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General Numerical Treatment of Competitive Binding Kinetics: Application to Thrombin–Dehydrothrombin–Hirudin

Petr Kuzmič

BioKin Consulting, P.O. Box 8336, Madison, Wisconsin 53708

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This paper describes a general numerical method for the determination of rate constants that characterize the binding of a ligand *L* simultaneously and competitively to two different receptor molecules, R_1 and R_2. The experimental data consist of changes in the concentration of one receptor (e.g., R_1) monitored over time. An example problem is represented by hirudin (*L*) binding to thrombin (R_1) and to a chemical mutant of thrombin (R_2). The published experimental data [Wedemeyer *et al.* (1997) *Anal. Biochem.* 248, 130–140], previously analyzed by using an appropriate algebraic method, were reanalyzed here by numerical integration [Kuzmic (1996) *Anal. Biochem.* 237, 260–273]. This general numerical method offers the following advantages. (1) It provides an estimate for the lower limit on feasible values of association rate constants. (2) It is many orders of magnitude more accurate. (3) It is easily extensible to more complicated reaction mechanisms. (4) It uses a simpler formalism and it is thus more accessible to nonmathematicians. (5) A suitable computer program for the analysis of competitive binding kinetics can be obtained via the Internet (http://www.biokin.com).

Key Words: **enzyme kinetics; progress curves; rate constants; differential equations; enzyme inhibition; slow binding; tight binding; competitive binding; thrombin; hirudin.**

Thrombin is an enzyme that causes blood clotting when the vascular system is injured, thus preventing the loss of blood. Conversely, thrombin inhibitors are very important in preventing the formation of blood clots inside veins and arteries. One important inhibitor of thrombin is hirudin secreted by medicinal leech. Hirudin has been widely studied for its ability to bind with thrombin in a time-dependent fashion ("slow binding"), yielding a very strongly bound molecular complex ("tight binding"). This "slow, tight" binding between thrombin and hirudin leads to a rather complex mathematical model for the reaction time-course.

In this study, the mathematical complexity is further increased by observing the simultaneous (competitive) binding of hirudin to two different forms of thrombin, namely, the natural form and a chemically mutated form. The paper describes a general numerical method for the determination of rate constants that characterize the simultaneous, competitive binding of one biochemical ligand to two different receptor molecules. The thrombin–hirudin system is used as an illustrative example.

The published experimental data (1) were previously analyzed by using an *approximate algebraic method*. Problems which motivated the research reported here can be summarized as follows. The approximate algebraic method does not provide any estimate fore the association rate constants; it is insufficiently accurate for least-squares regression analysis; it cannot be extended to more complex reaction mechanisms; it is exceedingly complicated despite being only an approximation (the original report contains 97 numbered equations and 3 mathematical appendices); and the corresponding computer program is unavailable to the scientific public.

In contrast, the *general numerical method* described here can be used to determine the lower limit on feasible values of association rate constants; it is 13 orders of magnitude more accurate which makes it suitable for least-squares regression analysis; it can be extended to an arbitrarily complex binding mechanism; it is comparatively simple to use because it requires as input from the investigator only chemical equations by no mathematical equations; and the corresponding computer program (2) is available to the scientific public via the Internet (http://www.biokin.com).

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EXPERIMENTAL DATA

The experiments that are analyzed in this paper have been described elsewhere (1). Professor H. A. Scheraga (Cornell University) is gratefully acknowledged for supplying the published data in electronic format. Briefly, thrombin (178 nM) and hirudin (208 nM) were incubated until equilibrium was achieved. The total volume was 90 μl. The equilibrated mixture was then diluted with 10 μl dehydrothrombin so that the total concentration of the mutated enzyme was 165 nM. In a complementary experiments, dehydrothrombin (183 nM) and hirudin (208 nM) were incubated in 90 μl of buffer, and 10 μl thrombin was added so that the final concentration was 160 nM. Thus the final concentrations of all components were identical in both experiments; only the order of addition was different. The concentration of free thrombin was followed over time by using a kinetic assay described elsewhere (1).

KINETIC ANALYSIS

The statistical analysis of the thrombin competitive binding kinetics was accomplished by using the program DYNAFIT (2), a general tool for the study of biochemical kinetics and equilibria. The presumed reaction mechanism for simultaneous (competitive) binding of thrombin and dehydrothrombin to hirudin is shown in Scheme 1. From the input data shown in Fig. 1, DYNAFIT automatically derived all the required differential and algebraic equations.

