Experimental approach to the kinetic study of unstable site-directed irreversible inhibitors: kinetic origin of the apparent positive co-operativity arising from inactivation of trypsin by *p*-amidinophenylmethanesulphonyl fluoride

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Experimental characterization of enzyme inactivation by unstable irreversible inhibitors has only previously been carried out by using discontinuous methods involving preincubation, removal of samples and further residual activity assays. A continuous method for the kinetic study of these inhibitors in the presence of an auxiliary substrate was recently proposed in a theoretical study. This method was based on approximate expressions for the evolution of the product concentration, which contained series expansions with five or more exponential terms, seriously complicating their use in practice. In the present paper, a new experimental method has been developed for the kinetic study of unstable and site-directed irreversible inhibitors, considering two different ranges of inhibitor concentration. Thus at low inhibitor concentrations, the system evolves from an

initial to a final steady state, the rates of which are described by exact analytical equations. At high inhibitor concentrations, however, the product accumulation can be described by an exact uniexponential equation. This simple and efficient method has been applied to the kinetic study of trypsin inactivation by *p*-amidinophenylmethanesulphonyl fluoride, near the optimum pH of the enzyme. The dependence of the final steady-state rate on the substrate concentration shows apparent positive co-operativity which has not previously been reported. The kinetic origin of this type of co-operativity is predicted by one of the exact analytical equations derived here. The instability of new protein and non-protein irreversible inhibitors has to be carefully characterized to prevent true unstable irreversible inhibitors being wrongly described as allosteric reversible inhibitors.

INTRODUCTION

The regulation of enzymic activity is of great interest for biochemical research purposes and for a number of applications in medicine, physiology, pharmacology, toxicology, agriculture and industry. Inactivation of enzymes from any biological source can be achieved by several agents, such as inactivating substrates and irreversible inhibitors, inactivators with increasing selectivity and minor secondary effects. The most irreversible inhibitors are directed to the active site of the enzyme, because of their structural similarities to substrates, and show fully competitive kinetic behaviour (Silverman, 1988; Sandler and Smith, 1989; Sigman and Boyer, 1990).

Enzyme inactivation by site-directed irreversible inhibitors has been mainly characterized by using discontinuous methods. Each inactivation assay involves preincubation of the enzyme with the inhibitor and removal of samples for measurement of residual activity (Kitz and Wilson, 1962; Malcom and Radda, 1970). The reliability of this method is limited by the inactivation continuing to take place in the assay sample. Another drawback is that its use is limited to slow inactivation processes (Tsou, 1988; Tipton, 1989). Better alternative methods exist based on mixing the enzyme with the inhibitor in the presence of an auxiliary substrate and continuously recording a detectable species such as the product of the reaction. Thus continuous methods involving a single auxiliary substrate (Tian and Tsou, 1982; Tsou, 1988) or a series of coupling reagents in steps catalysed or not by enzymes (Teruel et al., 1986, 1987) have been developed and experimentally applied.

Kinetic study of unstable site-directed irreversible inhibitors has also been carried out by using discontinuous methods (Purdie and Heggie, 1970; Ashani et al., 1972; Rakitzis, 1974, 1985, 1987; Topham, 1985, 1986, 1987, 1988). 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 kinetics of these irreversible inhibitors, in the presence of an auxiliary substrate, can be described by the reaction mechanism shown in Scheme 1.

A continuous method for the study of these inhibitors has been proposed in a theoretical work (Topham, 1990) and discussed in other theoretical papers (Topham, 1992; Varón et al., 1992). This method is based on approximate expressions of the evolution of the product concentration and involves series expansions with five or more exponential terms, which severely complicates their use in practice. In fact, no experimental studies involving unstable and site-directed irreversible inhibitors exist which can be applied to continuous methods.

