Analysis of progress curves by simulations generated by numerical integration

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A highly flexible computer program written in FORTRAN is presented which fits computer-generated simulations to experimental progress-curve data by an iterative non-linear weighted least-squares procedure. This fitting procedure allows kinetic rate constants to be determined from the experimental progress curves. Although the numerical integration of the rate equations by a previously described method [Barshop, Wrenn & Frieden (1983) *Anal. Biochem.* **130**, 134–145] is used here to generate predicted curves, any routine capable of the integration of a set of differential equations can be used. The fitting program described is designed to be widely applicable, easy to learn and convenient to use. The use, behaviour and power of the program is explored by using simulated test data.

INTRODUCTION

Data associated with monitoring a chemical or enzymatic reaction to completion are often referred to as a 'full time course' or 'progress curve'. Progress curves are a potentially powerful tool in the estimation of kinetic parameters and in differentiation between kinetic mechanisms. Despite this potential, the difficulties encountered in model diagnostics and computer implementation of the complex algorithms involved in the determination of rate constants from progress curves have limited its general use. In this paper we present the description of a recently developed computer program named FITSIM that analyses progress-curve data for a wide variety of kinetic mechanisms. This program is designed to be user-friendly and highly flexible in its operation.

Although previously reported computer programs have allowed the analysis of full-time-course reactions to specific mechanisms (i.e. Canela & Franco, 1986; Gordon *et al.*, 1986; Cox & Boeker, 1987), FITSIM is designed to allow, at least in principle, the analysis of nearly any user-entered mechanism. Examples of the use of this program are given by Zimmerle *et al.* (1987), Kurz *et al.* (1987), Sekhar & Plapp (1988) and Lee *et al.* (1988).

The flexibility of the system described is due, in part, to the operation of the kinetic simulation routines utilized by FITSIM. These kinetic simulation routines are derived from the program KINSIM (Barshop *et al.*, 1983), which explicitly establishes the differential equations for a reaction mechanism that are then numerically integrated to yield outputs at given time intervals. This approach eliminates the need for steady-state approximations, overcoming some experimental limitations and errors associated with this approximation (Farrow & Edelson, 1974). Furthermore, the simulation routines used can, in principle, monitor any physical parameter known to be proportional to the reaction time course (e.g. fluorescence, absorbance, activity, radioactivity, etc.).

FITSIM is written in FORTRAN 77 and is anticipated to be largely machine-independent, highly flexible in its operation and relatively easy to use. In this paper we will present the major options available to the user and show how this program can be used in the analysis of full-time-course reactions.

THEORY

In general, the utilization of progress curves in kinetic analyses has mostly been limited to relatively simple Michaelis–Menten type kinetics and has involved fitting data to an analytically derived integrated rate equation (e.g. Kellershohn & Laurent, 1985; Cox & Boeker, 1987) or directly to the rate equation (e.g. Canela & Franco, 1986; Koerber & Fink, 1987). The program described here fits experimental progress curve data to curves generated by the KINSIM program (Barshop *et al.*, 1983). The method of numerical integration used by KINSIM to generate the predicted progress curves utilizes both the Gear (1971) method and the flux-tolerance method to keep the numerical solution within the bounds set by the user. This approach was described in detail previously (Barshop *et al.* 1983; Barshop, 1984). The structure of FITSIM is such, however, that any routine capable of generating a simulated progress curve can be used and calls to the KINSIM subroutine could be redirected to any subroutine capable of generating the predicted time course.

In FITSIM, each point at a given time in the simulated progress curve is then numerically compared with the corresponding time point in the experimental curve by calculating the summation of the squared differences (SSQ) between the two. The overall SSQ value between all the simulations and experimental progress curves is determined by eqn. (1),

$$SSQ = \sum_{s=1}^{n_s} w_s \left[\sum_{t=1}^{t=m} w_t (\text{exp}_t - \text{cal}_t)^2 \right] \quad (1)$$

Abbreviation used: SSQ, sum of squares.

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where n_s is the number of data sets, or reaction time courses, m is the maximum simulation time, whereas exp_i and cal_i are the experimental and calculated points at the reaction time, t , for a given simulation parameter set, s . Unequal variance of the experimental data at time t can be compensated for by the weighting factor w_i . The term w_i is routinely used to normalize each full time course to a common value. This compensates for differences in the number of data points or overall scale in different progress curves and prevents over-weighting of one reaction progress curve over another. In the present implementation of FITSIM it is assumed that all progress curves are of equal weight.

