Temperature Jump Studies of the Binding of Bromphenol Blue to \(\beta\)-Lactoglobulin in the Vicinity of the N\(\rightarrow\)R Transition*

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SUMMARY

Temperature jump studies of the binding of bromphenol blue to \(\beta\)-lactoglobulin at pH 8.5 and pH 9.0 are characterized by two concentration-dependent relaxation times. The data are fit by a mechanism of the form:

\[
\begin{align*}
P + D & \rightleftharpoons P^* + D^* \\
R^+ + H & \rightleftharpoons R + H^+
\end{align*}
\]

Since the protein itself is known to undergo an isomerization in this pH range, a slightly more complicated mechanism is proposed which is consistent with the observations available in the literature.

Several investigators have studied the equilibrium properties of \(\beta\)-lactoglobulin over a wide range of pH (1–14). Below pH 3.5 the protein, which exists in the native state as a dimer of molecular weight 35,500, dissociates into two identical monomer units of molecular weight 17,750 (1–3). Between pH 3.5 and pH 5.2 tetramerization of the dimer units has been observed for the more common genetic variants (4–9). At pH 7.5 the titration of one anomalous carboxyl group per monomer unit (10) is accompanied by changes in optical rotation (11–13) and sedimentation coefficient (14) but not in molecular weight (12). Above pH 9.4, the protein undergoes slow irreversible denaturation (13).

The present study concerns the reversible transition between pH 7 and pH 9, which has been labeled the “N \(\rightarrow\) R” transition (12), where N represents the native form dominant at pH 7 and R represents the reversibly denatured form present at pH 9.

The discussion and preliminary results reported below show how dye binding may be used as a probe in spectrophotometric temperature jump studies of the isomerization of \(\beta\)-lactoglobulin.

THEORY

For the purposes of this treatment, it is convenient to discuss the rate equations for the isomerization separately and then to investigate the effects of dye binding. The basic methods used here are outlined in several sources (17–21).

Rate Equations for Isomerization—With the use of the model suggested by Tanford and Taggart (12), the mechanism for the isomerization of \(\beta\)-lactoglobulin in the absence of dye may be written
Table I

Summary of mechanisms treated in this report for case (R) \( \gg \) (D), \( \langle \bar{N} \rangle \), \( \gg \) (D), and \( \langle P \rangle \rangle \) (D)

In the derivation of the expressions below, the bimolecular steps are assumed to equilibrate much more rapidly than the unimolecular steps and \( K'_1 = K_1/(1 + K_0) \). For other definitions see the text.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>( K_{eq} )</th>
<th>Special assumptions</th>
<th>( 1/r_1 )</th>
<th>( 1/r_2 )</th>
<th>( 1/r_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. [ R + D \xrightarrow{k_1 \frac{k_{-1}}{k_0}} RD ]</td>
<td>( \frac{K_1}{1 + K_0} )</td>
<td>( k_{-1} + \frac{k_1}{1 + K_0} ) (P)_0</td>
<td>( k_{-1} + k_0 )</td>
<td>( k_{-1} + k_0 )</td>
<td></td>
</tr>
<tr>
<td>II. [ R + D \xrightarrow{k_1 \frac{k_{-1}}{k_0}} RD ]</td>
<td>( K_1(1 + K_0) )</td>
<td>( k_{-1} + k_0 )</td>
<td>( k_{-1} + k_0 )</td>
<td>( k_{-1} + k_0 )</td>
<td></td>
</tr>
<tr>
<td>III. [ R + D \xrightarrow{k_1 \frac{k_{-1}}{k_0}} RD ]</td>
<td>( \frac{K_1(1 + K_0)}{1 + K_0} )</td>
<td>( k_{-1} + \frac{k_1}{1 + K_0} ) (P)_0</td>
<td>( k_{-1} + k_0 )</td>
<td>( k_{-1} + k_0 )</td>
<td></td>
</tr>
<tr>
<td>IV. [ R + D \xrightarrow{k_1 \frac{k_{-1}}{k_0}} RD ]</td>
<td>( K_1(1 + K_0) )</td>
<td>( k_{-1} + \frac{k_1}{1 + K_0} ) (P)_0</td>
<td>( k_{-1} + k_0 )</td>
<td>( k_{-1} + k_0 )</td>
<td></td>
</tr>
<tr>
<td>V. [ P + D \xrightarrow{k_1 \frac{k_{-1}}{k_0}} PD ]</td>
<td>( K_1(1 + K_0) )</td>
<td>( k_{-1} + \frac{k_1}{1 + K_0} ) (P)_0</td>
<td>( k_{-1} + k_0 )</td>
<td>( k_{-1} + k_0 )</td>
<td></td>
</tr>
</tbody>
</table>

\[ \text{RH} \xrightarrow{k''_0} \text{R + H} \]
\[ \text{NII} \xrightarrow{K'_0 \text{COOH}} \text{N + H} \]