Proteases are enzymes that are widely inactivated by physiological and synthetic irreversible inhibitors (Barrett and Salvesen, 1986; Beynon and Bond, 1989). Thus there are many irreversible inhibitors of serine proteases of the trypsin family, which include trypsin (Desnuelle et al., 1986; Neurath, 1989; Bode and Huber, 1992) and trypsin-like proteases involved in blood coagulation, fibrinolysis, activation of complement, generation of vasoactive products, development and self-assembly, as well as processing of bioactive peptides and protein hormones (Cunningham and Long, 1987; Festoff and Hantäi, 1990). Among these irreversible inhibitors, there are several sulphonyl fluorides (Gold, 1967;

Scheme 1

Wong et al., 1978; Tanaka et al., 1983). The most potent inhibitor of this class, *p*-amidinophenylmethanesulphonyl fluoride (APMSF), is a commercially available irreversible inhibitor with an inactivation efficiency much higher than that of diisopropyl fluorophosphate and phenylmethanesulphonyl fluoride. Trypsin, thrombin, plasmin and the complement enzymes C1r and C1s are inactivated by micromolar levels of APMSF in about 100 s (Laura et al., 1980). APMSF also inactivates human tryptases (Tanaka et al., 1983), but does not inhibit chymotrypsin or acetylcholinesterase. It stoichiometrically inactivates trypsin and thrombin, blocking the active-site serine residue (Laura et al., 1980). Irreversible inhibition of trypsin by APMSF has been carried out using a discontinuous method at pH 6.8 (Laura et al., 1980). The slowness of this method limits its reliability when used to study such a fast process, which furthermore involves the high instability of APMSF.

Therefore the aim of the present work was to develop a new experimental method for the kinetic study of unstable 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 rapid inactivation of trypsin by APMSF, taking fully into consideration its instability at pH 8.0, this inactivation being demonstrated for the first time in the literature.

MATERIALS AND METHODS

Reagents

Bovine pancreas trypsin (EC 3.2.21.4) treated with Tos-Phe-CH₂Cl to remove contamination by chymotrypsin (type XIII) was purchased from Sigma. The enzyme was active-site-titrated with *p*-nitrophenyl-*p*'-guanidinobenzoate (Chase and Shaw, 1969) obtained from Sigma. The irreversible inhibitor, APMSF, the substrate *N*- α -carbobenzoxy-L-lysine thiobenzyl ester (ZLSBE), and the coupling reagent, 4,4'-dithiodipyridine, were also obtained from Sigma. Other reagents were of analytical grade and supplied by E. Merck.

Stock solutions of trypsin, APMSF and ZLSBE contained 1 mM HCl, whereas 4,4'-dithiodipyridine was dissolved in dimethyl sulphoxide. The assay medium was 0.1 M Tris/HCl buffer, pH 8.0, with 1% dimethyl sulphoxide, 0.3 mM 4,4'-dithiodipyridine and 0.1 M NaCl at 25 °C.

Spectrophotometric and stopped-flow assays

The instability of APMSF was directly recorded at 260 nm (Laura et al., 1980). The instability of ZLSBE plus 4,4'-dithiodipyridine caused by non-enzymic hydrolysis was followed

by monitoring the appearance of 4-thiopyridone at 324 nm (Castillo et al., 1979; Green and Shaw, 1979).

Spectrophotometric assays of trypsin activity on ZLSBE and trypsin inactivation of APMSF in the presence of ZLSBE were followed by recording the appearance of 4-thiopyridone at 324 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 exported in ASCII format and imported by using the Sigma Plot 5.0 program (Jandel Scientific, 1992) 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

Inactivation of enzymes by unstable irreversible inhibitors in the presence of auxiliary substrate (Scheme 1) can be analysed by making the following assumptions: 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. $k_1 \ll k_{-1}^I, k_{+1}^I$ $[I]_0 \rightarrow K_I = k_{-1}^I/k_{+1}^I$ in Scheme 1 (Brocklehurst, 1979; Cornish-Bowden, 1979); rapid equilibrium or steady state for the action of S on E, i.e. $K_m = K_s = k_{-1}^S/k_{+1}^S$ and $k_{cat} = k_{+2}^S$ or $K_m = (k_{-1}^S + k_{+2}^S)/k_{+1}^S$ and $k_{cat} = k_{+2}^S$ respectively; restricted steady state for the species included within the dashed box of Scheme 1, as 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 has been derived by using a factorization method (Cha, 1968).