Since the determined SSQ is a measure of the goodness-of-fit between the experimental and simulated data, decreases in this value should lead to an overall better fit. The value of the SSQ is decreased by using a non-linear least-squares regression algorithm. Such algorithms can be expressed as:

$$Y = F(\beta_0, \beta_1, \dots, \beta_k; X_1, X_2, \dots, X_n) + \epsilon \quad (2)$$

where ϵ is the residual error, β_i represents all the unknown non-linear parameters and X_i represents the various independent variables of a function, F . It is clear from this equation that an inadequate number of β parameters (k) will result in a larger ϵ , whereas if a model perfectly fits the observed data, ϵ is equal to zero. From the initial estimates of the β parameters, corrections are applied which result in a better fit of the observed to the simulated data as defined by a lower value of ϵ until corrections become negligible and no further reduction in the SSQ is possible.

PROGRAM OPERATION

Scheme 1 shows the relationship between the four major routines composing FITSIM. The arrows in Scheme 1 indicate input or output between the modules.

The REGRESS module contains a non-linear regression algorithm which calculates the changes in the kinetic constants believed to result in a better fit between the experimental data and the predicted data generated by the module KINSIM. Although in the present implementation the algorithm is based on one first described by Marquardt (1963), others could be incorporated here as well. The module KINSIM contains the kinetic simulator routines and is essentially identical with the program KINSIM, previously described by Barshop *et al.* (1983), running in supervised mode. Use of this module requires prior mechanism entry followed by compilation to set up the differential equations and simulation necessary for the numerical iteration of these equations. Other routines capable of calculating predicted data at a given reaction time may also be inserted at this point, but the use of the KINSIM program as the kinetic simulator enables many of the user-friendly features of the KINSIM program to be implemented in FITSIM. The module AUTOSIM is the communication linkage between the KINSIM and REGRESS modules and is also responsible for data input and output. The module FITDATA compiles the necessary information needed by the AUTOSIM routine into a sequential file read by the main program.

The present program is compiled with a VAX FORTRAN 77 compiler under VAX/VMS ver. 4.7. Since only a few VAX-specific RTL routines are used in the program, and these are unnecessary for the basic operation of the program, the program should be portable to other computer systems with a good FORTRAN 77 compiler. Of course system-specific modules such as file in/out routines and plotting routines will probably need to be modified.

The time required for program execution depends not only on the complexity of the mechanism, but also on the number of varied parameters, the number of curves, and the number of data points. Although generally less than 20 iterations are required to achieve convergence, for

Scheme 1.

Table 1. Files used in FITSIM

File name	File contents	Comment
Data.DAT Mechanism.MEC	Experimental time course Mechanism description Output equations	Generated from real data The proposed mechanism
Mechanism.SIM	Binary description of mechanism	Compiled mechanism needed for KINSIM simulations. Generated from KINSIM
Parameter.SAV	Initial kinetic parameters, reactant concentrations, timing and tolerance levels. Output factors	Generated by using KINSIM to give initial fitting using Mechanism.SIM
Autosim.FIT	Names of Data.DAT files, weighting options for fitting, linkages between rate constants	Created by file editor on FITDATA program
Log.LOG	Output file containing fitted kinetic constants and other information	Generated by FITSIM

each iteration the minimum number of generated kinetic simulations is equal to $(NV + 1) \cdot ND$ where NV is the number of freely varied constants and ND is the number of progress curves. Thus except for relatively simple models, FITSIM usually requires a large amount of computational time. The implementation of FITSIM used here requires approx. 300 kilobytes of memory, depending slightly on the terminal version compiled. In addition there is a separate command program available for VAX/VMS systems which integrates the simulation (KINSIM), analysis (FITSIM), and mechanism compiler programs with each other. This command procedure also allows an extensive amount of on-line help to be accessed.

A brief description of the files used in the input execution of FITSIM is given in Table 1. As noted, there is complete compatibility between the files used by the kinetic simulator program, KINSIM, and those used by FITSIM. This facilitates the use of both programs, since fits can easily be transferred between both programs. Entry of a specific mechanism is typed in using conventional chemical format and compiled in the same manner as described previously for KINSIM (Barshop *et al.*, 1983). Plots of the final fits obtained by FITSIM can be displayed using KINSIM. Although experimental data must be in a specific format to be able to be read in by the system, conversion into this format is relatively simple and requires only a minimal amount of FORTRAN programming. Any piece of computer-interfaced equipment should be able to generate data in a form usable by FITSIM.

METHODS

As indicated in Table 1, before a regression analysis of any progress-curve data can begin, initial values for each of the kinetic constants contained within a given mechanism must be specified. Although it is impossible to give any general guidelines, it is usually not difficult to estimate initial values for the kinetic constants based either on experimental evidence or preliminary simulations using KINSIM. The choice of initial values is more important when a large number of kinetic constants are being varied.