If the protolytic steps equilibrate extremely rapidly and, in addition, hydrogen ion is buffered, only a slow relaxation time corresponding to the unimolecular transition from \( N + NH \) to \( R + RH \) will be observed. In complete analogy to the considerations leading to Equation 26 of Reference 20 the rate equation for this step is

\[ \frac{d \delta([R] + [RH])}{dt} = -(k_{-app} + k_{app}) \delta([R] + [RH]) \]
the equilibrium value of the concentration variable \((R + RH)\) at time \(t\) and

\[
k_{\text{a.app}} = \frac{k_{-a} + \frac{k_a(\text{H})}{K_{\text{COOH}}}}{1 + (\text{H})/K_{\text{COOH}}}
\]

\[(3a)\]

\[
k_{\text{b.app}} = \frac{k_{b} + \frac{k_b(\text{H})}{K_{\text{COOH}}}}{1 + (\text{H})/K_{\text{COOH}}}
\]

\[(3b)\]

The solution to Equation 3 yields a relaxation time for the system given by

\[
1/\tau = k_{-a,\text{app}} + k_{a,\text{app}}
\]

\[(4)\]

At any particular pH, then, if the protolytic steps equilibrate more rapidly than all other reaction steps and the system is buffered, the isomerization may be represented simply by the reaction

\[
\begin{align*}
R & \xrightleftharpoons{\ k_{\text{app}} \ } N \\
R & \xrightarrow{\ k_{a,\text{app}} \ } N
\end{align*}
\]

\[(5)\]

where \(R\) represents all of the "unfolded" species and \(N\) represents all of the species in the native configuration. This notation is used throughout the rest of the report. It must be remembered, however, that \(k_{a,\text{app}}\) and \(k_{-a,\text{app}}\) are functions of pH and that it is this pH dependence which will permit the separation of the contributions of the two isomerization steps to the relaxation time.

**Rate Equations for Dye Binding**—The dye-binding steps which contribute to the mechanism are assumed to equilibrate much more rapidly than the unimolecular isomerization steps but much less rapidly than the steps involving protons so that the simplification described in the preceding section will hold. These assumptions seem reasonable since the relaxation time obtained by Takahashi (16) for the isomerization is much longer than that which has been observed for dye binding (22, 23) and, in general protolytic steps proceed at rates very near their diffusion controlled limits (24) and are much faster in buffered solutions than dye-binding steps. The protolytic equilibria of the dye are ignored here since the contribution of the low pH forms of both dye and complex (23) should be negligible in the experimental pH range.

The general mechanism for dye binding at constant pH is

\[
\begin{align*}
R + D & \xrightarrow{k_1} RD \\
k_{-1} & \xrightarrow{k_0} k_{-2} \xrightarrow{k_2} k_{-3} \xrightarrow{k_3} k_{-4} \\
N + D & \xrightarrow{k_1} ND \\
k_{-1} & \xrightarrow{k_0}
\end{align*}
\]

where the species \(RD\) and \(ND\) are defined in a manner completely analogous to \(R\) and \(N\) in Equation 5 and \(k_0, k_{-2}, k_{-3}, \) and \(k_{-4}\) are apparent rate constants.

Since this mechanism possesses three relaxation times, requiring the solution of a cubic equation, which is more readily accomplished numerically than in open analytical form, only special cases for which analytical expressions may readily be obtained are considered in this paper. Table I contains a summary of the expressions for the reciprocals of the relaxation times \(1/\tau_1 >> 1/\tau_2 >> 1/\tau_3\) for the mechanisms against which the data in this report were tested. (Note that for Mechanism IIIA, \(1/\tau_2 \geq 1/\tau_3\), and for Mechanism IV, \(1/\tau_1 \geq 1/\tau_3\), since these expressions were obtained exactly without using a series expansion.)

