Kinetic Study on the Inhibition of Type Ib Pencillinase by Imipenem and Thienamycin

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The mechanism of inhibition of type Ib penicillinase by imipenem (N-formimidoyl thienamycin) and thienamycin was studied. Imipenem and thienamycin. were found to inhibit the penicillinase in a progressive manner, but they differed from one another in the maximum level of enzyme inhibition, even with excess inhibitor. The maximum inhibitions by imipenem and thienamycin amounted to about 50% and 80%, respectively. To elucidate the mechanism of the equilibrium phenomenon, the rate constants in the proposed scheme were estimated. Computer-assisted simulation using the rate constants revealed that there is an upper limit to the extents of inactivation by imipenem and thienamycin.

Keywords——type Ib penicillinase; thienamycin; imipenem; progressive inhibition; computer-assisted simulation

Introduction

Since the discovery of clavulanic acid[1] in 1976, a variety of β-lactamase inhibitors with a novel β-lactam nucleus have been found. Although most of them, including clavulanic acid, sulbactam[2] and 6β-bromopenicillanic acid,[3] irreversibly inactivate β-lactamases in a time-dependent manner (progressive inhibition) through covalent-bond formation,[3,4] these compounds exhibit hardly any antibacterial potency. Thienamycin[5] and related carbapenem derivatives,[6-8] such as imipenem (N-formimidoyl thienamycin),[9,10] are interesting, for they have high activities as both antibacterial agents and β-lactamase inhibitors, although the inhibitory effect is generally reversible.[11-14]

In a previous paper,[15] we reported that imipenem inhibited Proteus vulgaris β-lactamase (cephalosporinase) in a progressive manner, and that the activity of the inactivated enzyme was not completely recovered after intact imipenem had been completely exhausted. During the course of studies on imipenem, as a β-lactamase inhibitor, we observed that type Ib penicillinase[16-18] (TEM-type penicillinase mediated by the R plasmid) was reversibly inhibited in a time-dependent manner by imipenem and thienamycin, but the maximum inhibition level reached an equilibrium point even when the inhibitor concentration was increased greatly.

This study was undertaken, as an extension of the previous study, to elucidate the mechanism of inhibition of the penicillinase by the thienamycins, and to elucidate the reason for the equilibrium phenomenon.

Materials and Methods

Enzyme——Type Ib penicillinase is a TEM-2 penicillinase according to Matthew's classification.[16] The penicillinase was produced in an *Escherichia coli* strain harboring R plasmid RGN823[17] and was purified by the
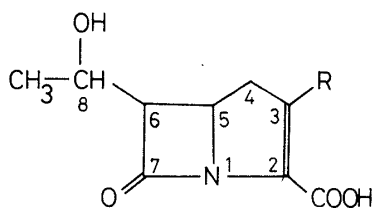
compounds	R
thienamycin	$-SCH_2CH_2NH_2$
imipenem	$-SCH_2CH_2NH-CH=NH_3$

Fig. 1. Structures of Thienamycin and Imipenem

procedure described previously.[18]

The actual specific activity of the enzyme used in the present study was 1913 units per mg of protein. The amount (mol) of active enzyme was calculated on the basis of the specific activity. One unit of penicillinase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of benzylpenicillin per minute at pH 7.0 and 30 °C.

β-Lactam Antibiotics——Imipenem and thienamycin were provided by Merck Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A. The chemical structures of the antibiotics are shown in Fig. 1. Benzylpenicillin was a commercial product of Banyu Pharmaceutical Co., Ltd., Tokyo, Japan.

Measurement of the Progressive Inhibition of the Penicillinase——Inhibition of the penicillinase by the thienamycins was measured by means of a modification of the dilution method of Fisher et al.[11] Three hundred microliters of the penicillinase solution (final concentration of the active enzyme, 1 μM) was preincubated with 15 μl of various concentrations of an inhibitor in 50 mM sodium-potassium phosphate buffer, pH 7.0, at 30 °C for an appropriate period of time. After the incubation, a 5-μl aliquot of the mixture was removed and immediately added to 3.0 ml of the phosphate buffer containing 200 μM benzylpenicillin. The substrate-containing buffer was warmed at 30 °C prior to mixing. Under these conditions, competitive inhibition by the inhibitors could be prevented since the inhibitors were diluted in excess substrate. The remaining penicillinase activity was estimated by a spectrophotometric method.[14] The enzyme reaction was monitored at 30 °C with a temperature-controlled spectrophotometer (Perkin Elmer 550). When the enzyme–inhibitor mixture was diluted in excess substrate, a very short lag time of about one minute was observed before the remaining activity was fully expressed. Therefore, the residual enzyme activity was calculated from the rate of benzylpenicillin hydrolysis after the short lag time. The degree of enzyme inactivation was expressed as the percentage of the remaining enzyme activity to the enzyme activity in the absence of an inhibitor.

