with $K_{4,3} = 1 \mu M$ for case Bc-1 and Bs-1 only, constant flow (solid curves) and stopped flow (dotted curves). Data not shown in Table VI are in Table III.
FIGURE 13. Model C with $c_1^0 = 0.2 \, \mu M$ only for constant flow (solid curves, case Cc-2) and stopped flow (dashed curves, case Cs-2). Data not shown in Table VII are in Table IV.

FIGURE 14. Stopped flow with $c_2^0 = 0.2 \, \mu M$ and $c_3^0 = 0 \, \mu M$. Solid curves refer to Model A (Table V), dashed curves to Model B (Table VI).
the models is in the shapes of $c_1$ and $c_2$. The equilibrium values for component 5 are also strikingly different. Comparing the latter (solid curve) with the corresponding one in Figure 10, one sees that the equilibrium value and the transient maximum for component 5 are much lower in Figure 10 where $c_2^0 = 1 \mu M$.

**Detection Strategy**

The numerical simulations reveal that the models are best distinguished by the position of the concentration curves for components 3 and 5. If the curve for component 3 starts with the curve for component 5, Model A determines the system. If the curve for component 3 starts visibly later than the one for component 5, Model B determines the system (but see below). Model C is most likely present if there is no indication of component 5. As stopped flow and constant flow data are similar, data for constant flow under otherwise identical conditions are similar to Figure 14.

The simulated concentration data guide the use of specific fluorophors. We incorporated the fast reaction step with fluorophor-labeled dGTP (component 6) to provide an instant measure of the amount of telomerase-telomere complex formed. But this is not enough to distinguish between mechanisms. If component 1 is also fluorophor-labeled and its fluorescence changes measurably upon conversion, one could follow the decrease in this concentration and compare it with the appearance of the concentrations of components 4 and 7. If the latter correlates directly with the former, mechanism C is indicated. If the correlation is not present, either mechanism A or B are present.

Figure 14 shows that components 4 and 7 appear earlier for Model A than for Model B. One may expect such behavior on the basis of simply comparing the original models. However, much is dependent upon the conditions for the life of component 5. If the transient concentration of component 5 is relatively large, there will be a delay in the appearance of components 4 and 7. The conditions for the life of component 5 are largely effected by the size of $c_2^0$ and $c_3^0$, plus their relation to the associated equilibrium constants. It is thus best to use a fluorescent indicator for free 5 in Model A (or for ternary complex 5 in Model B). One would then be able to quantitatively correlate all concentration changes along the path of fixed components. Obviously, one has to be careful that the indicator does not alter the kinetics significantly. If there is a free indicator binding with component 5, the conditions should be such that at most 20% of component 5 is bound to the indicator; otherwise, one would need to take into account the equilibrium constants involved in the binding of the indicator to component 5; for ease of detection this binding should be substantially faster than the binding of components 2 and 3.

One potential compound specific for free component 5 (unoccupied ssDNA) could possibly be a low molecular weight analogue of Pot1 (Loayza and de Lange, 2004), as Pot1 is specific for binding only to the ends of telomeres. Pot1 may be the protective agent (blocker) discussed above (Loayza and de Lange, 2004), deduced from work of Teixeira et al (2004).

Is there a way to distinguish between Models A and B without the use of an indicator for compound 5? In chemical relaxation one of us (Czerlinski, 1966) showed that the concentration dependence of the chemical relaxation times for Model B is inverse to those for Model A. By looking at Figure 14 it is also apparent that the transient concentration of component 5 decreases with increasing $c_2^0$ and $c_3^0$ for Model A, but increases with increasing $c_2^0$ and $c_3^0$ for Model B (not all data shown). This would be an indirect method of distinguishing between the two Models.
Consideration of Diffusion

So far we have ignored the fact that the observation volume in our mixing arrangement is not a closed system, but is surrounded by space without spatially fixed reactants. We have two cases. (1) Reactants are attached to the surface of an object glass. Then we have to take into account the fluid space above the fixed reactants. (2) Reactants are fixed near the surface of a cell nucleus. There is space all around the fixed reactants within the reaction volume. If one employs constant flow, the concentration at the reaction location can be kept constant, so we have an open system which is fully controlled. Stopped flow, on the other hand, requires consideration of reactants (components 2, 3 and 6) diffusing from outside the reaction volume into the reaction volume.

