



# On the validity of the quasi-steady state approximation of bimolecular reactions in solution

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## Abstract

Two-step binding kinetics are extensively used to study the relative importance of diffusion in biochemical reactions. Classical analysis of this problem assumes ad hoc that the encounter complex is at quasi-steady state (QSS). Using scaling arguments we derive a criterion for the validity of this assumption in the limit of irreversible product formation. We find that the QSS approximation (QSSA) of two-step binding is only valid if the total ligand and receptor concentrations are much smaller than  $(k_2 + k_{-1})/k_1$ , where  $k_1$  and  $k_{-1}$  are, respectively, the forward and reverse diffusion encounter rate constants and  $k_2$  is the chemical association rate constant. This criterion can be shown to imply that the average time between encounters is much longer than the half-life of the encounter complex and also guarantees that the concentration of the encounter complex is negligible compared to the reactant and product concentrations. Numerical examples of irreversible and reversible cases corroborate our analysis and illustrate that the QSS may be invalid even if  $k_{-2} \ll k_2$ . Our analysis of the irreversible case is shown to carry through to the more rigorous framework of the Smoluchowski theory of diffusion-controlled reactions. This work underscores the need for exercising greater caution in invoking the QSSA.

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## 1. Introduction

In the collision theory of gas phase reactions products are formed only if the collision is sufficiently energetic so that the encounter pair can overcome the energy barrier associated with the reactions (Atkins, 1994). If the collision is insufficiently energetic, then the colliding reactant molecules separate before any product is formed. Thus, in a dilute gas the number of collisions is equal to the number of encounters and the simple binding of a ligand and a receptor can be described as a reversible one step reaction (Atkins, 1994)



where  $k_f$  is the association rate constant and  $k_r$  is the dissociation rate constant. Encounters between reactants in solution occur in a very different manner from the idealized picture of gas phase collision theory. First, particles have to jostle their way through the solvent, so the encounter frequency is considerably less than in a gas. Second, because particle migration is slower, encountering molecules can remain near each other much longer in solution than in a gas; during a single encounter in solution molecules can undergo many collisions before they react with each other or separate. Consequently, binding of a ligand to a receptor in solution is a two-step reaction (Atkins, 1994; Eigen, 1974; Pecht and Lancet, 1977; Hammes, 1982; Lauffenburger and Linderman, 1993; Lee and Karplus, 1987)



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where  $L$  denotes ligand,  $R$  denotes receptor,  $k_1$  and  $k_{-1}$  are, respectively, the forward and reverse diffusion encounter rate constants,  $L \cdots R$  denotes the encounter complex in which the ligand and receptor form a part of the solvation sphere of each other with no elementary interactions formed,  $k_2$  and  $k_{-2}$  are the chemical association and dissociation rate constants, respectively and  $LR$  denotes the product.

Nevertheless, single-step binding reactions are widely used in the analysis of ligand–receptor interaction in well mixed solutions (Pecht and Lancet, 1977; Hammes, 1982; Lauffenburger and Linderman, 1993). In an attempt to resolve this seeming paradox, Eigen noted that when the encounter complex is at quasi-steady state (QSS) the observable kinetics correspond to kinetic scheme 1 with the effective rate constants (Atkins, 1994; Eigen, 1974)

$$k_f \equiv \frac{k_1 k_2}{k_{-1} + k_2} \quad (1a)$$

and

$$k_r \equiv \frac{k_{-2} k_{-1}}{k_{-1} + k_2}. \quad (1b)$$

These results are readily interpreted as a product of independent steady-state probabilities for encounter and activation (Shoup and Szabo, 1982) by noting that Eqs. (1a) and (1b) can be rewritten as

$$k_f = k_1 p \quad (2a)$$

and

$$k_r = k_{-2}(1 - p), \quad (2b)$$

where

$$p \equiv k_2 / (k_{-1} + k_2) \quad (3)$$

is the steady state probability for an encounter complex to cross the energy barrier and form a product. When  $k_{-1} \ll k_2$  and  $p \approx 1$ , the reaction is said to be diffusion controlled. In the opposite extreme  $k_{-1} \gg k_2$  and  $p \approx k_2 / k_{-1} \ll 1$ , the reactions is said to be activation controlled. This classification is only valid under QSS conditions.