All of the cases are considered in the limit of high protein to dye ratios since the present experiments were performed under those conditions. Thus, in the expressions in Table I, it is

**Table II**

<table>
<thead>
<tr>
<th>pH (mM)</th>
<th>Buffer (mM)</th>
<th>1/(\tau_1) (msec)</th>
<th>1/(\tau_2) (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>1.2</td>
<td>0.60 ± 0.04</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>8.5</td>
<td>3.3</td>
<td>0.55 ± 0.05</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>8.5</td>
<td>4.5</td>
<td>0.50 ± 0.04</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>8.5</td>
<td>6.3</td>
<td>0.45 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>8.5</td>
<td>7.5</td>
<td>0.43 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>8.5</td>
<td>8.8</td>
<td>0.40 ± 0.01</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>8.5</td>
<td>9.0</td>
<td>0.40 ± 0.01</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>8.5</td>
<td>12.0</td>
<td>0.35 ± 0.02</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>8.5</td>
<td>15.0</td>
<td>0.30 ± 0.03</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>9.0</td>
<td>1.9</td>
<td>0.78 ± 0.03</td>
<td>0.27 ± 0.10</td>
</tr>
<tr>
<td>9.0</td>
<td>3.0</td>
<td>0.75 ± 0.03</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>9.0</td>
<td>6.0</td>
<td>0.58 ± 0.02</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>9.0</td>
<td>7.5</td>
<td>0.53 ± 0.02</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>9.0</td>
<td>9.6</td>
<td>0.48 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>9.0</td>
<td>11.3</td>
<td>0.45 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>9.0</td>
<td>15.0</td>
<td>0.35 ± 0.05</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

The experimental errors represent the spread of the observed results.
assumed that $K_0 = k_0/k_a$ and $K_1 = k_1/k_{-1}$. It should be noted that $k_0$ and $k_{-1}$ are apparent rate constants whose pH dependence is given by Equation 3, $a$ and $b$, respectively.

Since Mechanism IIIA has not been treated explicitly in the literature, but is crucial to the discussions found later in this report, it is considered in some detail under "Appendix." The expression for $1/\tau_1$ in Mechanism IV, A and B, contains the apparent rate constants $k_{-1,app}$ and $k_{+1,app}$, where

$$k_{-1,app} = \frac{k_0 + k_{-2}K_s(D) + 1 + K_1(P)_o}{1 + K_1(D) + 1 + K_1(P)_o}$$ (7a)

and

$$k_{+1,app} = \frac{k_0 + k_{+1}K_s(D) + 1 + K_1(P)_o}{1 + K_1(D) + 1 + K_1(P)_o}$$ (7b)

When experiments are conducted at constant, low $D$ this relaxation time will have only a very weak dependence on $P$ if $K_{eq}, K_1$, and $K_1'$ (and, consequently, $K_0$ and $K_2$) do not differ too greatly, and these equations may be written

$$k_{-1,app} = \frac{k_0 + k_{-2}K_s(D)}{1 + K_1(D)}$$ (8a)

$$k_{+1,app} = \frac{k_0 + k_{+1}K_s(D)}{1 + K_1(D)}$$ (8b)

These equations are exact for the case when $K_1 = K'_1 = K_{eq}$ (implying $K_0 = K_2$).

The presence of a strong dependence of $\tau_1$ on protein concentration would tend to eliminate this mechanism.

Mechanism V is simply a shorthand representation of the two-step binding mechanism

$$R + D \xrightleftharpoons{k_1}{k_2} RD \xrightleftharpoons{k_3}{k_4} RD'$$

$$N + D \xrightleftharpoons{k'_1}{k'_2} ND \xrightleftharpoons{k'_3}{k'_4} ND'$$

written in Table I as it would appear in the very special case in which the dye binds equivalently to both isomers of the protein ($k_1 = k'_1, k_{-2} = k'_{-2}, k_2 = k'_2, k_{-3} = k'_{-3}$). In this case, then, the isomerization would not be observed in dye-binding experiments. It should be noted that Mechanisms II and V cannot be distinguished in experiments performed at constant pH.