The dynamic behavior of the thrombin–dehydrothrombin–hirudin system was internally represented in DYNAFIT (2) by a system of simultaneous, first-order, nonlinear, ordinary differential equations (ODE) (see Appendix). The numerical solution was accomplished by using a modification of the algorithm LSODE (3) (maximum local truncation error 10^{-17} nM). Equilibrium states of the thrombin–hirudin–dehydrothrombin system were internally represented by a system of simultaneous, nonlinear algebraic equations (see Appendix) which express the mass balance law for component reaction species. The numerical solution was accomplished by using a modification of the multidimensional Newton–Raphson method (4, 5) (absolute error tolerance 10^{-16} nM). Regression analysis was accomplished by using Reich's variation (6) of the Levenberg–Marquardt least-squares fitting algorithm (7). Standard errors of rate constants were computed from square roots of diagonal elements of the final variance–covariance matrix (8).

All rate constants were subjected to a determination of the asymptotic 95% confidence interval (9) by conducting a systematic search in the parameter space. This procedure consisted of many repeated least-squares regression analyses. The general principles of the confidence interval search are explained elsewhere (10–12). Briefly, each rate constant of interest was kept constant at its best-fit value, while all the remaining rate constants were optimized. The resulting value of weighted least squares was recorded. Subsequently the rate constant of interest was shifted to a lower or to a higher value, progressively away from the best-fit

```
[task]
   data  = progress
   task  = fit
   model = compet

[mechanism]
   E + L <===> EL  :  k1   k2
   F + L <===> FL  :  k3   k4

[constants]
   k1 = 0.001 ?? , k2 = 0.00005 ??
   k3 = 0.001 ?? , k4 = 0.00005 ??

[concentration]

[response]
   E = 1

[progress]
   directory data/thrombin
   extension txt
   delay     30

   file d1e | concentration F=165 ?
      equilibrate   E=178, L=208, dilute 90:100

   file d4e | concentration E=160 ?
      equilibrate   F=183, L=208, dilute 90:100

[settings]

   <filter>      | Scale = hours
   <constraints> | ConcError = 0.5
   <confidence>  | Level = 95
   <marquardt>   | Interrupt = 50

[end]
```

FIG. 1. DYNAFIT (2) script file for the least-squares fit of the thrombin (*E*)–dehydrothrombin (*F*)–hirudin (*L*) competitive binding data from Fig. 1 of Wedemeyer *et al.* (1).

			$k_{\rightarrow}$	$k_{\leftarrow}$
E + L	$\rightleftharpoons$	EL	k_1	k_2
F + L	$\rightleftharpoons$	FL	k_3	k_4

SCHEME 1

value. At each step, all the remaining rate constants were again subjected to least-squares optimization. As the rate constant of interest moved away from its best-fit value, the sum of least-square deviations naturally increased. Statistical theory gives us a critical value of this increase in the sum of squares for each probability level of the confidence interval. The sequence of values to be explored for each rate constant of interest was determined by an automatic algorithm to be described elsewhere.

DYNAFIT Script File

The complete DYNAFIT script file used for the thrombin–hirudin–dehydrothrombin data is shown in Fig. 1. In the `[mechanism]` section, the first rate constant after the colon always refers to the left-to-right arrow in each reversible reaction step, while the second rate constant refers to the right-to-left arrow. In the `[constants]` section, the values of rate constants are initial estimates. Those values that are followed by a question mark are selected for least-square optimization. Values followed by two question marks are selected for a 95% confidence interval search. In this example we have chosen to represent all concentrations in nanomolar units, thus the dimension of all bimolecular rate constants is $nM^{-1}\ s^{-1}$. The `[responses]` parameter links the concentrations of species to the observable physical quantity. In this case the free thrombin concentration was measured directly (see the original report (1) for details); therefore, the molar response coefficient of free thrombin is set to unity, while the other species are ignored. The `delay` parameter in the `[progress]` section defines the time in seconds that elapsed after the last component was added to the assay, and before the first measurement of thrombin concentration was made.

RESULTS

Changes in free thrombin concentration over time were fitted to the reaction mechanism shown in Scheme 1 by using the DYNAFIT script file displayed in Fig. 1. The adjustable parameters were all four rate constants that appear in the reaction mechanism. The total concentrations of thrombin and hirudin were kept constant in the least-squares fit because preliminary computations indicated that the nominal values (160 and 178 nM, respectively) were in good agreement with the available experimental data. On the other hand, the concentrations of dehydrothrombin (nominally 165 nM) were optimized separately for each data set because the concentration of the mutated enzyme was known less precisely than the concentration of the native enzyme.

