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EXPERIMENTAL METHOD FOR THE KINETIC STUDY OF UNSTABLE AND SITE-DIRECTED IRREVERSIBLE INHIBITORS AND ITS APPLICATION TO THE INACTIVATION OF CHYMOTRYPSIN BY PHENYLMETHYLSULFONYL FLUORIDE†

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The inactivation of enzymes by unstable irreversible inhibitors has only been experimentally characterized by means of discontinuous methods involving preincubation of the enzyme with the inhibitor and the removal of aliquots for further measurements of residual activity. A recent theoretical work proposed a continuous method for the kinetic study of these inhibitors in the presence of an auxiliary substrate. This method was based on approximate expressions for the evolution of the product concentration, which contained series expansions with five or more exponential terms, which severely complicates their use in practice. In this paper, a new experimental method has been developed for the kinetic study of unstable and site-directed irreversible inhibitors. This new method considers the operation of the enzymatic inactivation system in two different ranges of inhibitor concentration. Thus, at low inhibitor concentrations exact analytical equations describe the kinetic behaviour of the system from the rates of the corresponding initial and final steady states. At high inhibitor concentrations, however, the product accumulation follows an exact uniexponential equation. The simplicity and efficiency of the method are illustrated by the study of the inactivation of chymotrypsin by phenylmethylsulfonyl fluoride, whose instability has been seriously under estimated in the literature.

KEY WORDS: Enzyme kinetics, Irreversible inhibitors, Unstable inhibitors, Proteases, Chymotrypsin, Phenylmethylsulfonyl fluoride

INTRODUCTION

The regulation of enzymatic activity is of great interest for biochemical research and for a number of applications in medicine, physiology, pharmacology, toxicology, agriculture and industry. The inactivation of enzymes from humans, animals, plants and microorganisms can be carried out by several agents, among which are inactivating substrates and irreversible inhibitors, inactivators with increasing selectivity and minor

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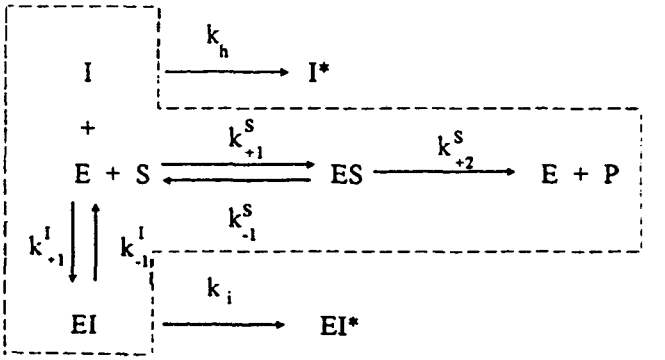
Abbreviations: PMSF = Penylmethylsulfonyl fluoride; SAAPFNA = N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; TLCK = N α-*p*-Tosyl-L-lysine chloromethyl ketone; Tris = Tris (hydroxymethyl) amino-methane.

secondary effects. Most irreversible inhibitors are directed to the active-site of the enzyme, due to their structural similarities with enzymatic substrates, and shows a fully-competitive kinetic behaviour.[1,2,3]

Enzymatic inactivation by site-directed irreversible inhibitors has been mainly characterized by using discontinuous methods. Thus, each inactivation assay involves preincubation of the enzyme with the inhibitor and removal of aliquots for further measurements of residual activity.[4,5] The reliability of this method is limited by the continuing enzymatic inactivation which take place in the aliquots. Another drawback of this method is its low applicability since it is limited to slow inactivation processes.[6,7] Better alternatively methods exist, based on mixing the enzyme with the inhibitor in the presence of an auxiliary substrate, which leads to continuous recording of a detectable species such as the product of the reaction. Thus, continuous methods involving a single substrate[6,8] or a series of coupling reagents in steps catalyzed or not by enzymes[9,10] have been developed and experimentally applied.

The kinetic study of unstable and site-directed irreversible inhibitors has also been carried out by using discontinuous methods.[11–19] These methods have the same limitations as described above for stable irreversible inhibitors, but they are even more serious for inhibitors of high instability. The kinetic study of these irreversible inhibitors, in the presence of an auxiliary substrate, could be described by means of the reaction mechanism:



Scheme I

A continuous method for the kinetic study of these inhibitors has been proposed in a recent theoretical work[20] and discussed in other theoretical papers.[21,22] This method is based on approximate expressions for the evolution of the product concentration and involves series expansions with five or more exponential terms, which severely complicate their use in practice. In fact, no experimental studies exist concerning unstable and site-directed irreversible inhibitors, which can be applied to continuous methods.