The kinetic analysis was carried out by using the transient-phase approach, previously applied to the study of enzyme inactivation by stable irreversible inhibitors (Teruel et al., 1986, 1987; Tudela et al., 1986, 1987a) and by inactivating substrates (Tudela et al., 1987b,c; García Cánovas et al., 1987, 1989; Escribano et al., 1989; Varón et al., 1990). Thus the disappearance of I follows a decreasing uniexponential $[I] = [I]_0 e^{-k_h t}$ which affects the evolution of the other species of the system (Scheme 1). The kinetic behaviour of the active enzyme species ($[E_a] = [E] + [ES] + [EI]$) can be described by the equation:

$$[E_a] = [E]_0 \left(\frac{1 + s + i e^{-k_h t}}{1 + s + i} \right)^{\frac{k_1}{k_h}} \quad (1)$$

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

$$[\dot{P}] = k_{+2}^S \int_{ES} [E_a] = \frac{k_{+2}^S [E]_0 s}{1 + s + i e^{-k_h t}} \left(\frac{1 + s + i e^{-k_h t}}{1 + s + i} \right)^{\frac{k_1}{k_h}} \quad (2)$$

Expressions equivalent to eqns. (1) and (2) have been previously derived by other authors (Topham, 1990). Eqn. (2) can be rearranged into the equation:

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

where $r = k_1/k_h$. This equation reveals that experimental recordings of [P] versus t begin with an initial ($t \rightarrow 0$) steady state and

evolve towards a final ($t \rightarrow \infty$) steady state, with V_0 and V_s rates respectively:

$$V_0 = \frac{k_{\text{cat}} [E]_0 s}{1 + s + i} \quad (4)$$

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

Note that eqn. (3), when $r = 1$, leads to an exact analytical equation:

$$[P] = \frac{k_{\text{cat}} [E]_0 s}{1 + s + i} t = V_0 t \quad (6)$$

which corresponds to a single steady state, where inhibition instead of inactivation of the enzyme takes place.

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

$$[P] = \frac{k_{\text{cat}} [E]_0 s}{(k_1 - k_h) i} [1 - e^{-(k_1 - k_h) t}] \quad (7)$$

where the term outside the square brackets on the right-hand side is the concentration of product obtained at the final time of the reaction ($[P]_\infty$).

This expression indicates that experimental recordings of $[P]$ versus t evolve from an initial steady state, with $V_0 = [P]_\infty (k_1 - k_h)$, to a final ($t \rightarrow \infty$) steady state without any enzyme activity ($V_s = 0$). Eqns. (3)–(7) have not previously been reported 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 1000 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 (Leatherbarrow, 1990; Brand and Johnson, 1992), using an improved Gauss–Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 5.0 program (Jandel Scientific, 1992). This program yields the values of the parameters, their standard deviations, statistics that describe the goodness of 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 (Mannervik, 1982; Bardsley et al., 1986). Initial estimations of the parameters were made 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 (Bevington, 1969).

RESULTS AND DISCUSSION

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

Assay conditions

The usual assay conditions for trypsin were chosen (see the Materials and methods section), involving a buffer near the optimum pH and ionic strength. The low proportion of organic cosolvent did not change the enzyme activity, whereas it improved the solubility of the coupling reagent. Concentrations of 0.3 mM 4,4'-dithiopyridine were used to secure first-order conditions for the coupling reaction as well as its fast operation without the introduction of an additional exponential term (Castillo et al., 1979; Green and Shaw, 1979).

Under these assay conditions, the instability of ZLSBE was negligible during the time of the activity and inactivation assays (results not shown). Activity assays of 1 nM trypsin on 10–300 μ M ZLSBE produced 15 data points of steady-state rate (V_0) versus $[S]_0$ (results not shown). From these assays, $[S]_0/V_0$ versus $[S]_0$ was plotted and fitted by linear regression; the intercept and slope yielded initial estimations of K_m and k_{cat} . These values were used in a further non-linear regression fit of V_0 versus $[S]_0$ to the Michaelis equation, which provided the final estimates of both kinetic constants (Table 1).