First, consider individual nucleotides on the surface of the cell nucleus diffusing to their reaction site. For simplicity we use the established diffusion constant of ATP (Diel et al., 1991), \( D = 3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \), and the relation \( x^2 = 2 D t \) where \( x \) is the mean diffusion distance and \( t \) the characteristic diffusion time (Moore, 1960). With a diffusion channel length of \( x = 10^{-6} \text{ cm} \) we compute \( t = 1.67 \times 10^{-5} \text{ sec} \) which is negligibly short compared to the relaxation process of the steps involving nucleotide binding (Figure 4). This even holds if the diffusion constant is a factor of ten slower in the channel (Rostoftseva and Bezrukov, 1998). Telomerase and blocker may have a smaller diffusion constant \( D \), but their kinetics is also slower than that of the nucleotide (Figure 3). Thus, diffusion through the pore will probably not affect the observed kinetics.

Next, consider reactants attached to the surface of a objective glass, case (1) above. The height of the flow volume above the surface is at least 10 \( \mu \text{m} \) (10\(^{-3} \text{ cm})\). One obtains \( t = 0.167 \text{ s} \) from the cited formula. To obtain homogeneity after diffusion, \( t \) should be multiplied by 10. That is 1.67 s for nucleotides (component 6), and longer for the other free reactants (components 2 and 3). The data of Figure 14 show that we reach this limit for stopped flow operation. Obviously, the results are worse for case (2) above. However, if the actual kinetics is slower than assumed by us, we may be able to use stopped flow successfully. Reducing the size of the space around the reaction volume then becomes of paramount importance.

Optimizing the Design

While for constant flow we only need to consider the chemistry (and possibly small volume flow which can be arranged by a narrow flow channel), stopped flow requires the design of a stopped flow chamber with as small a volume as possible. One possible configuration is shown in Figure 15. Obviously, special tools are required to fabricate such small reaction chambers. Besides very small volumes consumed, stopped flow allows one to follow the changes in the concentrations of free components (2, 3, 6).

In early equipment tests one may use pH (and other) indicators together with suitable buffers to observe the stability of temperature as well as changes in temperature, selecting components with the proper reaction enthalpies (Czerlinski, 1966, pp. 218–227). In reverse, one may also use fluorescent pH indicator systems as fast optical temperature indicators, possibly even for temperature control (stability as well as fast rise time for temperature jumps).
FIGURE 15. Object glass designs for small volumes. The top part shows a vertical cross-section in the plane of the central flow section where observation takes place; “beam” refers to the fluorescence emission beam collected by the microscope objective. Fluorescence excitation and emission beams propagate coaxially. The short lines on the objective indicate the desoxyribonucleotide chains attached to the surface of the objective. The flow moves from left to right. Distance d refers to the flow transit distance to the end of observation. Distance b covers the remainder of the attached chains. Distance e covers the width of the observation beam at the site of fluorescence emission, while a refers to the distance between top surface of objective to bottom surface of cover glass and w denotes the width of the object glass covered with the desoxyribonucleotide chains. A design for the smallest possible cell for stopped flow operation is shown in the middle portion. The four plungers are all moved together, providing a no-displacement closing valve system. The two-way valve on the left may have to be located further away than shown, as the central flow channel should only have a diameter of 10 µm. Such a small diameter bore may require advanced technology. The lower part shows a vertical cross-section in the circular plane of the bore. No cover glass is used on the left, but is required on the right to close off the groove. The required plungers are not shown here, but are different for the two types of bores.
References


Received December 1, 2005; accepted January 12, 2006.