Proteases are enzymes which are widely inactivated by physiological and synthetic irreversible inhibitors.[23–26] Thus, there are many irreversible inhibitors of serine-proteases of the chymotrypsin-superfamily, which include elastases, chymotrypsin and

chymotrypsin-like enzymes such as neutrophil cathepsin-G, skin chymase and mast cell proteinases.[27–29] Among these irreversible inhibitors there are several sulfonyl fluorides.[30–32] Phenylmethylsulfonyl fluoride (PMSF) is a commercially available irreversible inhibitor with a similar inactivation efficiency, but lower toxicity, than diisopropyl fluorophosphate. PMSF inactivates serine-proteases (such as chymotrypsin, trypsin and thrombin), cysteine-proteinases (such as papain) and hydrolases (such as acetylcholinesterase) with different degrees of inactivation efficiency. Thus, it is used in the prevention of unwanted proteolysis in homogenates from cultured cells and tissues.[25] Previous studies of the irreversible inhibition of chymotrypsin by PMSF have been carried out using discontinuous[33,34] and continuous[8] methods. The kinetic constants obtained from these studies are biased by a serious under estimation of the instability of PMSF.

Therefore, the aim of this paper is to develop a new experimental method for the kinetic study of unstable and site-directed irreversible inhibitors. This method is based on exact and simple analytical equations that provide a sound and easy experimental application. The use of this continuous method is illustrated by means of the kinetic characterization of the inactivation of chymotrypsin by PMSF, taking fully into consideration its instability at pH 8.0 for the first time in the literature.

MATERIALS AND METHODS

Reagents

Bovine pancreas α-chymotrypsin (EC 3.4.21.1), treated with TLCK to remove contamination by trypsin (type VII), was purchased from Sigma (Germany). The enzyme was active-site titrated with N-trans-cinnamoyl-imidazole[35] obtained from Sigma. The irreversible inhibitor phenylmethylsulfonyl fluoride (PMSF) and the substrate N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAAPFNA) were also obtained from Sigma. Other reagents were of analytical grade and supplied by E. Merck (Germany).

Stock solutions of chymotrypsin contained 1 mM HCl, and 0.1 M $CaCl_2$, whereas PMSF and SAAPFNA were dissolved in dimethylsulfoxide. The solubility of PMSF was higher than 2 mM in 50 mM Tris-HCl buffer, pH 8.0 with 10% dimethylsulfoxide, 10 mM $CaCl_2$ and 0.1 M NaCl at 25°C.

Spectrophotometric and stopped-flow assays

The instability of PMSF was directly recorded at 262 nm.[36] The instability of SAAPFNA caused by non-enzymatic hydrolysis was followed by monitoring the appearance of *p*-nitroaniline at 410 nm.[37,38]

Activity assays of chymotrypsin using SAAPFNA and the inactivation of chymotrypsin by PMSF in the presence of SAAPFNA were followed by recording the appearance of *p*-nitroaniline at 410 nm in the above mentioned assay medium. Experiments were carried out with a Perkin Elmer Lambda-2 spectrophotometer or, when full assay time was less than 100 s, with a Bio-Logic SFM-3 stopped-flow spectrophotometer. Both instruments were on-line controlled by IBM-XT-compatible

computers through RS232C serial interfaces. Absorbance (Perkin Elmer) and transmittance (Bio-Logic) data were produced in ASCII format and converted by using the Sigma Plot 5.0 program[39] in an IBM-486-compatible computer for further data analysis. All the slow and rapid kinetic assays were carried out at 25°C, by means of a Haake-D1G circulating bath equipped with a heater/cooler, and controlled by a Cole-Parmer digital thermometer with a precision of ±0.1°C.

Kinetic analysis

The inactivation of enzymes by unstable irreversible inhibitors, in the presence of auxiliary substrate (Scheme I), can be analyzed by taking into account the following considerations:

- No interference of S and I on their respective isolated actions on E.
- Negligible consumption of S and I in their reaction with E, *i.e.*, $[S]_0 \gg [E]_0$, $[P]_\infty \ll [S]_0$ as well as $[I]_0 \gg [E]_0 \rightarrow [EI^*]_\infty \ll [I]_0$.
- Rapid equilibrium for the action of I on E, *i.e.*, $k_i \ll k^I_{-1}$, $k^I_{+1}[I]_0 \longrightarrow K_I = k^I_{-1}/k^I_{+1}$ in Scheme I.[40,41]
- Rapid equilibrium or steady state for the action of S on E *i.e.*, $K_M = K_S = k^S_{-1}/k^S_{+1}$ and $k_{cat} = k^S_{+2}$ or $K_M = (k^S_{-1} + k^S_{+2})/k^S_{+1}$ and $k_{cat} = k^S_{+2}$, respectively.
- Restricted steady state for the species involved within the tracing box of Scheme I, since their transformation steps occur in a time range very much shorter than the steps controlled by k_h and k_i. The molar fraction of each species have been derived by using a factorization method.[42]