The best-fit values of rate constants k_1 through k_4 are summarized in Table 1. The dissociation of the thrombin–hirudin complex is governed by the rate constant $k_2 = 22$ [15, 40] μs^{-1}, while the dehydrothrombin–hirudin complex is marginally faster at $k_4 = 30$ [25, 39] μs^{-1}. The best-fit values of the *association* rate constants and the associated 95% confidence intervals are $k_1 = 0.69$ [0.17, ∞] $\mu M^{-1}\ s^{-1}$ for thrombin and $k_3 = 0.38$ [0.06, ∞] $\mu M^{-1}\ s^{-1}$ for dehydrothrombin.

TABLE 1

Rate Constants for Thrombin–Dehydrothrombin–Hyrudin Interactions (see Scheme 1) and in Square Brackets the Corresponding 95% Confidence Intervals

Set	Parameter		Best fit	Ref. (1)
	k_1	$\mu M^{-1}\ s^{-1}$	0.68 [0.17, ∞]	—
	k_2	μs^{-1}	22 [15, 40]	19 [16, 23]
	k_3	$\mu M^{-1}\ s^{-1}$	0.38 [0.06, ∞]	—
	k_4	μs^{-1}	30 [25, 39]	29 [26, 32]
	k_1/k_3		1.8	1.7 [1.3, 2.5]
	k_1k_4/k_2k_3		2.7	2.6 [2.4, 2.9]
1	$\hat{c}_F$	nM	137.4 ± 14.6	—
2	$\hat{c}_F$	nM	139.5 ± 18.9	—
3	$\hat{c}_F$	nM	125.7 ± 12.9	—
4	$\tilde{c}_F$	nM	186.4 ± 2.5	—
5	$\tilde{c}_F$	nM	185.6 ± 2.5	—
6	$\tilde{c}_F$	nM	191.0 ± 2.5	—

Note. The "hat" accent ($\hat{c}$) represents the best-fit concentration of dehydrothrombin (nominal value 165 nM) after thrombin was added to the assay. The "tilde" accent ($\tilde{c}$) represents the best-fit initial concentration of dehydrothrombin (nominal value 183 nM) in the inverse experiment.

The graphical results of the least-squares fit are shown in Fig. 2. It can be seen that the data and the theoretical fitting model are in very good agreement, similar to the original report (1). The results of the confidence interval search are summarized in Fig. 3 and in Table 1.

The 95% confidence interval limits for the *dissociation* rate constants were more or less in agreement with the formal standard errors. For example, the best-fit value and the formal standard error of the rate constant k_2 was (22 ± 3) μs^{-1}, which corresponds to the predicted 95% confidence interval $k_2 = 22$ [16, 28] μs^{-1}. The confidence interval found in the systematic search was $k_2 = 22$ [15, 41] μs^{-1}. The confidence interval is nonsymmetrical and wider than the standard errors suggest, but at least it is closed both from above and from below.

In contrast, the 95% confidence interval limits for the *association* rate constants are open-ended on one side. Only the lower limit could be determined, but (disregarding the physical constraints imposed by diffusion) the upper limit on these association rate constants is infinity (see Fig. 3a). The lower 95% confidence interval limit seems quite well determined by the experimental data. The thrombin association rate constant certainly is higher than 0.7 $\mu M^{-1}\ s^{-1}$, while