Determination of Kinetic Parameters (K_i, K_m, and k_{cat})——The K_i values of the penicillinase for the thienamycins were determined by the procedure described previously.[14] The rate constants for hydrolysis (k_{cat}: molar activity) of imipenem and thienamycin by the penicillinase were determined from the initial velocity of hydrolysis with substrate concentrations sufficiently higher than the respective K_i values. The hydrolysis of the thienamycins was measured by following the change in absorption at 299 nm for imipenem and at 297 nm for thienamycin.

Computer-Assisted Simulation——The validity of the scheme proposed for the penicillinase inhibition by the thienamycins was established by a modified Runge-Kutta-Gill numerical integration method, as described previously.[13]

Results

Progressive Inhibition of Type Ib Penicillinase by the Thienamycins

Progressive inhibition of type Ib penicillinase by imipenem and thienamycin was measured by preincubating the enzyme with the inhibitors for different times according to the procedure described under Materials and Methods.

The results are shown in Fig. 2A, B. The rate of inactivation of the enzyme by imipenem was relatively lower than that by thienamycin, but showed a similar pattern to that in the case of thienamycin. The maximum inactivation obtained with imipenem corresponded to about 50% inhibition even when the molar ratio of the inhibitor to the enzyme was increased to 500. A similar phenomenon was observed with thienamycin, the maximum inactivation level corresponding to about 80% inhibition. The inactivated enzyme resulting on treatment with the thienamycins gradually regained its activity, and the full activity was restored after
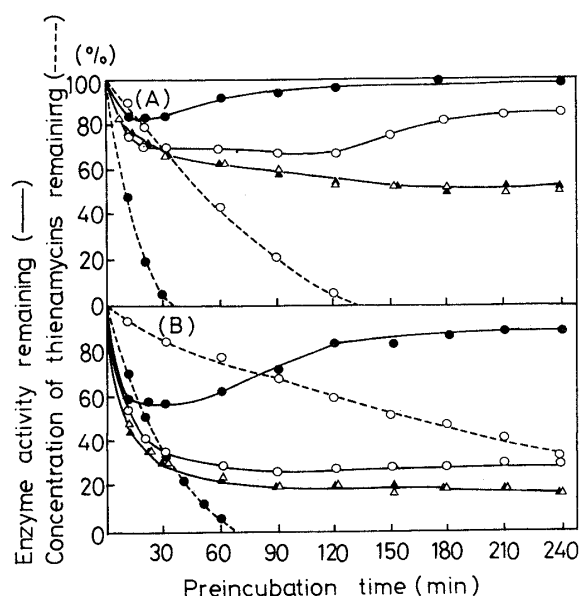
Fig. 2. Effect of the Preincubation Time with the Thienamycins on the Activity of Type Ib Penicillinase, and the Hydrolysis of the Thienamycins by the Penicillinase

Type Ib penicillinase (1 μM) was incubated at 30 °C with (A) (●) 10, (○) 50, (▲) 250 or (△) 500 μM imipenem, and (B) (●) 10, (○) 50, (▲) 100 or (△) 250 μM thienamycin. The enzyme activity remaining (solid line) was measured by the dilution method described under Materials and Methods. The concentrations of the intact inhibitors (dotted lines) in the reaction mixtures were spectrophotometrically monitored.

incubation of 16 h (data not shown). It can be presumed from Fig. 2 that the reactivation starts just after complete consumption of the inhibitors. The k_{cat} values of the penicillinase for the thienamycins were estimated from the experimental data in Fig. 2A, B. The k_{cat} for imipenem and thienamycin were 0.62 and 0.42 min^{-1}, respectively. The K_i of imipenem and thienamycin for the penicillinase, which were determined by using benzylpenicillin as the substrate, were 6.1 and 3.3 μM, respectively.