The kinetic analysis was carried out by using the transient phase approach, previously applied to the study of enzymatic inactivation by stable irreversible inhibitors.[9,10,43,44] and by inactivating substrates.[45–50] Thus, the disappearance of I follows a decreasing uniexponential: $[I] = [I]_0\, e^{-k_h t}$, that affects the evolution of the other species of the system (Scheme I). The kinetic behaviour of the active enzymatic species ($[E_a] = [E] + [ES] + [EI]$) can be described by equation (1):

$$[E_a] = [E]_0 \left(\frac{1 + s + i\, e^{-k_h t}}{1 + s + i}\right)^{\frac{k_i}{k_h}} \tag{1}$$

where $s = [S]_0/K_M$ $i = [I]_0/K_I$. The differential equation corresponding to the accumulation of the product has no exact analytical solution:

$$[\dot{P}] = k^S_{+2}\, f^*_{ES}\, [E_a] = \frac{k^S_{+2}\, [E]_0\, s}{1 + s + i\, e^{k_h t}} \left(\frac{1 + s + i\, e^{k_h t}}{1 + s + i}\right)^{\frac{k_i}{k_h}} \tag{2}$$

Expressions equivalent to equations (1) and (2) have been previously derived by other authors.[20] Equation (2) can be rearranged into equation (3);

$$[\dot{P}] = \frac{k_{cat}\, [E]_0\, s}{1 + s + i} \left(\frac{1 + s + i\, e^{-k_h t}}{1 + s + i}\right)^{r-1} \tag{3}$$

where $r = k_i/k_h$. This equation reveals that experimental recordings of $[P]$ *vs.* t begin with an initial steady state and evolve towards a final $(t \rightarrow \infty)$ steady state with V_0 and V_s rates, respectively:

$$V_0 = \frac{k_{cat}\,[E]_0\; s}{1 + s + i} \tag{4}$$

$$V_s = V_0 \left(\frac{1+s}{1+s+i}\right)^{r-1} \tag{5}$$

Furthermore, at high $[I]_0$ such as $i\; e^{k_h\, t} \gg (1+s)$, which also implies that $i \gg (1+s)$, the differential equation (3) acquires an exact analytical solution:

$$[P] = [P]_\infty\,(1 - e^{-\lambda t}) = \frac{k_{cat}\,[E]_0\; s}{(k_i - k_h)\; i}\left[1 - e^{-(k_i - k_h)\; t}\right] \tag{6}$$

This expression indicates that experimental recordings of $[P]$ *vs* t evolve from an initial steady state, with $V_0 = [P]_\infty\;\lambda$, to a final $(t \rightarrow \infty)$ steady state without any enzymatic activity $(V_s = 0)$. The equations (3) to (6) have not been reported previously and are the basis of the efficient and simple experimental method detailed below.

Data analysis

All the experimental assays were carried out in triplicate, with one thousand data points per instrumental recording. Mean values of the appropriate kinetic parameters are shown in the figures, whereas the reciprocals of their variances were used as weighting factors in further statistical analysis. Data fittings to linear and non-linear equations were carried out by non-linear regression,[51,52] using an improved Gauss-Newton algorithm[53] implemented in the Sigma Plot 5.0 program.[39] This program yields the values of the parameters, their standard deviations, statistics which describe the goodness of the fit and any possible overdetermination, as well as residual plots. These data have been used when needed to estimate the reliability of the fit and to discriminate among alternative equations by using the F test.[54,55] Initial estimations of the parameters were taken as indicated in the Results and Discussion section. The reliability of the kinetic constants calculated from others is revealed by their standard deviations, which were evaluated taking into account their propagation of errors.[56]

RESULTS AND DISCUSSION

Within this section are detailed the steps of the experimental method developed for the kinetic study of unstable and site-directed irreversible inhibitors, as well as its application to the inactivation of chymotrypsin by PMSF.

TABLE I
Kinetic constants which characterize the irreversible inhibition of chymotrypsin by PMSF, in the presence of SAAPFNA

Constant	Value
$K_M(M)$	$(9.02\pm0.26)\times10^{-5}$
$k_{cat}(s^{-1})$	$(4.68\pm0.15)\times10$
$k_{cat}/K_M\ (M^{-1}\ s^{-1})$	$(5.18\pm0.32)\times10^{5}$
$K_I\ (M)$	$(2.43\pm0.06)\times10^{-4}$
$k_i\ (s^{-1})$	$(1.17\pm0.02)\times10^{-1}$
$k_i/K_I\ (M^{-1}\ s^{-1})$	$(4.81\pm0.20)\times10^{2}$
$k_h\ (s^{-1})$	$(2.37\pm0.01)\times10^{-3}$
$r(=k_i/k_h)$	$(4.94\pm0.11)\times10$

Assay Conditions

The usual assay conditions for chymotrypsin were chosen (see Materials and Methods section), involving a buffer near the optimum pH, ionic strength and stabilization by Ca^{2+}. The low proportion of organic co-solvent did not change the enzymatic activity, whereas it improved the solubility of the inhibitor. Concentrations up to 1 and 2 mM PMSF were obtained, such as are used for the prevention of unwanted proteolysis.[25]