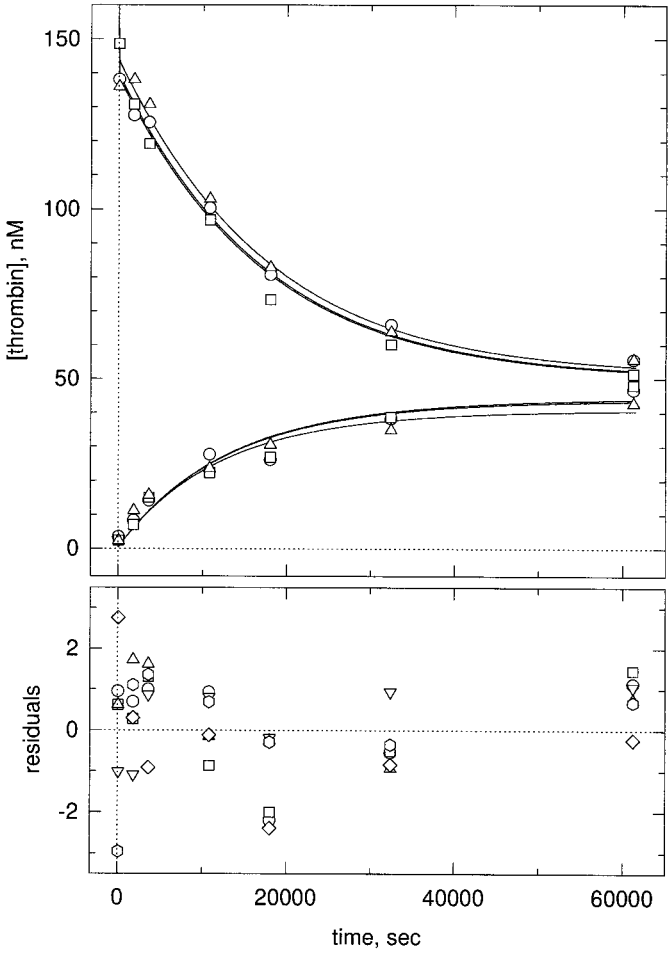

FIG. 2. Results of least-squares fit of the thrombin (E)–dehydrothrombin (F)–hirudin (L) competitive binding data from Fig. 1 of Wedemeyer *et al.* (1) to the reaction mechanism shown in Scheme 1 (DYNAFIT) (2) script file in Fig. 1).

for dehydrothrombin the lowest reasonable value of the association rate constant k_3 is 0.4 μM^{-1} s^{-1}.

DISCUSSION

It has been known for decades that fitting progress curves permits the estimation of individual rate constants, whereas methods based on pseudo-equilibrium hypotheses only permit estimates of kinetic constants that are combination of rate constants. However, the challenging feature of the thrombin–dehydrothrombin–hirudin system is that each experiment contains both an equilibrium phase, during which the components are incubated until reaching stationary state, and a dynamic phase during which the composition changes over time. Thus in this case a generalized numerical analysis of the reaction progress is inseparable from the numerical simulation of preexisting equilibria. It is the dual nature of the problem at hand which, in a more traditional approach to data analysis, creates rather exceptional algebraic complexity (1). In this work all algebraic complexity was circumvented simply by invoking the mass action law both for the equilibrium and for the dynamic phase of the experiment.

The problem of competitive simultaneous binding of two different ligands to the same receptor has been treated in the literature mostly from the equilibrium point of view. For example, we have previously studied the tight binding of dihydrofolate reductase with the antileukemic drug, methotrexate, and simultaneously with one of its metabolites, methrotrexate–α-aspartate (13). We also designed an assay for the determination of ligand–receptor binding constants by using a fluorescent ligand (a fluorescent analog of the immunosuppressant cyclosporin A) as a reporting agent, which competed for recombinant human cyclophilin with a cyclosporin A analog (14).

Even such relatively simple systems, including two ligands binding simultaneously to the same receptor, are quite difficult to describe mathematically. Although Wang (15) was able to derive an exact mathematical expression for competitive binding of two different ligands, using our cyclosporin data (14) as a test example, the final algebraic expressions are quite complicated. It appears simpler to calculate the equilibrium composition by using iterative computations, based in certain special cases on simple recurrent formulas (13–16), or in the general case on matrix computations (5).

In this paper we have examined a case similar to those discussed above, namely, the case of two receptor molecules (thrombin and one of its chemical mutants) binding simultaneously to the same ligand (hirudin). The kinetic analysis must include solving a system of differential equations. One well-known biochemical software system designed for this purpose consists of the programs KINSIM/FITSIM (17–19). A reviewer has suggested that the rate constants for competitive binding could in fact be solved by using those programs, but this statement is incorrect. The programs KINSIM/FITSIM were not designed to perform computations on biochemical systems at equilibrium. Thus if the kinetic phase of a biochemical assay is preceded by an equilibrium phase, during which an arbitrary number of components is equilibrated, KINSIM/FITSIM cannot be used for data analysis.