The decomposition of APMSF followed first-order kinetics, through an increasing uniexponential, $[I^*] = [I]_0 (1 - e^{-k_h t})$ (Figure 1, curve a), which enabled k_h to be determined (Table 1). The determination of the high instability of APMSF, $t_{1/2} = 15.7$ s and $t_{90} = 104.4$ s at pH 8.0, required the use of stopped-flow instrumentation. Each curve was submitted to logarithmic linearization in order to obtain initial estimates of $[I]_0$ and k_h values used in further non-linear regression fits of $[I^*]$ versus t to the above increasing uniexponential. The reliability of the fits was supported by several criteria of goodness of fit such as the low values of the sum of the squared residuals and the random pattern of the corresponding residual plot (Mannervik, 1982; Brand and Johnson, 1992). Furthermore, the fits had no overdetermination and were not improved by additional exponential terms, according to the F test (Mannervik, 1982; Bardsley et al., 1986).

Inactivation assays: experimental recordings

Experiments on trypsin inactivation by APMSF in the presence of ZLSBE 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, i.e. eqns. (3)–(5) and eqn. (7) respectively.

Low $[I]_0$

Instrumental recordings (Figure 1b) were submitted to successive logarithm linearizations in order to obtain initial estimates for the parameters of the multiexponential equations proposed by Topham (1990). A minimum of five exponential terms was considered in subsequent non-linear regression fits of $[P]$ versus t to eqn. (24) of that paper (Topham, 1990). The fits show overdetermination, as reported for equations with three or more exponential terms, as several sets of parameters could provide the same calculated data (Zierler, 1981; Bardsley et al., 1986).

From the kinetic analysis developed here [eqns. (3)–(5)], each experimental recording (Figure 1, curve b) involved separate linear fits of their initial (50–100 data points) and final (200–300 data points) portions of the curve (1000 data points). Their slopes were denoted by V_0 [eqn. (4)] and V_s [eqn. (5)], respectively. The V_0 and V_s values were significantly different from the non-enzymic hydrolysis of the substrate. Therefore both V_0 and V_s values are used below in further kinetic data analysis. The criteria of goodness of fit mentioned above were used to detect

Table 1 Kinetic constants that characterize the irreversible inhibition of trypsin by APMSF in the presence of ZLSBE

Constant (units)	Value
K_m (μM)	15.94 ± 0.52
k_{cat} (s^{-1})	55.25 ± 0.75
k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	3.47 ± 0.16
K_i (μM)	1.76 ± 0.08
k_i (min^{-1})	18.10 ± 0.30
k_i/K_i ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)	10.28 ± 0.64
k_h (min^{-1})	2.65 ± 0.01
$r (= k_i/k_h)$	6.83 ± 0.06

Figure 1 Experimental recordings of the instability of the inhibitor, and enzyme inactivation

(a) Spontaneous hydrolysis of APMSF. Conditions were as described in the Materials and methods section with $100 \mu\text{M}$ APMSF. —, Experimental data; ----, non-linear regression fit of experimental data to an increasing uniexponential equation. (b) Inactivation of trypsin by APMSF in the presence of ZLSBE. Conditions were as described in the Materials and methods section with 1.3 nM trypsin, $50 \mu\text{M}$ ZLSBE and $1.8 \mu\text{M}$ APMSF. —, Experimental data; ----, linear regression fits of the first [eqn. (4)] and final [eqn. (5)] portions of the experimental recording.

unreliable fits to eqn. (7), based on experimental data with low values of V_s [eqns. (3)–(5)].

The initial linear portion (Figure 1, curve b) corresponded to the initial steady state restricted to the active enzyme species ($[E_a] = [E] + [ES] + [EI]$), before the significant contribution of the enzyme 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-state transient phase, defined by the simultaneous operation of the enzyme activity and the enzyme inactivation ($[E]_0 = [E_a] + [EI^*]$), as well as the non-enzymic decomposition of the irreversible inhibitor ($[I]_0 = [I] + [I^*]$). The last linear portion corresponded to the final steady state, in which there were portions of inactive ($[EI^*]_{\infty}$) and active enzyme

($[E_a]_{\infty}$), which gave rise to significant values of V_s (Figure 1, curve b). During this final phase, $[I] \rightarrow 0$.