Estimation of the Rate Constants for the Enzyme Inhibition by Imipenem and Thienamycin

As mentioned above, imipenem and thienamycin were poor substrates and acted on the enzyme as progressive inhibitors. However, the inactivated enzyme became completely reactivated after consumption of the inhibitors in the medium, and the maximum level of the progressive inhibition reached an equilibrium point. These phenomena could not be observed in the case of the progressive inhibition of *Proteus vulgaris* cephalosporinase by imipenem.[15] In order to elucidate the inhibitory mechanism as regards the kinetic aspects of the enzyme inhibition, the scheme shown in Fig. 3 is proposed. In this scheme, the transiently stable complex, X, branches off from the normal intermediate, M, which is probably the acyl-enzyme.[19]

If the inhibition kinetics follow the proposed scheme, we can estimate the respective rate constants in the scheme by means of the following mathematical analysis. The reaction velocities of I, E, E·I, M and X in the scheme are expressed by the following set of differential equations while the reaction proceeds.

$$d[\text{I}]/dt = -k_1[\text{E}][\text{I}] + k_{-1}[\text{E·I}] \tag{1}$$

$$d[\text{E}]/dt = -k_1[\text{E}][\text{I}] + k_{-1}[\text{E·I}] + k_3[\text{M}] \tag{2}$$

$$d[\text{E·I}]/dt = k_1[\text{E}][\text{I}] - (k_{-1} + k_2)[\text{E·I}] \tag{3}$$

$$d[\text{M}]/dt = k_2[\text{E·I}] - (k_3 + k_4)[\text{M}] + k_{-4}[\text{X}] \tag{4}$$

$$d[\text{X}]/dt = k_4[\text{M}] - k_{-4}[\text{X}] \tag{5}$$

Equational Expression of k_{cat}——Since the initial rate of enzymatic hydrolysis of imipenem and thienamycin was sufficiently faster than the inactivation rate, it is reasonable to assume that the k_3 value was much larger than that of k_4. Therefore, in the early stage of the enzymatic reaction, we can neglect the X-formation reaction in the calculation of the initial

Fig. 4. Semi-logarithmic Plot of Initial Inactivation of Type Ib Penicillinase by Imipenem and Thienamycin

The data in Fig. 2A, 2B were replotted. Penicillinase (1 μM) was incubated at 30 °C with 500 μM imipenem (●) or 250 μM thienamycin (○).
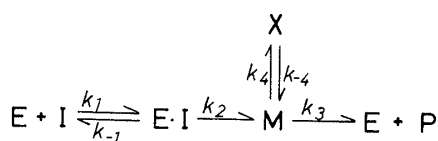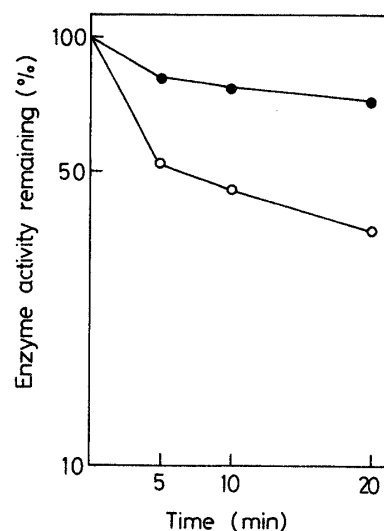

Fig. 3. Possible Minimal Scheme for the Progressive Inhibition of Type Ib Penicillinase by Imipenem and Thienamycin

E, E·I and M are the free enzyme, Michaelis complex and acyl intermediate, respectively. X represents to the unstable complex. P represents the hydrolyzed product of the thienamycins.

molar activity of the enzyme (k_{cat}). The following equation was taken from ref. 20:

$$k_{cat} = k_2 k_3 / (k_2 + k_3)$$ (6)

Equational Expression of the Inactivated Enzyme Fraction (F)——The branch from intermediate M to intermediate X should decrease the enzyme activity in the medium. When excess thienamycin or imipenem was present in the reaction mixture, there was a steady state at which the apparent enzyme activity remained constant (Fig. 2). In this state, the formation and degradation of the X-intermediate should be in equilibrium. Therefore, we can reasonably postulate the following conditions in this state:

$$d[E]/dt = d[E \cdot I]/dt = d[M]/dt = d[X]/dt = 0$$ (7)

Inactive fraction F is the sum of intermediates X and M, as verified in the Discussion, and is expressed as follows.

$$F = ([X] + [M])/[E_0]$$ (8)

E_0 represents total enzyme.