When the QSS approximation (QSSA) is justified, the reaction problem is neatly separated into two independent parts, one depending only on the reaction mechanism and the other depending on the distribution and geometry of reactive sites and the properties. The encounter rate constants can therefore be estimated using the steady state diffusion theory of dilute solutions (Atkins, 1994; Eigen, 1974; Lee and Karplus, 1987; Shoup and Szabo, 1982; DeLisi and Wiegel, 1981; DeLisi, 1983). Using these theoretical estimates in Eq. (1) also enables an experimental estimation of the intrinsic binding rates (Pecht and Lancet, 1977). Eq. (1) has formed the basis of numerous studies on the role of diffusion in biochemical reactions (Pecht and Lancet,

1977; Hammes, 1982; Lauffenburger and Linderman, 1993; DeLisi, 1983). However, the validity of the QSS assumption behind these equations has received little attention. Weller(1957) argued that the steady state description is valid only for measurements performed  $1/k_{-1} \approx 10^{-10}$  s after the perturbation since this ensures that any initial transients have already died out. We now use scaling analysis to study two-step binding reactions in closed systems. Remarkably, we find that the validity domain of the QSSA of scheme 2 is a negligible fraction of the validity domain of the Michaelis–Menten approximation and is limited to low total reactant concentrations such that  $\max(L_{tot}, R_{tot}) \ll (k_2 + k_{-1})/k_1$ . At higher initial reactant concentrations significant depletion of the reactants occurs before steady state is attained and Weller’s argument breaks down.

## 2. Analysis

For closed systems, two-step binding reactions imply the following conservation relationships

$$LR \equiv R_{tot} - R - L \cdots R, \quad (4)$$

$$L \equiv L_{tot} - L \cdots R - LR = L_{tot} - R_{tot} + R \quad (5)$$

and differential equations

$$\frac{dR}{dt} = -k_1 L \cdot R + k_{-1} L \cdots R, \quad (6)$$

$$\frac{dL \cdots R}{dt} = k_1 L \cdot R - (k_{-1} + k_2) L \cdots R + k_{-2} LR. \quad (7)$$

Here  $L_{tot}$  and  $R_{tot}$  are the total ligand and receptor concentrations, respectively. We shall consider the solution of these equations subject to initial conditions typical of rapid mixing experiments (Hammes, 1982, pp. 61–64)

$$(L, R, L \cdots R, LR) = (L_{tot}, R_{tot}, 0, 0), \quad t = 0. \quad (8)$$

To simplify the algebra we shall henceforth restrict our time-scale analysis to the irreversible case  $k_{-2} = 0$ , in which case Eqs. (4), (5) and (7) yields the nonlinear equation

$$\begin{aligned} \frac{dL \cdots R}{dt} &= k_1(L_{tot} - L \cdots R - LR) \\ &\quad \times (R_{tot} - L \cdots R - LR) - (k_{-1} + k_2) \times L \cdots R. \end{aligned} \quad (9)$$

A more complete analysis of kinetic scheme 2 will be given elsewhere. Restricting our attention to sufficiently short times such that

$$LR + L \cdots R = R_{tot} - R \ll R_{tot} \quad (10a)$$

and

$$LR + L \cdots R = L_{tot} - L \ll L_{tot} \quad (10b)$$

we obtain the following approximate equation for the initial transient rise of the encounter complex

$$\frac{dL \cdots R}{dt} \approx k_1 L_{tot} R_{tot} - (k_{-1} + k_2) L \cdots R. \quad (11)$$

Integrating Eq. (11) subject to initial condition 8 we find

$$L \cdots R_{ITA} = \overline{L \cdots R} (1 - e^{-t/t_{ITA}}), \quad \overline{L \cdots R} \equiv \frac{L_{tot} R_{tot}}{K_M}, \quad (12)$$

where the subscript *ITA* reminds us that Eq. (12) is an initial transient approximation (ITA) and we introduced the notations

$$K_M \equiv \frac{k_{-1} + k_2}{k_1} \quad (13)$$

and

$$t_{ITA}^{-1} \equiv k_{-1} + k_2. \quad (14)$$

Self consistency of the ITA requires its compatibility with inequalities (10a and b). The latter can be rewritten in the more compact form

$$\varepsilon \equiv \frac{R_{tot} - R_{ITA}}{\min(L_{tot}, R_{tot})} \ll 1. \quad (15)$$

As emphasized by Segel (1972), inconsistent approximations are generally invalid, while consistent approximations are generally valid unless the problem is ill conditioned. By ill conditioned we mean that that a small error in the problem can lead to large errors in the solution. Thus, Eq. (15) is a necessary condition for the validity of the ITA, and very likely a sufficient condition as well, since this problem does not seem to be ill conditioned. Noting that the *mean value theorem* of differential calculus (Courant and John, 1965) guarantees the existence of  $\theta \in (0, t)$  such that  $R(t) = R_{tot} + (dR/dt)_\theta t$ , we can rewrite Eq. (15) as

$$\varepsilon \approx \left( \frac{k_1 L(\theta) \cdot R(\theta) - k_{-1} L \cdots R(\theta)}{\min(L_{tot}, R_{tot})} \right) t_{ITA}, \quad \theta \in (0, t_{ITA}). \quad (16)$$