None of these mechanisms may be eliminated simply because fewer than three relaxation times are observed in temperature jump experiments. The missing relaxation times may be either too fast or too slow to be observed, or the mode of detection may be insensitive to the reaction coordinate which corresponds to a particular relaxation time in the experimental concentration range. Of course, if three relaxation times are observed, Mechanisms I, II, and V may safely be eliminated.

The actual choice of possible mechanisms will be made on the basis of fit to the kinetics results, agreement with equilibrium measurements and with the relaxation time for the isomerization observed by Takahashi (16), and the requirement that the pH dependence of the unimolecular steps be consistent with that observed in the equilibrium studies of Tanford and Taggart (12).

### EXPERIMENTAL

Pentex crystallized β-lactoglobulin, Lot 32, was used without further purification. For the preparation of solutions the molecular weight was taken to be 17,750 and thus all of the data are reported in terms of monomer concentration. The dye used is bromphenol blue (23), obtained from E. Merck AG, Darmstadt.

All solutions were made with water which was first deionized and triple distilled from glass and then freshly boiled just before use to prevent cavitation in the temperature jump experiments.

A Cary model 14 recording spectrophotometer equipped with a thermostatted cell compartment was used for the equilibrium measurements and the pH of each solution was adjusted with a
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RESULTS

Binding isotherms plotted according to the equation (23)

\[
\frac{1}{r} = \frac{1}{(PD)} - \frac{1}{n} + \frac{1}{nK_{eq} \langle D \rangle}
\]

where \(PD\) is the total equilibrium concentration of all complex species, show that there is only one site per monomer unit and that the equilibrium behavior is simple. The equilibrium results (25) at pH 8.5 and pH 9.0 are presented in Table III. The strength of dye binding increases with pH in this range.

The results of the kinetics experiments are listed in Table II. A plot of \(1/\tau_{fast}\) against \(P_0\) gives a straight line (Fig. 2) as predicted by all of the mechanisms listed in Table I. The slope of this line is called \(k_{1,app}\) and the intercept, \(k_{-1,app}\). These plus their ratio \(K_{1,app} = k_{1,app}/k_{-1,app}\) are listed in Table III.

The slow relaxation time is also a function of concentration (Fig. 3). Neither Mechanism I nor Mechanism IV predicts such a concentration dependence for a step which equilibrates in this time range, so these mechanisms may safely be eliminated.

In all of the remaining mechanisms, it is appropriate to analyze a concentration-dependent slow relaxation time with a plot of \(1/\tau_{slow}\) against \(P_0\) (Fig. 4) which is being observed indirectly through its effect on dye binding.

To explain all of the available experimental evidence, therefore, Mechanism III was chosen. Mechanism III, B and C, were eliminated immediately because the pressure jump result at pH 8.4 in the absence of dye yields \(k_{-0} + k_0 \approx 10 \text{ sec}^{-1}\) which, together with the results for pH 8.5 given in Table III, means that \(k_{-0} + k_{0} \approx k_{2} + k_{0}\). Thus, since Mechanism IIIA does not predict a slow relaxation time in the absence of dye, the dye-binding experiments must be used to obtain values of \(k_{0}\) and \(k_{-0}\), the apparent rate constants for the isomerization.

Unfortunately, \(k_{-0}\) is too small to determine accurately in temperature jump experiments. In fact, the slow relaxation time for pH 8.4, \(P_0 = 1.9 \times 10^{-4} \text{ M}\), could not be measured because of convection in the temperature jump cell. It is necessary to go to protein concentrations at least a factor of 10 lower and to use the concentration jump technique. The construction of a stopped flow apparatus suitable for such work is nearing completion now. A detailed analysis of the pH dependence of \(k_{0}\) and \(k_{-0}\) and comparison with the results of Tanford and Taggart will have to wait until the experiments have been extended over a wider range of pH and meaningful values of \(k_{-0}\) can be obtained.

A few additional points may be made now, however. The
first question that comes to mind centers around the choice of the species R as the keystone for Mechanisms I, II, and III. Obviously, the system is completely symmetrical in N and R and thus N and R can be interchanged without changing the functional dependence of any of the relaxation times on the concentration variables. Only the identity of the constants would be altered. Why then, choose R?

The basis for the choice is the observation that the experimentally determined $k_0$ decreases with pH, just as the mechanism chosen would predict. In this mechanism $k_0$ represents the rate at which the R form reverts to give the N form. Clearly, this step will proceed faster at low pH when the carboxyl group possesses no charge than it will at high pH when the charged carboxylate group must be buried in a hydrophobic region of the protein.