Under these assay conditions, the instability of SAAPFNA was negligible during the time of the activity and inactivation assays (data not shown). Activity assays of 10 nM chymotrypsin on 20 to 400 μM SAAPFNA led to fifteen data points of steady state rate (V_0) *vs* $[S]_0$ (data not shown). From these assays $[S]_0/V_0$ *vs.* $[S]_0$ data were plotted and fitted by linear regression, the intersection and slope yielding initial estimations of K_M and k_{cat}. These values were used in a further non-linear regression fit of V_0 *vs.* $[S]_0$ data to the Michaelis equation, that provided the final estimations of both kinetic constants (Table I).

Instability assays of I

The disappearance of PMSF followed first order kinetics through a decreasing uniexponential (data not shown), which enabled k_h to be determined (Table I). The high instability of PMSF, $t_{1/2} = 4.9$ min at pH 8.0, agreed with that obtained by other authors, $t_{1/2} = 30$ and 4 min at pH 7.0 and 8.0, respectively.[36] These data were overlooked in the commercial literature related with PMSF, which reported $t_{1/2} = 110$ and 35 min at pH 7.0 and 8.0 respectively.[57] These values where under estimated four- and nine-fold, respectively, due to the use of second order kinetics ($[E]_0/[I]_0 = 1/1$), and to the discontinuous measurement of enzymatic activity with a solid substrate (azocoll).

Inactivation assays: Experimental recordings

Experiments on the inactivation of chymotrypsin by unstable PMSF, in the presence of SAAPFNA, were carried out under two sets of assay conditions, hereafter termed "low $[I]_0$", and "high $[I]_0$". They correspond to the two experimental situations considered in the kinetic analysis, equations (3)–(5) and equation (6), respectively.

Low $[I]_0$. Instrumental recordings (Figure 1A) were submitted to successive logarithmic linearizations in order to obtain initial estimations for the parameters of the multiexponential equations proposed by other authors.[20] A minimum of five exponential terms was considered in further non-linear regression fits of $[P]$ *vs* t data to equation (24) of Topham's paper.[20] The fits showed overdetermination, as reported for equations with three or more exponential terms, since several sets of parameters could provide the same calculated data.[55,58]

From the kinetic analysis developed here (equations (3)–(5)), each experimental recording (Figure 1A) involved separate linear fits of their initial (50 to 100 data points) and final (300 to 200 data points) portions of the curve (1000 data points). Their slopes were denoted by V_0 (equation (4)) and V_s (equation (5)), respectively. The V_s values were not significantly different from the non-enzymatic hydrolysis of the substrate. Therefore, only V_0 values were used in further kinetic data analysis.

The initial linear portion (Figure 1A) corresponded to the initial steady state restricted to the active enzymatic species ($[E_a] = [E] + [ES] + [EI]$), prior to the significant contribution of the enzymatic inactivation towards EI^* ($[E]_0 \approx [E_a]$), and that of the instability of I towards I^* ($[I]_0 \approx [I]$). The intermediate non-linear portion was an inter-steady transient phase, defined by the simultaneous operation of the enzymatic activity and the enzymatic inactivation ($[E]_0 = [E_a] + [EI^*]$) as well as the non enzymatic decomposition of the irreversible inhibitor ($[I]_0 = [I] + [I^*]$). The last linear portion corresponded to the final steady state, in which the enzymatic inactivation removed a significant portion of the active enzyme ($[E]_0 = [E_a]_\infty + [EI^*]_\infty$), and the irreversible inhibitor did not originate more enzymatic inactivation ($[I]_0 = [I]_\infty + [I^*]_\infty$, taking into account $[I]_\infty \ll K_I$, which evolved non-enzymatically towards $[I]_0 \approx [I^*]_\infty$). In this case (Figure 1A), the negligible values of V_s indicated that $[E_a]_\infty \approx 0$ and $[E]_0 \approx [EI^*]_\infty$.

High $[I]_0$. Under these assay conditions (equation (6)), increasing uniexponential curves were obtained (Figure 1B). Each curve was submitted to logarithmic linearization in order to obtain initial estimations of $[P]_\infty$ and λ, values which were used in further non-linear regression fits of $[P]$ *vs* t data to equation (6). The reliability of the fits was supported by the low value of the sum of the squared residuals and by the random pattern of the corresponding residual plot.[52,54] Furthermore, the fits had no overdetermination and were not improved by additional terms, according to the F test.[54,55] These criteria were used for detection of unreliable fits to equation (6), from experimental data at low $[I]_0$ with $V_s \approx 0$ (equations (3)–(5)).