Since  $k_1 L(\theta) \cdot R(\theta) - k_{-1} L \cdots R(\theta) \leq k_1 L_{tot} R_{tot}$  we obtain the following criterion for the validity of the *ITA*

$$1 \gg \varepsilon \approx \left( \frac{k_1 L_{tot} R_{tot}}{\min(L_{tot}, R_{tot})} \right) t_{ITA} = \frac{L_{tot} R_{tot}}{K_M \min(L_{tot}, R_{tot})} = \frac{\max(L_{tot}, R_{tot})}{K_M}. \quad (17)$$

Assuming that inequality (17) is satisfied, we note that Eq. (11) implies that  $L \cdots R$  increases and in a time of order  $t_{ITA}$  approaches the maximal asymptotic value implied by the initial conditions,  $\overline{L \cdots R}$ . This signals the onset of a QSS such that  $dL \cdots R/dt \approx 0$  and

$$L \cdots R \approx \frac{L \cdot R}{K_M}. \quad (18)$$

Substituting this into Eq. (6) yields the following QSSA

$$\frac{dR}{dt} \approx -k_f L \cdot R + k_r LR \approx -\frac{dLR}{dt} \quad (19)$$

with the irreversible limits of Eq. (1),  $k_f = k_2/K_M$  and  $k_r = 0$ . We therefore conclude that in the irreversible case, the criterion for the validity of the ITA, inequality (17), guarantees that ITA is succeeded by the QSSA of kinetic scheme 2. In order for Eq. (19) to be *uniformly* valid in time we must further require that the total change in receptor concentration during the initial transient be negligible with respect to its maximal variation (Segel, 1988)

$$1 \gg \frac{R_{tot} - R(t_{ITA})}{R_{tot} - R_{eq}}, \quad (20)$$

where the subscript “*eq*” denotes the equilibrium value, and that the duration of the initial transient is negligible compared to that of the QSS phase,  $t_R$

$$1 \gg \delta \equiv t_{ITA}/t_R. \quad (21)$$

Since

$$R_{tot} - R_{eq} = \min(L_{tot}, R_{tot}), \quad k_{-2} = 0 \quad (22)$$

the first requirement is equivalent to  $\varepsilon \ll 1$  and therefore guaranteed by inequality (17). The time-scale of the QSS can be estimated as the ratio of the total change in  $R$  during the QSS and  $\max |dR/dt|$  during the QSS (Segel, 1972, 1988)

$$t_R \approx \frac{R_{tot} - R_{eq}}{(k_2/K_M) L_{tot} R_{tot}} = \frac{K_M}{k_2 \max(L_{tot}, R_{tot})}. \quad (23)$$

Substituting this estimate into Eq. (21) we obtain the inequality

$$\delta \approx \left( \frac{\max(L_{tot}, R_{tot})}{K_M} \right) \left( \frac{k_2}{k_2 + k_{-1}} \right) = p\varepsilon \leq \varepsilon. \quad (24)$$

This implies that inequality (17) is the *sole* criterion for the validity of the QSSA of irreversible two step binding.

We tested the validity of this criterion by comparing the numerical solution of Eqs. (6)–(8) to the numerical solution of Eq. (19) for a range of representative cases (Figs. 1–3). To simplify the comparison between the various cases the normalized product concentration,  $LR/L_{tot}$ , is plotted as a function of normalized time,  $t/t_R$ . Fig. 1 depicts three cases for which  $\varepsilon = 0.01$ . The QSSA is very good for the irreversible case ( $R^2 = 1.000$ ), the slightly reversible case  $k_{-2}/k_2 = 0.1$  ( $R^2 = 0.996$ ), and the reversible case  $k_{-2}/k_2 = 0.5$  ( $R^2 = 0.966$ ). Fig. 2 depicts three cases for which  $\varepsilon = 0.1$ . Again, the QSSA is very good for the irreversible case ( $R^2 = 0.992$ ) and the slightly reversible case  $k_{-2}/k_2 = 0.1$  ( $R^2 = 0.977$ ), but is only fair for  $k_{-2}/k_2 = 0.5$  ( $R^2 = 0.911$ ). Finally, Fig. 3 illustrates three cases for which  $\varepsilon = 1.0$ . As expected, the QSSA is poor for the irreversible case ( $R^2 = 0.797$ ), the slightly reversible case  $k_{-2}/k_2 = 0.1$  ( $R^2 = 0.764$ ), and the reversible case  $k_{-2}/k_2 = 0.5$  ( $R^2 = 0.652$ ).