Under the condition of excess [I], the following equation can be derived (see Appendix):

$$1/F = 1 + k_3 k_{-4} / k_2 k_4$$ (9)

Equational Expression of k_{inact}——The time course of the inactivation was found to be biphasic, as shown in Fig. 4, indicating the existence of at least two steps in the inactivation process. The faster process may correspond to the formation of the acyl-enzyme (M-intermediate), which reaches an equilibrium state within 5 min. The faster process may be followed by the slow conversion of the M-intermediate to the X-intermediate, which is far more stable than the M-intermediate, although the formation of X is essentially reversible. With the assumption described above, the k_{inact} value in the second process is expressed as follows:

$$d[X]/dt = k_{inact}[E_0]$$ (10)

TABLE I. Calculated Rate Constants for the Computer-Assisted Simulation

Rate constants[a]	Imipenem	Thienamycin
K_i $(\mu M)^{b)}$	6.1	3.3
k_2 (min^{-1})	0.82	0.79
k_3 (min^{-1})	2.58	0.90
k_4 (min^{-1})	0.025	0.056
k_{-4} (min^{-1})	0.009	0.013

a) The rate constants are those for the minimal scheme in Fig. 3. b) $K_s(=k_{-1}/k_1)=K_i$.
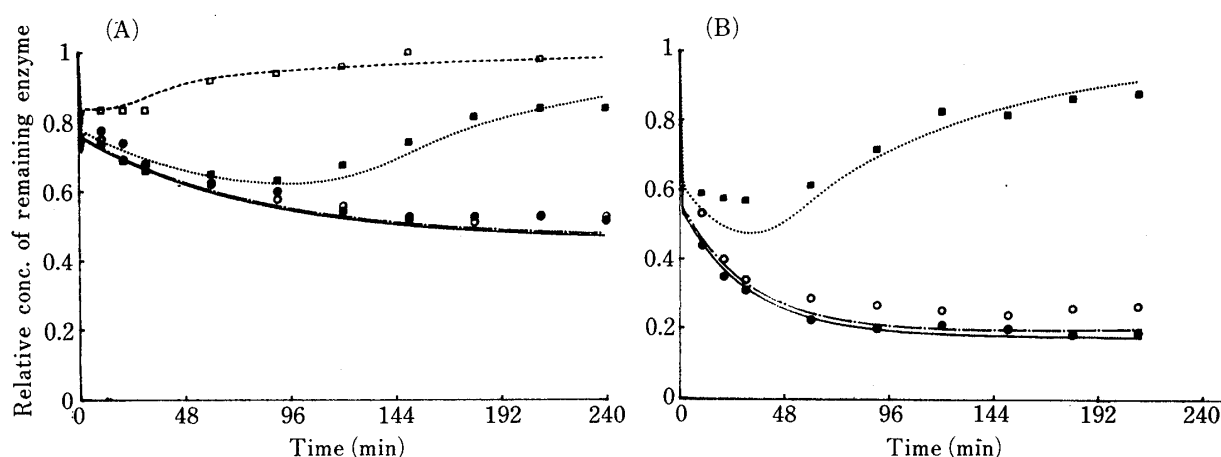

Fig. 5. Simulation of the Progress Curve for Type Ib Penicillinase Inactivation by Imipenem (A) and Thienamycin (B) with Various Initial I/E Ratios

Progress curves for $[E]+[E \cdot I]$ calculated by numerical integration were compared with experimental values of the enzyme activity remaining (symbols), obtained from Fig. 2. The rate constants used for the calculation are listed in Table I. The initial enzyme concentration was 1 μM. The initial inhibitor concentrations were as follows: (A) (□) 10, (■) 50, (○) 250, (●) 1000 μM; (B) (■) 10, (□) 50, (●) 250 μM.

The k_{inact} values for imipenem and thienamycin were estimated from the experimental data to be 0.006 and 0.026 min^{-1}, respectively. If the X-intermediate is still negligible in the equilibrium stage between the formation and degradation of the M-intermediate, the k_{inact} value can also be expressed as follows (see Appendix):

$$k_{inact} = k_2 k_4/(k_2 + k_3) \tag{11}$$

Estimation of k_{-4}———The reactivation constant (k_{react}) could be estimated from the results shown in Fig. 2. The estimated values of k_{react} for imipenem and thienamycin were 0.009 and 0.013 min^{-1}, respectively. The possibility that the reactivated enzyme is directly derived from the X-intermediate cannot be ruled out. However, in any case, the experimental value of k_{react} may be approximately equal to the k_{-4} value.