Neither can competitive kinetics be investigated by those general-purpose, commercial software packages which do not require full-scale compute programming on the part of the investigator, for example, by computer programs Axum, EnzFitter, FigP, Keleidagraph, Origin, Prism, SigmaPlot, UltraFit, WinNonlin, WinZyme, and others that were examined as part of this research. Some commercial software systems for data analysis do allow the user to write and compile sepa-

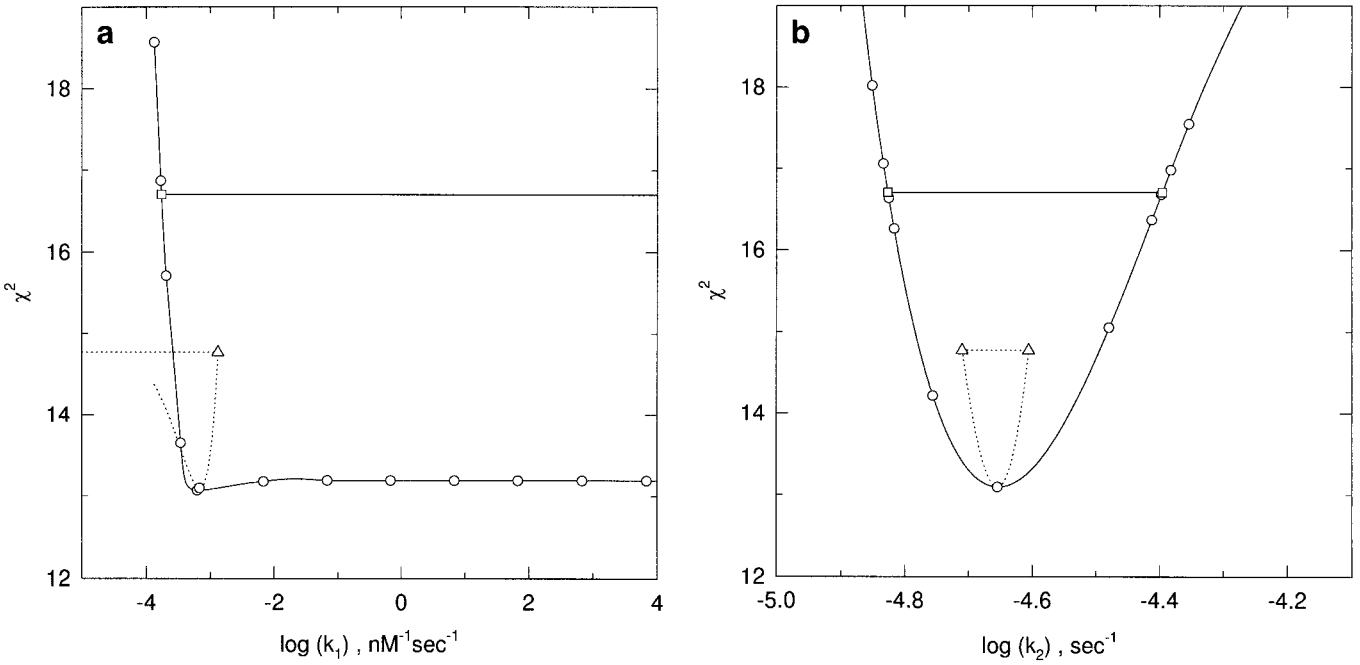

FIG. 3. Results of confidence interval estimation for rate constant k_1 (a) and k_2 (b). Similar results were obtained for rate constants k_3 and k_4, respectively. The solid horizontal line marks the allowed increase in the weighted sum of square corresponding to the 95% confidence level. The limits of the 95% confidence interval are represented by squares, while the limits of the formal standard error by triangles. The dotted curve is a parabola computed from the formal standard errors.

rate software modules in programming languages, such as FORTRAN or C, but most practitioners of experimental enzyme kinetics lack expertise in this kind of software development.

For example, Taylor *et al.* (20) studied the competitive binding of collagenase to TIMP-1 ("slow, tight" inhibitor of collagenase) and simultaneously to a radiolabeled analog of TIMP-1. This problem is a simpler variant of the competitive binding studied here because TIMP-1 and labeled TIMP-1 can be assumed to have the same binding constant. The authors used the software system Scientist (MicroMath Scientific Software), supplemented by a specially compiled software module representing the differential equation solver EPISODE (21). The authors then used their proprietary software system to fit progress curves for "slow, tight" inhibition of collagenase. Interestingly, they never attempted a quantitative treatment of the TIMP/collagenase competitive binding data, which is only displayed but not analyzed in their paper. Thus, the performance of Taylor's software for the task at hand remains unknown.