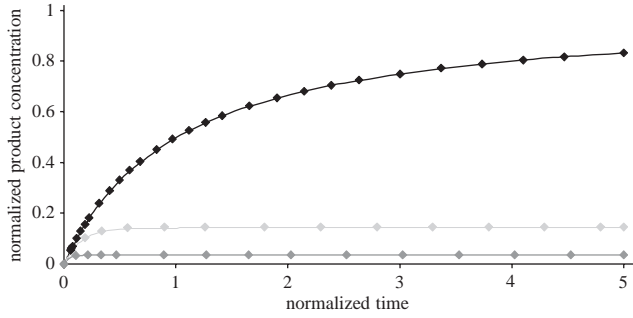


Fig. 1. Normalized concentration ( $LR/L_{tot}$ ) at low ligand and receptor concentrations. Numerical predictions (solid lines) are contrasted with the corresponding QSSA (solid diamonds) for  $L_{tot} = R_{tot} = 0.1$ ,  $k_1 = 0.001$ ,  $k_{-1} = k_2 = 0.01$  ( $K_M = 10$ ) and  $k_{-2} = 0$  (black),  $k_{-2} = 0.1k_2 = 0.001$  (light gray) or  $k_{-2} = 0.5k_2 = 0.005$  in arbitrary units. The time axis is normalized to the QSS time-scale,  $t_R = 10000$ .

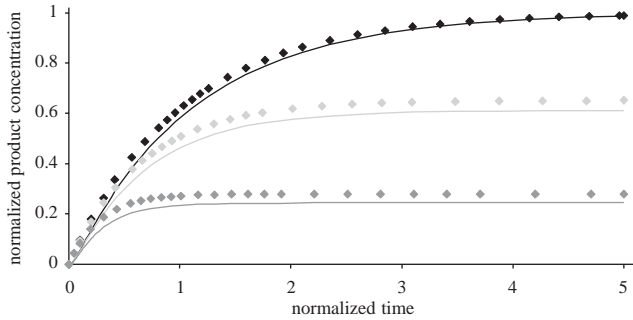


Fig. 2. Normalized concentration ( $LR/L_{tot}$ ) at low ligand concentration and intermediate receptor concentrations. Numerical predictions (solid lines) are contrasted with the corresponding QSSA (solid diamonds) for  $L_{tot} = 0.1$ ,  $R_{tot} = 1$ ,  $k_1 = 0.001$ ,  $k_{-1} = k_2 = 0.01$  ( $K_M = 10$ ) and  $k_{-2} = 0$  (black),  $k_{-2} = 0.1k_2 = 0.001$  (light gray) or  $k_{-2} = 0.5k_2 = 0.005$  in arbitrary units. The time axis is normalized to the QSS time-scale,  $t_R = 1000$ .

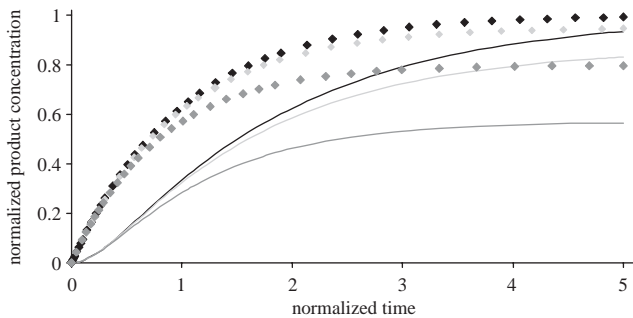


Fig. 3. Normalized concentration ( $LR/L_{tot}$ ) at low ligand concentration and high receptor concentration. Numerical predictions (solid lines) are contrasted with the corresponding QSSA (solid diamonds) for  $L_{tot} = 0.1$ ,  $R_{tot} = 10$ ,  $k_1 = 0.001$ ,  $k_{-1} = k_2 = 0.01$  ( $K_M = 10$ ) and  $k_{-2} = 0$  (black),  $k_{-2} = 0.1k_2 = 0.001$  (light gray) or  $k_{-2} = 0.5k_2 = 0.005$  in arbitrary units. The time axis is normalized to the QSS time-scale,  $t_R = 100$ .