These assay conditions (equation (6)) led to a negligible instability of the irreversible inhibitor during the assay period ($[I]_0 \approx [I]_\infty$), and to a saturation of the enzyme by the inhibitor ($[S]_0 \leq K_M$, $[I]_0 \gg K_I$). Thus (Figure 1B), the reaction

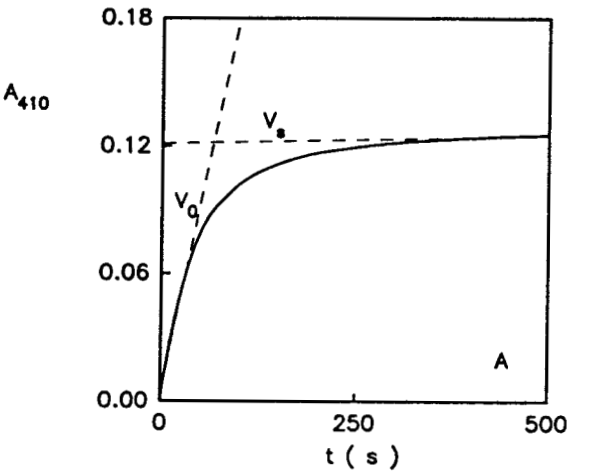


FIGURE 1(A)

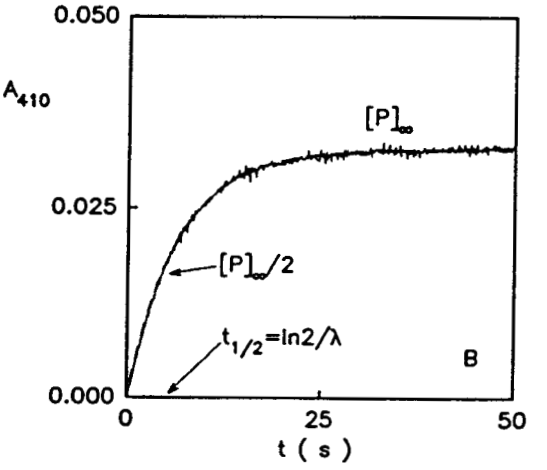


FIGURE 1(B)

FIGURE 1 (A) Course of chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at low $[I]_0$. Conditions are as described in Materials and Methods with 20 nM chymotrypsin, 280 μM SAAPFNA and 1 mM PMSF. (——) Experimental data. (------) Linear regression fits of the initial (equation (4)) and final (equation (5)) portions of the experimental recordings. (B) Course of chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at high $[I]_0$. Conditions are as described in Materials and Methods with 90 nM chymotrypsin, 70 μM SAAPFNA and 2 mM PMSF. (——) Experimental data. (------) Non-linear regression fit of experimental data to equation (6).

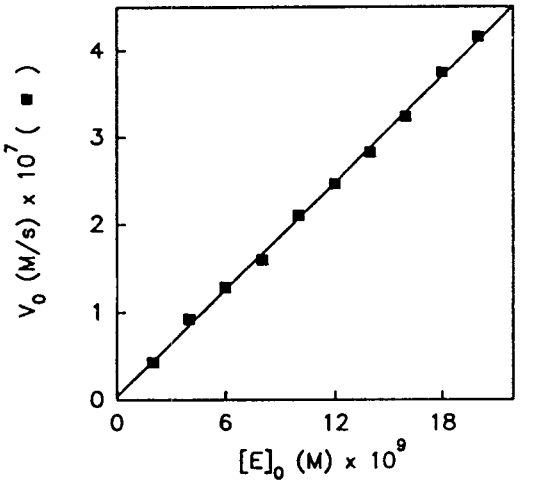


FIGURE 2 Effect of $[E]_0$ on chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at low $[I]_0$. Conditions are as described in Materials and Methods with 2–20 nM chymotrypsin, 360 μM SAAPFNA and 1 mM PMSF. (■) Experimental data of V_0 *vs.* $[E]_0$. (——) Linear regression fit of V_0 *vs.* $[E]_0$ data (equation (4)).

started with an initial steady state ($[E]_0 \approx [E_a]$), and continued with a post-steady transient phase with partial enzymatic inactivation ($[E]_0 \approx [E_a] + [EI^*]$), towards a final state with complete inactivation of the enzyme ($[E]_0 \approx [EI^*]_\infty$).

Inactivation assays: Effect of $[E]_0$

Low $[I]_0$. The increase in $[E]_0$ originated higher product formation and duration of the inter-steady transient phase (data not shown). This led to a linear dependence of V_0 on $[E]_0$ (Figure 2), in accordance with the prediction of the kinetic analysis (equation (4)).

High $[I]_0$. Greater $[E]_0$ enhanced the level of $[P]_\infty$, which was attained at the same time in the post-steady transient phase (data not shown). The dependence of $[P]_\infty$ and V_0 on $[E]_0$ was linear and there was no variation of λ with $[E]_0$ (Figure 3), which agreed with the kinetic analysis (equation (6)).