There are several options available to tailor the output and/or regression analysis to the needs of the user. For

instance, to decrease the number of freely varied rate constants or to maintain a given ratio between rates, ratios between rate constants (i.e. linked rate constants) can be defined. For any given group of linked rate constants, only one of the rate constants is allowed to vary, while the value for all other rate constants in the linkage group are calculated according to ratios entered by the user. In addition, different options exist within the program for setting the weights associated with the data in an individual progress curve (w_i in eqn. 1), although usually this parameter is assigned a value of 1, making all the data points in a given curve equal weight. This appears to be the best option to use for most data sets, and it is desirable to edit data files to eliminate data after the reaction is essentially complete.

Executing FITSIM creates output in the form of a sequential disk file named by the user (Log.LOG of Table 1). This file includes a description of the mechanism and initial values for the kinetic constants, the names associated with the data files being fitted, the regression options selected, the kinetic constants varied, and the simulation parameters used. Log.LOG also contains the results of the fitting procedure, i.e. the final SSQ, the weighted mean SSQ, standard deviation of the fit and the estimated kinetic parameters and their associated errors. All of these values are important when comparing the quality of fits achieved by rival kinetic mechanisms. A good model gives meaningful values for the kinetic constants with low standard deviations (generally less than 50%), a low value for the SSQ, and a value for the weighted mean SSQ compatible with the experimental variance (Mannervik, 1982).

As with nearly any other non-linear regression procedure, it is always possible that the final fit obtained is not the best one. Since only relatively small changes in the kinetic constants occur after each iteration, it is possible that a much larger change in the kinetic constants at a given point would lead to a much better fit. To prevent this problem, repeat runs of FITSIM should be performed with different initial starting values for the kinetic constants.

RESULTS

A simple kinetic mechanism, described by Scheme 2, is used here to demonstrate several features of the program.

This mechanism is essentially the same as that used previously to fit progress curves associated with a Mg^{2+} -induced conformational change in actin (Zimmerle *et al.*, 1987). With this scheme the binding of A induces a conformational change in the protein, P.

By using Scheme 2, simulations were generated by KINSIM using different concentrations of A, P and AP' and using the kinetic parameters $1 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ for k_{+1} , 1000 s^{-1} for k_{-1} , 0.1 s^{-1} for k_{+2} and 0.02 s^{-1} for k_{-2} . Random error was then introduced into these simulated progress curves by the Monte Carlo method to generate pseudo-experimental data. This involved the addition of a random number, derived from a Gaussian error distribution of 60 %, to the simulated progress curves. This pseudo-experimental data was subsequently used to test the operation of the program.

Fitting of the data was initiated with initial estimates of 5000 s^{-1} for k_{-1} , 0.5 s^{-1} for k_{+2} and 0.1 s^{-1} for k_{-2} . The value of k_{+1} was held constant at $1 \mu\text{M}^{-1} \cdot \text{s}^{-1}$. Table 2 summarizes the final parameter values obtained using simulated experimental data with maximum relative errors of 0, 20, 60 and 100 % respectively. Although the final SSQ increased considerably as the amount of random error increased, little difference in the final parameter estimates obtained by FITSIM is found. Thus random error has only a small effect on the accuracy of the final parameter estimates obtained. Fig. 1 shows the simulated experimental data with 60 % relative error along with the predicted progress curve of best fit. In addition, Fig. 1(b) demonstrates the potential of FITSIM not only to fit experimental progress curves following the concentration of AP', but also to fit simultaneously experimental data which follows the concentration of P.

Table 3 shows that the accuracy of the final parameter estimates is altered when a 10 % underestimation of the concentration of A occurs. This underestimation of the concentration of A results in an overestimation of the affinity of P for A. No increases in the errors associated with the final parameters are obtained; thus such error, at least in this case, might go undetected. A large increase in the errors associated with the final parameter estimates occurs with either over-parameterization of a model or

an insufficient amount of data. Table 3 clearly demonstrates the effect of the latter.

The KINSIM subroutine is capable of simulating more than one output at a time. This allows the simultaneous fitting of experimental data obtained by two different methods, i.e., data which follow the appearance of product can be analysed at the same time as data which follow the disappearance of substrate. This capability is demonstrated in Fig. 1.