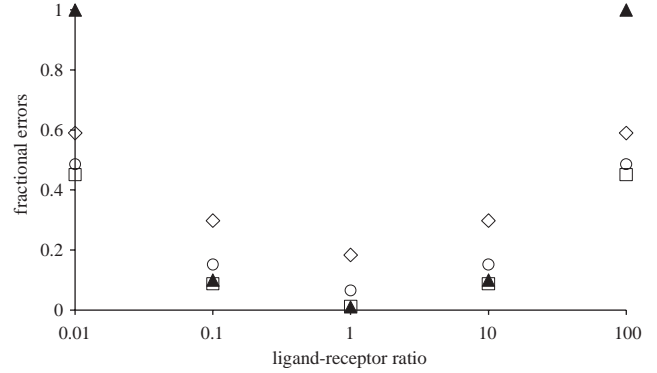


Fig. 4. Fractional error associated with the QSSA as a function of the ligand–receptor ratio  $L_{tot}/R_{tot}$  for the data depicted in Figs. 1–3. A priori error estimates according to Eq. (17) (solid triangles) are contrasted with the a posteriori error measures for  $k_{-2}/k_2 = 0$  (squares),  $k_{-2}/k_2 = 0.1$  (circles) and  $k_{-2}/k_2 = 0.5$  (diamonds). The excess ligand cases  $L_{tot}/R_{tot} = 10, 100$  correspond to the cases depicted in Figs. 2 and 3. This is made possible by the symmetry of product kinetics under the permutation of initial  $L_{tot} \leftrightarrow R_{tot}$  (see Appendix A for details).

Since product concentration is invariant under the permutation of initial conditions  $L_{tot} \leftrightarrow R_{tot}$  (see Appendix A), Figs. 2 and 3 also test our analysis for six additional cases such that  $L_{tot} > R_{tot}$ . Fig. 4 shows a plot of the fractional errors associated with the QSSA in Figs. 1–3. As in our analysis of the total QSSA of reversible Michaelis–Menten reactions (Tzafiriri and Edelman, 2004), fractional errors are estimated as  $\Delta LR/LR \equiv \sqrt{1 - R^2}$ . Inspection of Fig. 4 reveals that a priori error estimates according to Eq. (17) are good predictors of the a posteriori error measures for the irreversible case  $k_{-2}/k_2 = 0$  only for moderate ligand–receptor ratios. Overestimation of the errors for the extremes of excess ligand ( $L_{tot}/R_{tot} \gg 1$ ) and excess receptor ( $L_{tot}/R_{tot} \ll 1$ ) is due to the invalidity of the QSSA for these cases and hence of the estimates leading to Eq. (17). The errors associated with the QSSA are consistently higher for the reversible cases  $k_{-2}/k_2 = 0.1, 0.5$  than for the irreversible case, which suggests that in the reversible case Eq. (17) can be used as a necessary condition for the validity of the QSSA.

### 3. Discussion

The degree to which diffusion limits chemical reactions is a question of general importance and particularly for biochemical reactions such acid-base catalysis (Hammes, 1982; Rini et al., 2003), catalysis by immobilized enzymes (Nelesstuen and Martinez, 1997; Gaspers et al., 1994, 1995) or by enzyme aggregates in non-aqueous solutions (Klibanov, 1997), and ligand binding to cell-bound (Pecht and Lancet, 1977; Lauffenburger and Linderman, 1993) and internalized

(Schoeberl et al., 2002) receptors. Since its introduction by Eigen, kinetic scheme 2 and its QSSA have been widely accepted as a framework for studying diffusion influenced reactions in solution (Eigen, 1974; Pecht and Lancet, 1977). That the QSSA is a wonderful tool for simplifying kinetic problems is evident from its huge success in enzyme kinetics. Presumably, the similarity between the basic Michaelis–Menten scheme and kinetic scheme 2 led Eigen and others to believe that the QSSA would be equally successful in the latter case. Our analysis shows that this is not the case, and underscores the need for exercising caution when applying the QSSA to new problems. Briggs and Haldane (1925) showed that the QSSA of irreversible Michaelis–Menten kinetics is valid for excess substrate conditions  $L_{tot} \ll R_{tot}$ . Segel (1988) used scaling analysis to show that the Briggs–Haldane criterion is too strict and can be replaced by  $L_{tot} \ll K_M + R_{tot}$ . Using similar scaling analysis for irreversible two-step binding we obtained Eq. (17), which is even more restrictive than the Briggs–Haldane criterion. This surprising result is supported by error analysis (see Fig. 4) and can be traced back to the fact that in contrast to the Michaelis–Menten scheme, kinetic scheme 2 implies that the concentration of product is invariant under the permutation of initial conditions  $L_{tot} \leftrightarrow R_{tot}$  (see Appendix A). Since many bimolecular reactions involve additional intermediate reaction steps due to conformation changes of the encounter complex and or rearrangement of the solvation shell (Hammes, 1982; Rini et al., 2003; Hill, 1975) it is worth noting that this symmetry between the ligand and receptor is a property of general multi-step bimolecular reactions with several intermediates. This implies that, like inequality (17), the criterion for the validity of general multi-step bimolecular reactions with several intermediates must be invariant under the permutation  $L_{tot} \leftrightarrow R_{tot}$  and therefore rules out the applicability of the Briggs–Haldane criterion for this class of reactions.