Under both low and high $[I]_0$ assay conditions an appropriate $[E]_0$ must be chosen to ascertain whether the original planning considerations of the kinetic analysis were obeyed. Thus, a careful control of the improvement of the signal/noise ratio on the instrumental recordings was required, in order to keep substrate consumption negligible ($[P]_\infty \ll [S]_0$) in all the experimental assays.

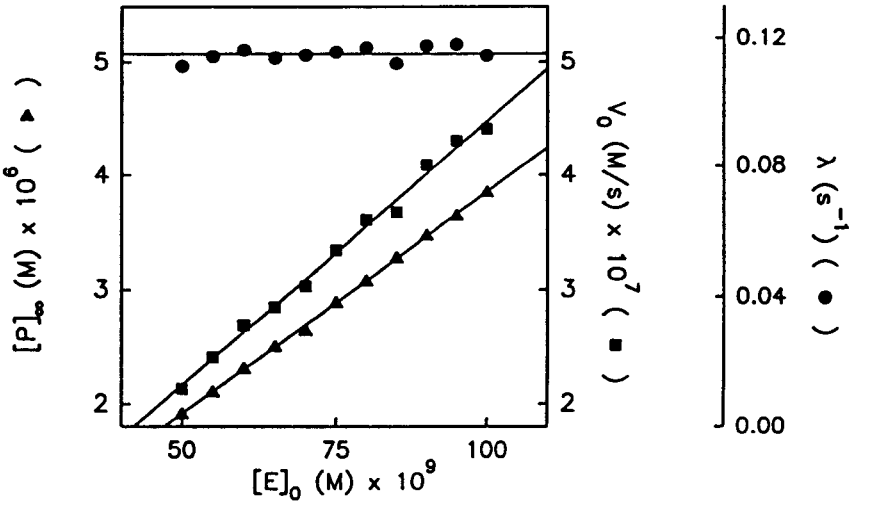


FIGURE 3 Effect of $[E]_0$ on chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at high $[I]_0$. Conditions are as described in Materials and Methods with 50–100 nM chymotrypsin, 70 μM SAAPFNA and 2 mM PMSF. (▲, ■, ●) Experimental data of $[P]_\infty$, V_0 and λ *vs.* $[E]_0$, respectively. (——) Linear regression fits corresponding to $[P]_\infty$, V_0 and λ *vs.* $[E]_0$ data (equation (6)).

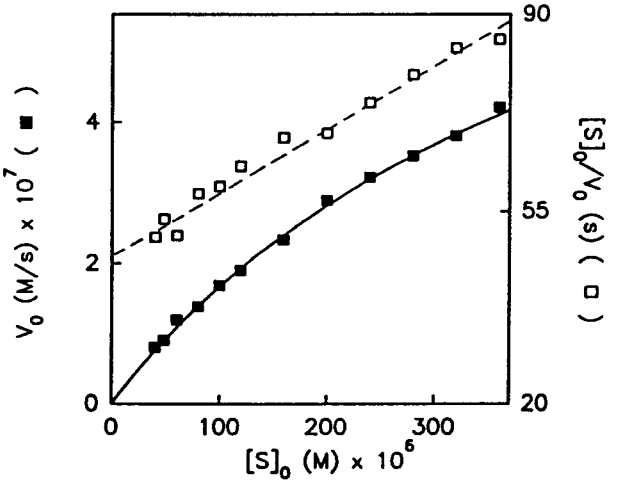


FIGURE 4 Effect of $[S]_0$ on chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at low $[I]_0$. Conditions are as described in Materials and Methods with 20 nM chymotrypsin, 40–360 μM SAAPFNA and 1 mM PMSF. (■,□) Experimental data of V_0 and $[S]_0/V_0$ *vs.* $[S]_0$, respectively. (------) Linear regression fit of $[S]_0/V_0$ *vs.* $[S]_0$ data (Hanes-Woolf plot). (——) Non-linear regression fit of V_0 *vs.* $[S]_0$ data to equation (4).