High $[I]_0$

Under these assay conditions [eqn. (7)], increasingly uniexponential curves can be obtained. These assay conditions can lead to a negligible instability of the irreversible inhibitor throughout the assay time ($[I]_0 \approx [I]_{\infty}$), and to a saturation of the enzyme by the inhibitor ($[S]_0 \leq K_m$, $[I]_0 \gg K_i$). Thus the reaction would start with an initial steady state ($[E]_0 \approx [E_a]$) and follow a post-steady-state transient phase with partial enzyme inactivation ($[E]_0 \approx [E_a] + [EI^*]$) towards a final state with complete inactivation of the enzyme ($[E]_0 \approx [EI^*]_{\infty}$). The fastest inactivation process occurs under the high $[I]_0$ conditions with an apparent constant of inactivation equal to $k_i - k_h$ [eqn. (7)], increasing the possible requirement of rapid kinetics instrumentation.

In practice, the low $[I]_0$ assay conditions are always useful for obtaining V_0 values, which can lead to the determination of K_i [eqn. (4)]. The determination of k_i , however, can only be carried out in these assay conditions when significant values of V_s can be obtained [eqn. (5)]. Otherwise (when $V_s \approx 0$), high $[I]_0$ assay conditions are required for the determination of k_i from eqn. (7). The application for low $[I]_0$ conditions [eqns. (3)–(5)] would involve two linear regression fits per ‘slow’ recording, for one series of ‘low’ concentrations of each reagent (E, S and I). The use of high $[I]_0$ conditions [eqn. (7)] would require one non-linear regression fit per ‘fast’ recording, for one more series of ‘high’ concentrations of each reagent. Thus greater quantities of possibly expensive reagents or the involvement of cumbersome processes of isolation and purification from biological sources would be needed. Our experimental system (Figure 1, curve b) showed significant values of V_0 and V_s , so that high $[I]_0$ assays were not necessary. Therefore the application of an experimental approach based on the low $[I]_0$ assay conditions only has been detailed below.

Inactivation assays: effects of $[E]_0$

Under any assay conditions, appropriate $[E]_0$ must be chosen to ascertain whether the original planning considerations of the kinetic analysis were obeyed. Thus 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.

The increase in $[E]_0$ originated higher product formation and longer duration of the inter-steady-state transient phase (results not shown). This led to a linear dependence of V_0 [eqn. (4)] and V_s [eqn. (5)] on $[E]_0$ (Figure 2), in accordance with the kinetic analysis.

From eqns. (4) and (5) the prediction of the shape of the progress curves for inactivation assays was possible, according to the value of the ratio $r = k_i/k_h$. When $r > 1$, enzyme inactivation would prevail over inhibitor decomposition, leading to a burst phase ($V_0 > V_s$) such as that observed here (Figure 1b). When $r = 1$, enzyme inactivation and inhibitor breakdown would be compensated, originating a linear accumulation of product ($V_0 = V_s$). When $r < 1$, a large part of I would be in the EI species, the relative concentration of which within E_a ($[E]_a = [E] + [ES] + [EI]$) would decrease with the disappearance of I . Thus the relative increase in ES species within E_a would prevail over the inactivation of E_a towards EI^* species, generating a lag period ($V_0 < V_s$). These predictions and explanations were not described from the multiexponential equations developed by other authors, although analytical and numerical simulations of the three shapes ($r > 1$, $r = 1$ and $r < 1$) were empirically reported (Topham, 1990).

Figure 2 Effect of $[E]_0$ on trypsin inactivation by APMSF in the presence of ZLSBE

Conditions were as described in the Materials and methods section with 0.52–5.2 nM trypsin, 50 μ M ZLSBE and 1.8 μ M APMSF. ●, ▲, Experimental values of V_0 and V_s versus $[E]_0$ respectively; —, linear regression fits corresponding to V_0 [eqn. (4)] and V_s [eqn. (5)] versus $[E]_0$.