The numerical tests depicted in Figs. 1–4 illustrate the validity of inequality (17) as a sufficient condition for the validity of the QSSA of two-step binding reactions in closed systems. These figures illustrate the effect of increasing  $\varepsilon$  and  $k_{-2}/k_2$  for a borderline case where the diffusional dissociation rate constant is equal to the chemical association rate constant,  $k_{-1} = k_2$ . In Fig. 1,  $\varepsilon = 0.01$  and the QSSA is an excellent approximation for  $k_{-2}/k_2 = 0–0.5$ . In Fig. 2,  $\varepsilon = 0.1$  and discrepancies between the QSSA and the numerical solution are observable in all three case and their relative magnitude increases with  $k_{-2}/k_2$ . In Fig. 3,  $\varepsilon = 1.0$  and the QSSA is seen to be a poor approximation for  $k_{-2}/k_2 = 0–0.5$ . The examples depicted in Fig. 3 correspond to excess substrate conditions such that  $R_{tot}/L_{tot} = 100$  and therefore refute that the Briggs–Haldane criterion guarantees that the “encounter complex is nearly

constant during most of the course of the reactions” (McCammon et al., 1986). Moreover, the examples shown in Fig. 3 refute previous singular perturbation analysis that suggested that the inequality  $k_{-2}/k_2 \ll 1$  guarantees the validity of the QSSA of two-step binding (Perelson et al., 1980). Nevertheless, the converse statement is true. Namely, Fig. 4 illustrate that regardless of the value of  $\varepsilon$  the QSSA eventually breaks down as  $k_{-2}/k_2$  is increased.

Using the definition of  $K_M$  (Eq. (13)) we can rewrite inequality (17) as

$$k_1 \max(L_{tot}, R_{tot}) \ll k_{-1} + k_2. \quad (25)$$

This implies that the QSSA is valid only if the forward encounter rate is *always* small compared to the *total* rate of decay of the encounter complex. In other words, the QSSA is valid if at every time interval formation of new encounter complexes is significantly outweighed by dissociation of existing encounter complexes. In this nomenclature the QSSA is justified whenever the average time between encounters is large compared to the half-life of the encounter complex,  $(k_{-1} + k_2)^{-1}$ , during the *entire* course of the reaction, including the fast initial transient phase. This reformulation of inequality (17) is intuitive and explains why inequality (17) is a necessary but insufficient condition in the reversible case. Namely, in the reversible case the half-life of the encounter complex is longer than  $(k_{-1} + k_2)^{-1}$  due to the back reaction, but  $[k_1 \max(L_{tot}, R_{tot})]^{-1}$  is still the average time between encounters during the initial transient. This extension of the encounter complex half-life by the back reaction will be minimized by any process such as metabolism or endocytosis that eliminates the product (ligand–receptor complex). Since the rate constants may not be known a priori it is useful to note that Eqs. (12) and (18) are both valid whenever inequality (17) is satisfied and imply that  $L \cdots R \ll \min(L_{tot}, R_{tot})$ . In turn, this implies that the QSSA is only valid when the concentration of the encounter complex is negligible compared to the ligand and receptor concentration during the entire course of the reaction (and can therefore be neglected in Eqs. (4) and (5)). This observation can be used as a simple operational criterion for the validity of Eq. (1) for fast mixing experiments.

A noteworthy implication of our analysis is that separation of time-scales, e.g.  $\delta \ll 1$ , does not guarantee the validity of the QSSA of two-step binding, since Eq. (24) implies that  $\delta \leq \varepsilon$  in the irreversible case. This result was obtained using an order of magnitude estimate of the time-scale of the QSS phase,  $t_R$ . The simulations depicted in Figs. 1–3 illustrate the validity of this estimate. It is instructive to compare our a priori estimate of  $t_R$  to the exact time-scale obtained from the analytical solution of Eq. (19) (see Appendix B). In the irreversible limit the exact QSS time-scale reduces to

$K_M/(k_2|R_{tot} - L_{tot}|)$  while  $t_R \approx K_M/(k_2 \max(L_{tot}, R_{tot}))$  according to Eq. (23). This implies that Eq. (23) underestimates  $t_R$  and consequently that  $\delta$  is even smaller than implied by Eq. (24). Moreover, the analytical solution of Eq. (19) implies that  $t_R$  is maximal in the irreversible limit. The decrease of  $t_R$  with  $k_{-2}$  is consistent with our numerical tests, which illustrates that increasing the ratio  $k_{-2}/k_2$  results in larger errors and an eventual breakdown of the QSSA.