To demonstrate the versatility of the program and its ability to analyse complex kinetic schemes, analysis of pseudo-experimental data with 20 % relative error to Scheme 3 was undertaken. Scheme 3 is the mechanism proposed by Frieden & Goddette (1983) for the polymerization of actin, where A represents actin, A'Mg represents Mg^{2+} -activated actin, A'Ca represents actin with bound Ca^{2+} and AMg_2 represents actin with two molecules of Mg^{2+} bound. K_{Mg1} and K_{Mg2} describe the initial binding to the first and second Mg^{2+} sites respectively, K_{dimer} , K_{trimer} , and K_{tet} the formation equilibrium for dimer, trimer and tetramer formation respectively, and k_{-e}/k_{+e} the dissociation constant for filament formation. The pseudo-experimental data follows a fluorescence increase upon the formation of dimer and higher- M_r species.

Scheme 3.

A problem with the analysis of complex mechanisms with a large number of freely varied rate constants is that large errors may be associated with the final determined values. This is to be expected, owing to the more

Table 2. Effect of random error on the final parameter estimates by FITSIM

Errors were randomly introduced by the maximum percentage shown into four progress curves generated by kinetic simulations to Scheme 2. Errors introduced followed a Gaussian distribution.

Parameter varied	No error	Relative error ... 20 %	60 %	100 %
$k_{-1} (\text{s}^{-1})$	1000.3	992.4 ± 9	976.5 ± 24.2	959.7 ± 40.0
$k_{+2} (\text{s}^{-1})$	0.100	0.099	0.098 ± 0.001	0.097 ± 0.001
$k_{-2} (\text{s}^{-1})$	0.0200	0.0200	0.0200 ± 0.0003	0.020 ± 0.004
Weighted mean SSQ	4×10^{-8}	9×10^{-5}	8×10^{-4}	2×10^{-3}

Fig. 1. Example of progress curve analysis using the FITSIM program, Scheme 2 and pseudo-experimental data which follows the concentration of AP'

Kinetic values taken from Table 2 in which there was an initial 60% relative error. In (a) the continuous lines represent the best fits to pseudo-experimental data with $[A] = 5 \text{ mM}$ (●), $500 \mu\text{M}$ (▲) and $50 \mu\text{M}$ (■) in the presence of $10 \mu\text{M}$ -protein P. In (b) the continuous line represents the best fit through the pseudo-experimental data for the reverse conformational change when $[AP']$ is $10 \mu\text{M}$. The broken line follows the generation of P by this reaction and demonstrates the potential of FITSIM to follow both product accumulation and substrate depletion in the same analysis. Pseudo-experimental data were generated by randomizing simulated progress curves produced by KINSIM as described in the text.

numerous degrees of freedom in the model. Furthermore, reported standard errors cannot be taken too seriously, as they are based on linearizing assumptions and always underestimate the true uncertainty (Motulsky & Ransnos, 1987). The extent to which a particular error value is underestimated depends upon the particular mechanism, progress-curve data and weighting scheme utilized. The best method to determine the amount of errors associated with a particular analysis is to systematically generate data with a known amount of error and then to analyse this pseudo-experimental data, as was done here.

Table 4 demonstrates parameter values are poorly determined when a particular method used in monitoring the reaction is relatively insensitive to changes in these rate constants. This occurs even when a presumably wide range of concentrations are analysed. Thus the kinetic parameters with the largest errors in Table 4 are those which only indirectly alter the increase in fluorescence seen, constants, such as K_{Mg} . However, since the ratio of the Mg^{2+}/Ca^{2+} -binding affinity at the first site does directly influence the fluorescence increase upon the polymerization of actin, this ratio is nearly constant at 5.2. As shown by analysis C of Table 4, simply setting the correct value for k_{+1} gives a considerably better end result. Therefore, particularly in analyses where a large number of kinetic parameters are varied, care should be exercised in the interpretation of both the final values obtained and their associated errors.

DISCUSSION

The method described here to fit progress-curve data allows great flexibility and ease of use. Progress-curve simulations generated by using numerical-integration techniques are used to create predicted curves. The kinetic parameters used in creating these predicted curves are then adjusted in an iterative manner by non-linear regression techniques to obtain the best fit between the experimental and predicted curves. Although fits to experimental data have been obtained previously by manually comparing simulated and experimental progress curves (e.g. Penner & Frieden, 1987), this method was tedious, time-consuming and allowed no parameter errors to be obtained. The program described here, FITSIM, eliminates such limitations while maintaining and expanding upon the strengths of this approach to analysing progress curves. FITSIM can fit experimental progress-curve data to widely different mechanisms and to test the resultant fits by statistical methods.