Figure 3 Dependence of V_0 on $[S]_0$ for trypsin inactivation by APMSF in the presence of ZLSBE

Conditions were as described in the Materials and methods section with 5 nM trypsin, 5–50 μ M ZLSBE and 1.8 μ M APMSF. ●, ○, Experimental values of V_0 and $[S]_0/V_0$ versus $[S]_0$ respectively; —, non-linear regression fit of V_0 versus $[S]_0$ to eqn. (4); —, linear regression fit of $[S]_0/V_0$ versus $[S]_0$ (Hanes–Woolf plot); —, non-linear regression fit of V_0 versus $[S]_0$ to eqn. (4).

Inactivation assays: effects of $[S]_0$

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 (results not shown). The hyperbolic dependence of V_0 on $[S]_0$ appeared to be confirmed by the linearization with the Hanes–Woolf transformation $[S]_0/V_0$ versus $[S]_0$ (Figure 3), in accordance with the kinetic analysis [eqn. (4)]. The fit of these data by linear regression gave values of intercept and slope, which were used as a source of initial estimates for the non-linear regression fit of V_0 versus $[S]_0$ to eqn. (4) (Figure 3).

Figure 4 Dependence of V_s on $[S]_0$ for trypsin inactivation by APMSF in the presence of ZLSBE

Conditions were as detailed in Figure 3. ▲, △, Experimental values of V_s versus $[S]_0$ and that of $\log [V_s/(V_{s_{app}} - V_s)]$ versus $\log [S]_0$ (Hill plot) respectively. —, Linear regression fit of data from the Hill plot with ordinate values within the -1 to $+1$ range; —, non-linear regression fit of V_s versus $[S]_0$ to the Hill equation; ···, non-linear regression fit of V_0 versus $[S]_0$ to eqn. (5) with final estimations of the kinetic constants (Table 1).

Enhancement of $[S]_0$ originated greater V_s values with a sigmoid pattern (Figure 4). From these data and choosing the apparent maximum rate, $V_{s_{app}} = 5 \times 10^{-8}$ M/s, a Hill plot was obtained (Figure 4). This plot was a true curve with the highest linear portion for data points within the -1 to $+1$ ordinate range (Dahlquist, 1978; Ricard and Cornish-Bowden, 1987). The fit of these data by linear regression provided values of intercept and slope, which were used to obtain initial estimates of the kinetic parameters of the Hill equation. Then the further non-linear regression fit of V_s versus $[S]_0$ to the Hill equation: $V_s = V_{max} [S]_0^h / (K^h + [S]_0^h)$, gave rise to a successful convergence with $V_{max} = 11.91 \times 10^{-8}$ M/s, $K = 64.56 \times 10^{-6}$ M and $h = 1.87$. Therefore these sigmoid data (Figure 4) showed apparent positive co-operativity, according to the usual criterion $h > 1$ (Dahlquist, 1978; Neet, 1980; Ricard and Cornish-Bowden, 1987). Kinetic studies on new protein and non-protein irreversible inhibitors must involve careful characterization of their instability. Thus new irreversible inhibitors with high instability that has not been properly characterized could be assayed with slow spectrophotometers, but only linear recordings with intercepts near the origin of the final steady-state would be obtained (Figure 1b). The corresponding true V_s values might be erroneously considered as V_0 values with apparently co-operative behaviour. Therefore true unstable irreversible inhibitors could be wrongly described as allosteric reversible inhibitors. The apparent positive co-operativity obtained here (Figure 4) is a new type of apparent kinetic co-operativity not previously described (Neet, 1980; Ricard and Cornish-Bowden, 1987). The kinetic origin of this apparent co-operativity was the reaction mechanism for a single-site enzyme (Scheme 1) under the above assay conditions for the kinetic analysis (see the Materials and methods section). This apparently co-operative behaviour was not predicted by other authors in theoretical papers on enzyme inactivation by unstable irreversible inhibitors in the presence of an auxiliary substrate (Topham, 1990).