Weller (1957) argued that the steady state description is valid only for measurements performed  $1/k_{-1} \approx 10^{-10}$  s after the perturbation since this ensures that any initial transients have already died out. Our analysis implies that the validity domain is in fact much more restrictive than suggested by these heuristic arguments. When inequality (17) is valid, an initial transient with a lifetime of  $1/(k_{-1} + k_2) \leq 1/k_{-1} \approx 10^{-10}$  s precedes the QSS. However, when inequality (17) is not valid, due to high ligand concentrations ( $L_{tot} \gg K_M$ ) or more surprisingly due to high receptor concentration ( $R_{tot} \gg K_M$ ) the notions of an initial transient and a QSS phase are no longer valid, so that Eqs. (1a) and (1b) are an invalid even at long times ( $\gg 1/k_{-1}$ ). Namely, fast mixing experiments that do not satisfy inequality (17) cannot be analysed in terms of the QSSA even for times  $\gg 1/k_{-1} \approx 10^{-10}$  s.

The current analysis is based on the classical chemical kinetics approach to bimolecular reactions in solution, as embodied by scheme 2. However, strictly speaking, diffusion influenced reactions should be modeled using diffusion equations coupled to surface or bulk reactions. The wide and enduring usage of kinetic scheme 2 as a framework for studying reactions in solution stems from its simplicity and intuitive appeal as it correctly interpolates between the diffusion controlled and reaction controlled regimes. For example, Eqs. (1) and (19) can also be derived from Smoluchowski's steady-state diffusion theory (Atkins, 1994; Eigen, 1974; Shoup and Szabo, 1982; Smoluchowski, 1917; Collins and Kimball, 1949). However, it should be noted, that while Smoluchowski's theory is a very good approximation for irreversible reactions (Szabo et al., 1988; Szabo, 1989) it fails to capture certain many body effects for reversible reactions (Agmon and Szabo, 1990). The current analysis suggests the need to reevaluate the validity of using steady state diffusion models for studying the relative influence of diffusion on transient reactions. This critique is supported by the analysis of a transient diffusion model, which shows that Eq. (19) has to be modified by additional transient terms (Goldstein and Dembo, 1995). Moreover, the relevance of our analysis to the Smoluchowski theory of diffusion controlled irreversible reactions is illustrated in Appendix C. Using a closed form solution of the transient Smoluchowski (diffusion) equation with radiation boundary conditions, we show there that when

inequality (17) is *not* satisfied most of the ligand is depleted before the bimolecular rate constant for the overall reaction reaches its observable steady state value. This analysis of irreversible binding directly illustrates that steady state Smoluchowski theory is *only* applicable to fast mixing experiments that satisfy inequality (17).

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## Appendix A

Kinetic scheme 1 implies that

$$\frac{dLR}{dt} = k_2 L \cdots R - k_{-2} LR. \quad (\text{A.1})$$

Similarly, we can use Eqs. (4) and (5) to rewrite Eq. (7) as

$$\frac{dL \cdots R}{dt} = k_1(L_{tot} - L \cdots R - LR)(R_{tot} - L \cdots R - LR) - (k_{-1} + k_2)L \cdots R + k_{-2}LR. \quad (\text{A.2})$$

Eqs. (A.1) and (A.2) along with Eqs. (4) and (5) fully determine the dynamics of the system. For fast mixing experiments these equations are solved subject to the initial condition

$$(L \cdots R, LR) = (0, 0), \quad t = 0. \quad (\text{A.3})$$

It is easy to verify that Eqs. (A.1) and (A.3) are invariant under the permutation  $L_{tot} \leftrightarrow R_{tot}$ . Clearly this line of argument is not restricted to two-step binding reactions and will hold general multi-step binding reactions with an arbitrary number of intermediates.

## Appendix B

In the general reversible case the steady state assumption  $dL \cdots R/dt \approx 0$  implies

$$0 \approx k_1 L \cdots R - (k_{-1} + k_2)L \cdots R + k_{-2}LR. \quad (\text{B.1})$$

Solving for the encounter complex and using Eqs. (4) and (5) to simplify we find

$$L \cdots R \approx \frac{k_1(L_{tot} - R_{tot} + R)R + k_{-2}(R_{tot} - R)}{k_{-1} + k_2 + k_{-2}}. \quad (\text{B.2})$$

Substituting Eq. (B.2) into Eq. (3) yields the following QSSA equation for the receptor

$$\frac{dR}{dt} \approx -a_f(L_{tot} - R_{tot} + R)R + a_r(R_{tot} - R), \quad (B.3)$$

where

$$a_f \equiv \frac{k_1(k_2 + k_{-2})}{k_{-1} + k_2 + k_{-2}}, \quad a_r \equiv \frac{k_{-1}k_{-2}}{k_{-1} + k_2 + k_{-2}}. \quad (B.4)$$