It is quite possible that a given rate constant may yield a wide range of values under certain experimental conditions, especially when dealing with relatively complex mechanisms. As demonstrated by Frieden & Goddette (1983), this occurs particularly when an insufficiently wide range of experimental conditions is analysed. Not only can this usually be avoided by selection of a proper range of experimental conditions, but an option in FITSIM has been implemented to check for such possibilities. Furthermore, insufficient data will cause large errors to be associated with the determined experimental rate constants, as shown in Table 3.

Little information is available for the justification of using the Marquardt (1963) method as the method of choice for non-linear regression analysis. It is possible

Table 3. Effect of insufficient data or absolute error on the final parameter estimates by FITSIM

A 60 % maximum relative error was introduced into progress curves generated by kinetic simulations to Scheme 1.

Parameter varied	10 % Underestimation of [A], four reaction profiles used	Two reaction profiles used	One reaction profile used
k_{-1} (s^{-1})	865.9 ± 24.7	988.9 ± 118.0	$10891 \pm 1.3 \times 10^5$
k_{+2} (s^{-1})	0.0985 ± 0.0007	0.0998 ± 0.002	0.265 ± 2.14
k_{-2} (s^{-1})	0.0203 ± 0.0003	0.0213 ± 0.001	0.021 ± 0.002
Weighted mean SSQ	8×10^{-4}	7×10^{-4}	7×10^{-4}

Table 4. Analysis of pseudo-experimental data to Scheme 2

The column labelled 'No error' represents the parameter values used to generate the pseudo-experimental data. Analysis A consisted of five progress curves spanning a 3-fold range in [B] and 4-fold in [Mg]. Analysis B consisted of seven progress curves spanning a 6-fold range in [B], 4-fold in [Mg] and 10-fold in [Ca]. Analysis C was identical with A, except k_{+1} was set equal to $0.14 s^{-1}$.

Varied parameter	No error	Analysis A	Analysis B	Analysis C
K_{Mg1} (μM)	1100	2400 ± 1200	2700 ± 310	1160 ± 100
K_{Ca} (μM)	15	25 ± 7	28 ± 2	16 ± 1
k_{+1} (s^{-1})	0.14	0.19 ± 0.04	0.18 ± 0.02	0.14 (set)
K_{Mg2} (μM)	2300	2150 ± 100	2150 ± 30	2300 ± 35
K_{dimer} (μM)	3×10^6	$(3.3 \pm 0.3) \times 10^6$	$(3.3 \pm 0.2) \times 10^6$	$(2.9 \pm 0.5) \times 10^6$
K_{trimer} (μM)	2	0.3 ± 0.3	2.3 ± 0.1	2.1 ± 0.2
k_{-e}/k_{+e} (μM)	0.15	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
Weighted mean SSQ	—	1.4×10^{-4}	1.5×10^{-5}	1.5×10^{-4}

that other methods may yield similar, or even quicker, convergence. Historically, the Marquardt (1963) method has appeared to be well-suited for the type of regression analysis needed by FITSIM. Matyska & Kovar (1985) reported that this was the best method for direct fitting of the Michaelis-Menten equation, and this method has generally been the one used most in other kinetic-analysis programs (Zimmerle, 1985; Franco *et al.*, 1986; Lutz *et al.*, 1986; Koerber & Fink, 1987). The design of FITSIM, however, is such that implementation of other regression methods should be relatively easy.

There is undoubtedly a practical limit to the number of parameters which can be varied before FITSIM cannot effectively solve for kinetic parameters which yield the best fit. The maximum upper limit for the number of varied parameters depends on the number of data files fitted, the quality of the data and the initial starting values. Although in the implementation we use the maximum number of varied parameters is 10 and the maximum number of data files is 8, these numbers can be increased relatively easily. Generally, in our experience with the program, the number of varied parameters should be equal to, or less than, the number of experimental progress curves entered for correct convergence and well-determined final parameters to be obtained. When the data are insensitive to changes in the kinetic constants being varied, little or no improvement in the SSQ will be obtained and the estimated kinetic parameters will have large errors associated with them. Thus the varied parameters should be tested to insure that the data are sensitive to the kinetic parameters being

varied. This test can be easily done through an option available in FITSIM.

FITSIM, along with its companion program, KINSIM, provides a powerful, yet relatively easy-to-use, system for the analysis and modelling of chemical kinetics. As with KINSIM, emphasis has been placed to make FITSIM 'user-friendly' and be able to clearly diagnose a multitude of input and/or output errors which could occur. The program has been designed to allow the user great flexibility in both mechanism entry and in the analysis of experimental data to a given mechanism. Furthermore, at least under VAX/VMS, the final fits of the data to the mechanism can easily be plotted either to a printer or to a terminal screen.

The present implementation of this program (version 1.63) along with program documentation and sample analysis files is available on request from C.F.

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