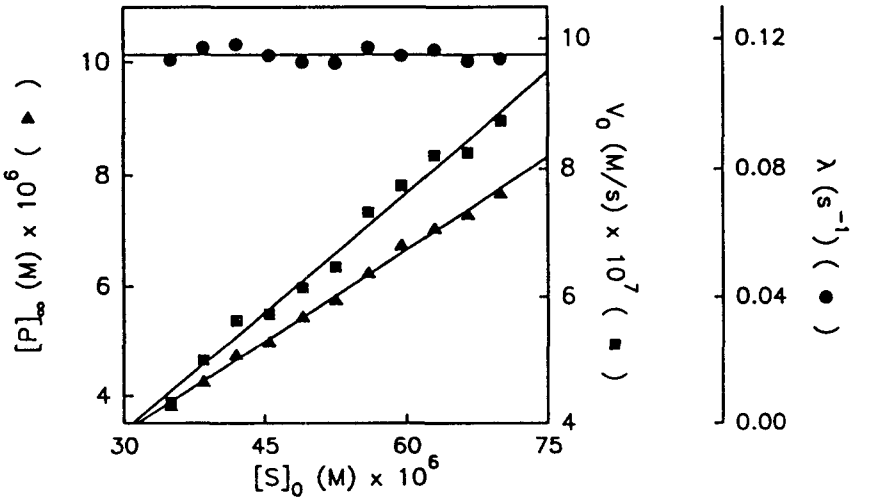


FIGURE 5 Effect of $[S]_0$ on chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at high $[I]_0$. Conditions are as described in Materials and Methods with 200 nM chymotrypsin, 35–70 μM SAAPFNA and 2mM PMSF. (▲, ■, ●) Experimental data of $[P]_\infty$, V_0 and λ *vs.* $[S]_0$, respectively. (——) Linear regression fits corresponding to $[P]_\infty$, V_0 and λ *vs.* $[S]_0$ data (equation (6)).

Inactivation assays: Effect of $[S]_0$

Low $[I]_0$. When $[S]_0$ rose, there was an increase in the quantity of product formed, whereas a longer time was required for the transient phase between the initial and the final steady states (data not shown). The hyperbolic dependence of V_0 on $[S]_0$ appeared to be confirmed by the linearization with the Hanes-Woolf transformation $[S]_0/V_0$ *vs* $[S]_0$ (Figure 4), in accordance with the kinetic analysis (equation (4)). The fit of these data by linear regression led to values of intercept and slope which were used as initial estimations for the non-linear regression fit of V_0 *vs* $[S]_0$ data to equation (4) (Figure 4).

High $[I]_0$. The enhancement of $[S]_0$ originated greater values of $[P]_\infty$ during a similar transient phase (data not shown). A linear dependence of $[P]_\infty$ and V_0 on $[S]_0$, as well as the non variation of λ with $[S]_0$ were obtained (Figure 5), as predicted by the kinetic analysis (equation (6)).

Inactivation assays: Effect of $[I]_0$

Low $[I]_0$. The increase of $[I]_0$ led to lower product formation and to a shortening of the inter-steady transient phase (data not shown). The non-linear decrease of V_0 with

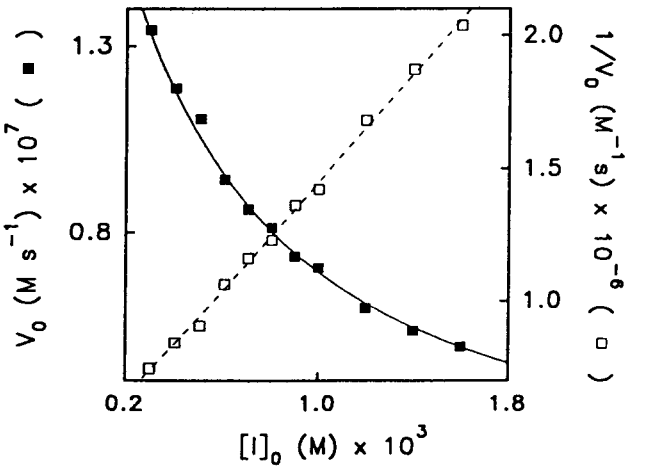


FIGURE 6 Effect of $[I]_0$ on chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at low $[I]_0$. Conditions are as described in Materials and Methods with 10 nM chymotrypsin, 80 μM SAAPFNA and 0.3–1.6 mM PMSF. (■,□) Experimental data of V_0 and $1/V_0$ *vs.* $[I]_0$, respectively. (------) Linear regression fit of $1/V_0$ *vs.* $[I]_0$ data (Dixon plot). (——) Non-linear regression fit of V_0 *vs.* $[I]_0$ data to equation (4).

$[I]_0$ was linearized with the Dixon transformation $1/V_0$ *vs* $[I]_0$ (Figure 6), according to the kinetic analysis (equation (4)). These data were fitted by linear regression, yielding values of intercept and slope, whose quotient was equal to $K_I[1 + ([S]_0/K_M)]$. This value of K_I was used as an initial estimation in a further non-linear regression fit of V_0 *vs* $[I]_0$ data to equation (4) (Figure 6), which led to the final value for this dissociation constant (Table I).

High $[I]_0$. When $[I]_0$ rose, $[P]_\infty$ was reduced and the duration of the post-steady transient phase appeared to remain constant (data not shown). Thus, λ did not depend on $[I]_0$, whereas both $[P]_\infty$ and V_0 showed a non-linear decrease against $[I]_0$, as well as a linear increase with regards to $1/[I]_0$ (Figure 7), as stated by the kinetic analysis (equation (6)). The value of k_i was calculated from the weighted average of λ *vs* $[I]_0$ (Figure 7), taking into account the value of k_h present in equation (6) (Table I). The efficiency of this procedure was supported by its simplicity and low propagation of errors. Another author[20] proposed the calculation of k_i/k_h from seven algebraic operations (equations (29) and (30) of that paper), involving parameters of the multiexponential equation, whose severe limitations were mentioned above.