The kinetic analysis of competitive binding was previously attempted by using an "approximate algebraic method," based on the derivation of algebraic formulas that are quite complicated, while still only approximations to the true solution. Both the approximate algebraic approach (1) and the general numerical approach (2) used here gave essentially identical estimates for the *dissociation* rate constant of the thrombin–hirudin complex. Thus Wedemeyer *et al.* (1) found $k_2 = 19$ [16, 23] μs^{-1}, while we have $k_2 = 22$ [15, 40] μs^{-1}. Similarly, for the chemical mutant of thrombin Wedemeyer *et al.* found $k_4 = 29$ [26, 32] μs^{-1}, while we have $k_4 = 30$ [25, 39] μs^{-1}.

The algebraic method (1) and the numerical method (2) used here also agree on various ratios of microscopic rate constants. For the ratio of overall affinities, Wedemeyer *et al.* (1) found $k_1k_4/k_3k_2 = 2.6$ [2.4, 2.9], while we have $k_1k_4/k_3k_2 = 2.7$, essentially an identical value. For the ratio of association rate constants, Wedemeyer *et al.* found $k_1/k_3 = 1.7$ [1.3, 2.5], while we have $k_1/k_3 = 1.8$. On the basis of these two rate constant ratios, both methods lead to the same conclusions which can be summarized as follows. First, the overall association equilibrium constant of dehydrothrombin is 2.6 times lower than thrombin, which corresponds (only) to about 0.6 kcal/mol difference in binding energy. Second, most of this difference between the native enzyme and the mutated enzyme comes from *association* rates. Only a small additional contribution comes from the difference in *dissociation* rates.

Because in most respects the two computational methods, analytical (1) and numerical (2), gave virtually identical answers, it is legitimate to ask what is the advantage in using either method for the analysis

of competitive binding kinetics. One advantage of the "approximate algebraic method" is that is does not require numerical integration of stiff systems of differential equations, and therefore it is probably faster than the numerical method used here. Even so, the least-squares regression analysis of the thrombin data by using the program DYNAFIT (2) took only 17 s on a standard desktop personal computer.

The numerical method presented here offers several major advantages: (1) it provides at least the lower limit for the association rate constants; (2) it is much more accurate than the approximate algebraic method; (3) it can be extended to more complex mechanisms; (4) the theoretical model is represented by a set of chemical equations, thus avoiding tedious derivation of algebraic and differential equations; and (5) the requisite computer program is publicly available. These advantages are now briefly discussed in their turn.

The first advantage of our numerical method is that can be used to estimate the lower limits for the association rate constants, $k_1 > 0.7\ \mu M^{-1}\ s^{-1}$ and $k_3 > 0.4\ \mu M^{-1}\ s^{-1}$ for thrombin and dehydrothrombin, respectively. In contrast, the authors of the approximate analytic method write, "it should be noted that . . . the fast-time rate constants [k_1 and k_3] are not individually determined, only their ratio" (1).

The infinite upper limit of the confidence interval for both association rate constants may appear unusual. Confidence intervals for adjustable model parameters usually are as considered symmetrical, and are most often estimated simply from formal standard errors. However, this simplified treatment of parameter uncertainty is only valid if the adjustable model parameters are not at all correlated, that is, if the best-fit values do not influence each other. Unfortunately, for any nonlinear fitting model such as the competitive binding kinetics described here, the assumption of parameter independence does not hold. Because biochemical kinetic model without exception are intrinsically nonlinear and contain mutually correlated parameters, many standard errors of parameters published in biochemical literature are strictly speaking meaningless. Ths problem of proper confidence interval estimation in biochemical kinetics is thoroughly explained in several practically oriented reviews (10–12).

The infinite upper bound on the association rate constants is caused by severe intrinsic ill-conditioning. As a matter of principle, no method of analysis could ever extract the upper limit from the data used in this study. However, if the association of thrombin and dehydrothrombin with hirudin were found on a much shorter scale (e.g., seconds instead of hours), then the association rate constants could be determined easily. Therefore, the ideal data for a complete investigation of the thrombin system would consist of several progress curves collected on disparate time scales.