Figure 5 Dependence of V_0 on $[I]_0$ for trypsin inactivation by APMSF in the presence of ZLSBE

Conditions were as described in the Materials and methods section with 1.3 nM trypsin, 20 μ M ZLSBE and 0.42–4.2 μ M APMSF. ●, ○, Experimental values of V_0 and $1/V_0$ versus $[I]_0$ respectively. ———, Linear regression fit of $1/V_0$ versus $[I]_0$ (Dixon plot); ———, non-linear regression fit of V_0 versus $[I]_0$ to eqn. (4).

Figure 6 Dependence of V_s on $[I]_0$ for trypsin inactivation by APMSF in the presence of ZLSBE

Conditions were as detailed in Figure 5. ▲, △, Experimental values of V_s versus $[I]_0$ and $\log(V_s/V_0)$ versus $\log[(1+s)/(1+s+i)]$ (logarithmic plot), respectively. ———, Linear regression fit of the logarithmic plot; ———, non-linear regression fit of V_s versus $[I]_0$ to eqn. (5).

Inactivation assays: effects of $[I]_0$

The increase in $[I]_0$ led to lower product formation and to a shortening of the inter-steady state transient phase (results not shown). The non-linear decrease in V_0 with $[I]_0$ was linearized with the Dixon transformation $1/V_0$ versus $[I]_0$ (Figure 5), according to the kinetic analysis [eqn. (4)]. These data were fitted

by linear regression and yielded values of intercept and slope with quotient equal to $K_i[1 + ([S]_0/K_m)]$. This value of K_i was used as an initial estimate in a further non-linear regression fit of V_0 versus $[I]_0$ to eqn. (4) (Figure 5), which gave rise to the final value of this dissociation constant (Table 1).

When $[I]_0$ rose, V_s was reduced in a non-linear way (Figure 6). Eqn. (5) predicted that a plot of $\log(V_s/V_0)$ versus $\log[(1+s)/(1+s+i)]$ is linear with a slope equal to $(r-1)$. Thus this plot was depicted from V_0 versus $[I]_0$ data (Figure 5) and V_s versus $[I]_0$ data (Figure 6), as well as from the previously determined values of K_m and K_i (Table 1). The slope of this linear plot (Figure 6) provided an initial estimate of r , the final value of which (Table 1) was obtained from the non-linear regression fit of V_s versus $[I]_0$ to eqn. (5) (Figure 6). The values of k_h and $r = k_i/k_h$ led to the calculation of k_i (Table 1). The efficiency of this procedure was supported by its simplicity and low propagation of errors. Topham (1990) proposed the calculation of k_i/k_h from seven algebraic operations [eqns. (29) and (30) of that paper], involving parameters of the multiexponential equation, the severe limitations of which were mentioned above.

The sigmoid pattern of the V_s versus $[S]_0$ plot arose from the protection of the substrate during inactivation of the enzyme by irreversible inhibitor [eqn. (5)], as well as from the rational nature of the values of r (Table 1). The values of the kinetic constants (Table 1) were used as initial estimates in the non-linear regression fit of V_s versus $[S]_0$ to eqn. (5). A successful fit, very similar to that obtained by using the Hill equation, was observed (Figure 4). Thus the prediction of our kinetic analysis was confirmed [eqn. (5)].

Conclusions

Discontinuous methods

The above-described continuous method is more reliable than discontinuous procedures, as it involves a single measurement of product formation per concentration of reagent, without the removal of samples 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 10–20 data points usually obtained in discontinuous methods, 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 (≥ 10) for the screening of the effect of each reagent. This makes it easier to carry out the experimental assays required to find the appropriate range of concentration of each reagent, below and above K_m and K_i , for instance (Bevington, 1969).

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 fitting of experimental recordings by simple linear regression and non-linear regression to uniexponential equations. The serious limitations of other theoretical methods (Topham, 1990) have been pointed out. The instrumentation required for these studies is determined by the assay medium, by the kinetic constants of the inactivation system and by the appropriate choice of concentration of the reagents [eqns. (3)–(7)]. This is the first report of the kinetic characterization of irreversible inhibition of trypsin by APMSF at pH 8.0. A new type of apparent

kinetic co-operativity was observed, in accordance with that predicted by one of the exact analytical equations derived here.

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