Eq. (B.3) can be rewritten in the canonical form

$$\frac{dR}{dt} \approx -a_f(R - R_+)(R - R_-), \quad (B.5)$$

where  $R_{\pm}$  are the roots of the quadratic equation

$$R^2 + (L_{tot} - R_{tot} + a_r/a_f)R - a_r/a_f R_{tot} = 0. \quad (B.6)$$

Note that  $R_+ > 0$  whereas  $R_- < 0$ , which implies that  $R_+ = R_{eq}$ . Using the identity

$$\frac{1}{(R - R_+)(R - R_-)} = \frac{1}{R_+ - R_-} \left( \frac{1}{R - R_+} - \frac{1}{R - R_-} \right)$$

to integrate Eq. (B.5) and performing some algebra we find

$$R \approx \frac{R_+(R_{tot} - R_-) - R_-(R_{tot} - R_+)e^{-t/\tau_R}}{R_{tot} - R_- - (R_{tot} - R_+)e^{-t/\tau_R}}, \quad (B.7)$$

where

$$\begin{aligned} \tau_R^{-1} &\equiv a_f(R_+ - R_-) \\ &= a_f \sqrt{(L_{tot} - R_{tot} + a_r/a_f)^2 + 4R_{tot}a_r/a_f}. \end{aligned} \quad (B.8)$$

In the irreversible case,  $a_r = 0$  and the latter result reduces to

$$\tau_R^{-1} = k_f |R_{tot} - L_{tot}| = (k_2/K_M) |R_{tot} - L_{tot}|. \quad (B.9)$$

Moreover, since  $a_r/a_f = (k_{-1}/k_1)k_{-2}/(k_2 + k_{-2})$ , Eq. (B.8) implies that the characteristic time-scale of the QSS phase is maximal in the irreversible case. Eq. (B.9) is only valid for  $L_{tot} \neq R_{tot}$ . For the irreversible case with initial conditions of equal ligand and receptor concentrations, Eqs. (B.3) and (19) reduces to

$$\frac{dR}{dt} \approx -k_f R^2 \quad (B.10)$$

with the solution

$$R = \frac{R_{tot}}{1 + t/\tau_R}, \quad \tau_R^{-1} \equiv k_f R_{tot}. \quad (B.11)$$

## Appendix C

Smoluchowski's theory of diffusion-controlled reactions assumes that the rate of encounters and subsequent reaction can be estimated by Fickian diffusion with appropriate boundary conditions (Atkins, 1994; Smoluchowski, 1917; Collins and Kimball,

1949). Within this framework the depletion rate of a minority concentration of ligand binding irreversibly with an excess concentration of receptors is given by (Rice, 1985)

$$\frac{dL}{dt} = -k(t)R_{tot}L \quad (C.1)$$

and

$$k(t) = 4\pi aD \left( 1 + \frac{2a}{\sqrt{\pi Dt}} \right). \quad (C.2)$$

Here  $a$  denotes the minimal encounter distance and  $D$  denotes the mutual diffusion coefficient of a ligand-receptor pair. At long times Eq. (C.2) reduces to the steady state observable rate constant

$$k \approx k_1 \equiv 4\pi aD, \quad t \gg 4a^2/\pi D. \quad (C.3)$$

Integrating Eq. (C.1) subject to Eq. (8) yields

$$L = L_{tot} \exp \left( -4\pi aD R_{tot} \left( 1 + \frac{2a}{\sqrt{\pi Dt}} \right) t \right). \quad (C.4)$$

For  $t = 40a^2/\pi D$  one obtains

$$k(t) = 1.1k_1 \quad (C.5)$$

and

$$L = L_{tot} \exp(-176a^3 R_{tot}). \quad (C.6)$$

Introducing the estimate (Eigen, 1974; Shoup and Szabo, 1982)

$$4\pi a^3/3 \approx k_1/k_{-1} \quad (C.7)$$

into Eq. (C.6) and noting that  $R_{tot} = \max(L_{tot}, R_{tot})$  we obtain

$$\begin{aligned} \frac{L}{L_{tot}} &\approx \exp \left( -38.6 \left( \frac{\max(L_{tot}, R_{tot})}{k_{-1}/k_1} \right) \right) \\ &\leq \exp(-38.6\epsilon). \end{aligned} \quad (C.8)$$

Consequently, if Eq. (17) is *not* satisfied then most of the ligand (minority species) is depleted during the transient phase, before the bimolecular rate constant attains its observable value. This is similar to our conclusion that Eq. (17) guarantees the ITA.

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