The second major advantage of our numerical method is that it is many orders of magnitude more accurate than the approximate analytical method. While Wedemeyer *et al.* (1) computed the changes of thrombin concentration with the uncertainty as high as 25×10^{-3} nM, the accuracy of the numerical solution used here (2) is 10^{-17} nM. To justify their approximate analytical method, Wedemeyer *et al.*, (1) make a direct comparison between the accuracy of the computations and the accuracy of available experiments. However, in the least-squares regression analysis the underlying theoretical model must be computed many orders of magnitude more accurately than the experimental data are measured, for the following reason.

Least-squares regression requires repeated computation of *derivatives* to the theoretical curve with respect to the optimized parameters. In their turn, these derivatives are obtained at each step of the iterative regression by the "finite-difference" method ([8], p. 186), that is, by computing two minutely different theoretical curves. The first is obtained by using the current values of the adjustable parameters, while the second curve is obtained by altering one of the adjustable parameters to a very small degree (typically by 0.01%). The difference between the two very closely matched curves, divided by the small increment in the parameter of interest, is approximately equal to the desired derivative. Understandably, in taking a very small difference between two very close numbers, both of these numbers must be computed very much more accurately than the desired result. The exact formulas governing the computational accuracy of the "finite-difference" method are given as Eq. [5.7.5] through [5.7.9] in the reference cited above (8).

With regard to the numerical accuracy of the present method, it should be noted that the local truncation error (10^{-17} nM) of the integration procedure accumulates over the entire progress curve. In the end, the global truncation error collected over many small integration steps could be larger than the local error, depending on the extent to which these small local errors mutually amplify or (occasionally) cancel. The important point is that both local and global truncation error control must be considered a very important part of any numerical simulation procedure.

The third advantage of the numerical method used here is that it can be easily extended to more complex reaction mechanisms. For example, if the competitive binding experiment were performed with an enzyme that dissociates into monomeric subunits, an algebraic model could not be derived as a matter of principle. In contrast the fitting model based on differential equations always exists.

The fourth advantage of the numerical method is that it relies on a comparatively simple mathematical formalism, which can be derived automatically by the

computer form the reaction stoichiometry. Thus, while Wedemeyer's paper (1) contains 97 numbered equations and 3 mathematical appendices, the entire input for program DYNAFIT consists of the script file in Fig. 1. Many practicing biochemists prefer writing $E + L \leftrightarrow EL : k_1\ k_2$; $F + L \leftrightarrow FL : k_3\ k_4$ over handling Bernoulli equations, "slow-time" and "fast-time" approximations, "quasi-equilibrium" solutions, or the "matched asymptotic expansion" method (1).

Finally, the fifth major advantage of the numerical method is that it is publicly available to any interested enzymologist. While the data-analysis software developed by the Cornell group (1) is currently unavaiable to the scientific public, the program DYNAFIT can be downloaded from the Internet at http://www.biokin.com. Researchers without access to the Internet can send a self-addressed stamped envelope including a blank formatted computer diskette to the author's address.

APPENDIX

From the symbolic representation of the reaction mechanism shown in Fig. 1, DYNAFIT (2) derived a system of differential Eq. [1] through [5], which describe the time course of the competitive binding experiment. The equations are solved numerically by using a modification of the algorithm LSODE [3].

$$\dot{c}_E = -k_1 c_E c_L + k_2 c_{EL} \quad [1]$$

$$\dot{c}_L = -k_1 c_E c_L + k_2 c_{EL} - k_3 c_L c_F + k_4 c_{FL} \quad [2]$$

$$\dot{c}_F = -k_3 c_L c_F + k_4 c_{FL} \quad [3]$$

$$\dot{c}_{EL} = k_1 c_E c_L - k_2 c_{EL} \quad [4]$$

$$\dot{c}_{FL} = k_3 c_L c_F - k_4 c_{FL} \quad [5]$$

DYNAFIT (2) also derived automatically the system of simultaneous nonlinear algebraic Eq. [6] through [8], which describe the mass balances of component species. The tilde accent ($\tilde{c}$) stands for total or analytic concentrations. Ratios of rate constants (e.g., k_1/k_2) are treated as unique association equilibrium constants. The algebraic equations are solved numerically by using a modification of the algorithm EQUIL (5).

$$\tilde{c}_E = c_E + \frac{k_1}{k_2} c_E c_L \quad [6]$$

$$\tilde{c}_F = c_F + \frac{k_3}{k_4} c_F c_L \quad [7]$$

$$\tilde{c}_L = c_L + \frac{k_1}{k_2} c_E c_L + \frac{k_3}{k_4} c_F c_L \quad [8]$$

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