In addition, this choice is reasonable because dye binding is known to be stabilized by hydrophobic interactions and thus the R form, which is partially unfolded and has more hydrophobic regions exposed to the solution, may be expected to bind dye more readily than the N form. Once the dye is bound, of course, the protein can refold, containing the dye in the now unexposed hydrophobic region. In other words, both rate constants for dye binding to the folded (N) species may be quite small as assumed in Mechanism III, the bimolecular constant because very few encounters result in a stable complex and the unimolecular one because the protein must first unfold before the dye can dissociate.

One may also ask why the third relaxation time is not observed. For the mechanism selected above $\tau_3$ falls well within the time range accessible to temperature jump measurements.

In order to answer this question it is first necessary to determine which reaction coordinate (or normal mode) possesses the relaxation time $\tau_2$. Without going into great detail, the procedure may be outlined as follows. First, one determines the eigenvalues of the matrix $A$ in Equation A-10. (This has diagonal matrix of eigenvalues) is obtained. Then this transformation matrix $T$ which takes $A$ into $A$ (the transformation matrix is applied to the concentration coordinates $N$ (Equation A-10) to give the set of orthogonal cordinates $N'$. With this procedure, the concentration variable corresponding to $\tau_2$ in Mechanism IIIA was found to be $\delta(N) + (ND)] = -\delta([R] + [RD])$. This result may be verified by writing the rate expression for this variable:

$$\frac{d\delta(N) + (ND)}{dt} = -k_{-\delta}[\delta(N) + (ND)] + k_\delta[\delta(R) + (RD)]$$

$$= -(k_{-\delta} + k_\delta)\delta(N) + (ND)]$$

$$= -\frac{K_0}{\tau_2}\delta(N) + (ND)])$$

This mode may be illustrated by considering the hypothetical process in which all N and ND molecules are removed instantaneously from an equilibrium mixture of protein and dye. The system then relaxes to a new equilibrium with no change in $[D]$. Thus $\tau_2$ is simply the relaxation time for the interconversion of N and ND with R and RD at constant $[D]$. In fact, it is precisely this relaxation time which would be observed for the isomerization in the absence of dye. If the spectra of the species ND and RD are the same, this mode of reaction will not be accompanied by a color change and will not be observable spectrophotometrically.

It may be verified that the reaction coordinate corresponding to $\tau_2$ is $K_0(D)\delta(N) - \delta(ND) = (1 + K_0(\delta) + (RD))\delta(D)$. Appearance or disappearance of free dye obviously involves a color change and can be observed spectrophotometrically.

The last question to be discussed is why the unimolecular relaxation time could not be observed in pressure jump studies on pure B-lactoglobulin below pH 8. The sensitivity function for the detection of the relaxation time for a system perturbed from equilibrium (25) contains the factor

$$\frac{dC}{d\ln K} = K \frac{dC}{dK}$$

where $C$ is the concentration variable which is observed and $K$ is the apparent equilibrium constant for the reaction step. For a simple unimolecular or pseudounimolecular process this factor is given by

$$\frac{dC}{d\ln K} = \frac{K_0}{(1 + K_0)^2} C_0$$

At all of the pH values treated here, $K_0 \gg 1$, so this equation reduces to

$$\frac{dC}{d\ln K} = \frac{(P_0)}{K_0}$$

since $K_0 = [(N) + ([NH])]/([R] + ([HR])]$ decreases with increasing pH, the sensitivity for detection of the unimolecular process increases with increasing pH, just as observed by Takahashi (16).

Acknowledgment—The author is indebted to Professor Robert A. Alberty for his support during the course of this research.