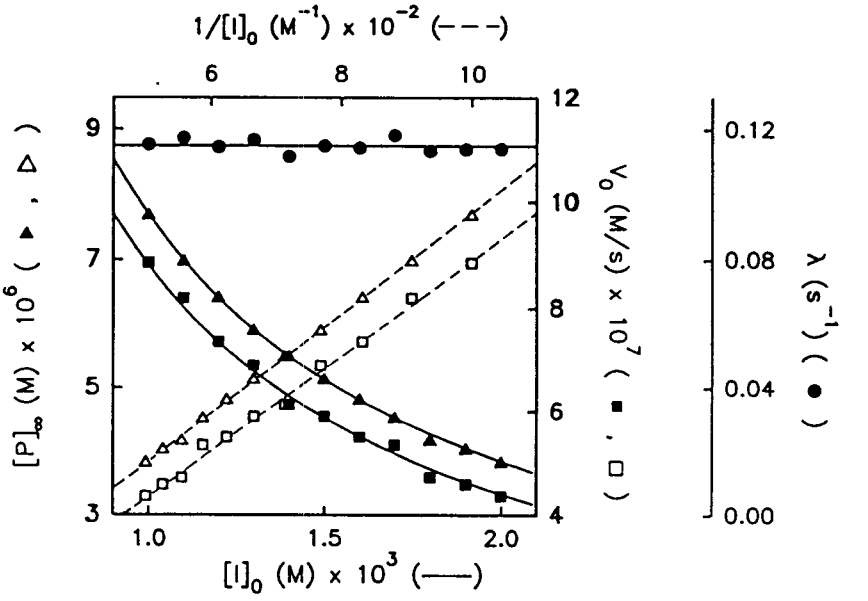


FIGURE 7 Effect of $[I]_0$ on chymotrypsin inactivation by PMSF in the presence of SAAPFNA, at high $[I]_0$. Conditions are as described in Materials and Methods with 200 nM chymotrypsin, 35 μM SAAPFNA and 1–2 mM PMSF. (▲, ■, ●) Experimental data of $[P]_\infty$, V_0 and λ *vs.* $[I]_0$, respectively. (△,□) Experimental data of $[P]_\infty$ and V_0 *vs.* $1/[I]_0$, respectively. (△---△, □---□) Linear regression fits of $[P]_\infty$ and V_0 *vs.* $1/[I]_0$ to equation (6), respectively. (▲—▲, ■—■) Non-linear regression fits of $[P]_\infty$ and V_0 *vs.* $[I]_0$ data to equation (6), respectively. (●—●) Weighted average of λ *vs.* $[I]_0$ data (equation (6)).

CONCLUSIONS

Discontinuous methods.

The continuous method described above is more reliable than discontinuous procedures since it involves one single measurement of product formation per concentration of reagent, without the removal of aliquots for further measurements of residual activity. The broad availability of chromogenic and fluorogenic substrates, as well as computer-controlled instrumentation, provides 1000 or more data points per experimental recording, in contrast with the approximately 10 to 20 data points usual in discontinuous methods, so enabling the study of inactivation processes even in the second and millisecond range. The simplicity and speed of this continuous method helps to obtain a significant number of data points ($\geq$ 10) for the screening of the effect of each reagent. This also reduces the number of assays required to find the appropriate range of concentration of each reagent: below and above K_M and K_I, for instance.[56]

Continuous methods.

The continuous method developed here has a simple and efficient experimental applicability based on exact analytical equations of steady state and transient phase kinetics as well as on reliable data fittings by simple linear regression and non-linear regression to uniexponential equations. The serious limitations of other theoretical methods[20] have been pointed out. The instrumentation required for these studies is determined by the assay medium, by the kinetic constants of the inactivation system, as well as by the appropriate choice of concentration of the reagents (equations (3)–(6)). Thus the fastest inactivation process occurs at high $[I]_0$ conditions with $\lambda = k_i - k_h$ (equation (6)).

Prevention of unwanted proteolysis.

The instability of PMSF has been severely underestimated in the commercial literature, and must be used with caution. The utility of PMSF is related to its inactivation power $(k_i[I]_0/K_I)$, to its instability (k_h), and to the catalytic power $(k_{cat}\,[S]_0/K_M)$ of proteins and other endogenous substrates of serine-proteases from cells and tissues. Appropriate experimental conditions should be chosen since, for instance, a decrease of pH and temperature reduces the instability of PMSF but also diminishes its inactivation power. Therefore, a better knowledge of the enzymes and substrates of the homogenates, as well as of optimal assay conditions, are desirable for improving the use of PMSF and other enzymatic inactivators.

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