APPENDIX

Three-Step Mechanism (III A)—This special case of the general mechanism is basically a combination of the simpler Mechanisms I and II. It is assumed here that both $k_1$ and $k_\gamma$ (see Equation 6) are extremely small so that, in the time that it takes for the other steps to equilibrate, essentially no material has passed from N to ND through this reaction path. Once the other three steps have equilibrated, of course, (N), (D) and (ND) will have attained their equilibrium values and this step will not have contributed at all. Thus it may be omitted and the mechanism becomes

$$R + D \xrightarrow{k_1} RD$$

$$N \xrightarrow{k_\gamma} k_0 \xrightarrow{k_\delta} k_2$$

Now

$$K_{eq} = \frac{[RD] + (ND)]}{[R] + (N)[D]} = \frac{K_0 (1 + K_0)}{(1 + K_0)}$$

(A-2)
Since it is assumed that the remaining bimolecular step equilibrates much more rapidly than do the other two steps, the fast relaxation time for this mechanism is given by

$$\frac{1}{\tau_1} = k_1 + k_1 \frac{[R]}{[D]}$$  \hspace{1cm} (A-3)$$

If, in addition, $[D] \ll [R]$ and $[D_p] \ll [R_p]$ then $[R] + [D] \approx [R] \approx [P]/(1 + K_s)$ and Equation A-3 may be rewritten

$$\frac{1}{\tau_1} = k_1 + \frac{k_1}{1 + K_s} [P]_0$$  \hspace{1cm} (A-4)$$

It is now necessary to derive the expressions for the other two relaxation times. Two rate equations may be written for the system

\[ \frac{ds(N)}{dt} = -k_{\text{eq}} s(N) + k_1 s(R) \]
\[ \frac{ds(ND)}{dt} = k_1 s(RD) - k_{\text{eq}} s(N) \]  \hspace{1cm} (A-5)$$

where the notation $s(\cdot)$ represents the displacement of the concentration in question from its equilibrium value.

In addition there are the relations for the conservation of protein

$$s(R) + s(N) + s(RD) + s(ND) = 0$$  \hspace{1cm} (A-7)$$

and conservation of dye

$$s(D) = -[s(RD) + s(ND)] = s(R) + s(N)$$  \hspace{1cm} (A-8)$$

The constraint which arises from the rapidly equilibrated dye binding step may be obtained by writing the equilibrium relation for that step in differential form (20)

$$s(RD) = K_1 [R][D]$$  \hspace{1cm} (A-9)$$

Combining Equations A-5 through A-9 yields two coupled linear differential equations which are, in matrix form,

\[ \begin{bmatrix} \frac{ds(N)}{dt} \\ \frac{ds(ND)}{dt} \end{bmatrix} = \begin{bmatrix} -k_{\text{eq}} & k_1 \\ k_1 K_1 [D] & -k_{\text{eq}} - k_2 K_1 [D] \end{bmatrix} \begin{bmatrix} s(N) \\ s(ND) \end{bmatrix} \]

or

\[ \frac{d}{dt} N = AN \]  \hspace{1cm} (A-10)$$

If $\lambda_i$ is an eigenvalue of the matrix $A$, then the corresponding relaxation time is $\tau_i = -1/\lambda_i$. Thus, for the $2 \times 2$ matrix

\[ (P)_0 = [R] + [N] + [RD] \sim [R] + [N] = [R] + (1 + K_s) [N] \]

A, the relaxation times are given by a quadratic equation whose solution is

$$\frac{1}{\tau_i} = -\text{tr} A \pm \sqrt{\text{tr}^2 A - 4 |A|} \hspace{1cm} (A-11)$$

where $\text{tr} A$ is the trace of the matrix $A$

$$\text{tr} A = k_{\text{eq}} + k_1 + k_1 [R]/(1 + K_s) + k_2 K_1 [D]/(1 + K_s)$$

and $|A|$ is the determinant of $A$

$$|A| = k_{\text{eq}} (1 + K_s) K_1 [R] + k_1 (1 + K_s) [R]/(1 + K_s) + k_2 K_1 [D]/(1 + K_s)$$  \hspace{1cm} (A-12)$$

Mechanism IIIA is that special case of the three-step mechanism in which $k_0 = k_2$ and $k_\text{eq} = k_\text{eq}$. Equation A-11 then becomes precisely

$$\frac{1}{\tau_1} = \frac{1}{\tau_2} = \frac{1}{\tau_3} = k_2 + k_{\text{eq}}$$  \hspace{1cm} (A-14)$$

or, if $[R] \gg [D]$ and $[P]_0 \gg [D]_0$

$$\frac{1}{\tau_1} = \frac{1}{\tau_2} = \frac{1}{\tau_3} = k_2 + k_{\text{eq}} + \frac{K_1 [R]}{1 + K_s}$$  \hspace{1cm} (A-15)$$

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