Derivation of Equations for Evaluating k_2, k_3, k_4 and k_5———Solution of Eqs. 6, 8, 9 and 11 leads to the following equations:

$$k_2 = k_{react} k_{cat}/(1/F-1)k_{inact} \tag{12}$$

$$k_3 = k_4 k_{cat}/k_{inact} \tag{13}$$

$$k_4 = k_2 k_{inact}/(k_2 - k_{cat}) \tag{14}$$
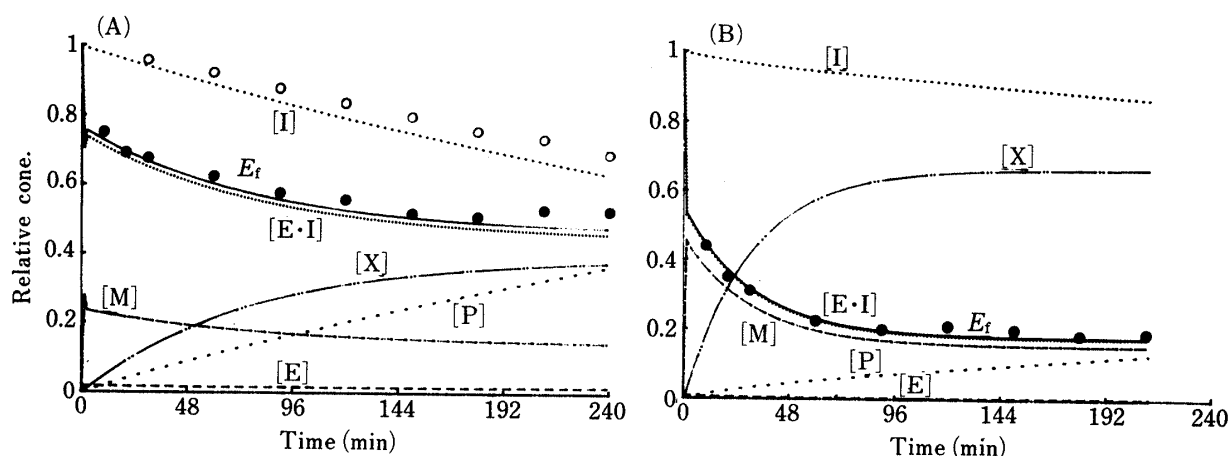
K_s can be obtained as follows:

Fig. 6. Numerical Solutions for All the Intermediates of the Reaction of Imipenem
(A) and Thienamycin (B) with the Enzyme Shown in Fig. 3

The initial molar ratio of the inhibitors to the enzyme (I/E) was 250. The experimental data plotted are: (●) residual activity, (○) concentrations of imipenem and thienamycin remaining unhydrolyzed in the reaction mixture. E_f represents the residual activity (E+E·I).

$$K_s \ (=k_{-1}/k_1)=K_m \ (\text{or} \ K_i) \tag{15}$$

The rate constants in the scheme (Fig. 3) could be estimated by using the above equations, and are listed in Table I.

Computer-Assisted Simulation of the Inhibitory Mechanism

The validity of our proposed scheme for the progressive inhibition of type Ib penicillinase by the thienamycins was verified by means of a modified Runge-Kutta-Gill numerical integration method[13] using the rate constants in Table I. A program, called YHS 1, for numerical integration of the set of rate equations derived from the scheme was developed using a HITAC M-180 computer and transferred to a personal computer, NEC PC9801. A copy of this program written in FORTRAN 77 or N88-BASIC can be obtained from the authors. The enzyme activity measured with various initial molar ratios of the inhibitor to the enzyme (I/E) was compared with the calculated value. The experimental values agreed very closely with the calculated values (Fig. 5).

Then, all the intermediates, M, X, P and I, were calculated using the rate constants obtained. The hydrolysis curves for substrate (I) were also consistent with the calculated values, within experimental error (Fig. 6).

Discussion

Type Ib penicillinase (TEM-2 penicillinase) is a common penicillinase mediated by the R plasmid and is widely distributed among gram-negative bacteria including *Pseudomonas*. The penicillinase was inhibited in a time-dependent manner by imipenem and thienamycin, and the results obtained with imipenem confirmed our preliminary observation.[14] However, imipenem and thienamycin differ from one another in the maximum level of enzyme inhibition in the presence of excess inhibitor. The equilibrium phenomenon and the difference in the inhibition level between imipenem and thienamycin can be explained by the results obtained in this study. Calculations based on the proposed scheme (Fig. 3) revealed that there is an upper limit to the extent of inactivation, which depends on the ratio of the k_2 and k_3 values. As shown in Table I, the k_3 value for imipenem is greater than the k_2 value. The higher rate of release of free enzyme from the acyl-intermediate (M) is probably the reason for the

$$X \xrightarrow{k_5} E + P'$$

$$\Big\uparrow k_4$$

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} M \xrightarrow{k_3} E + P$$
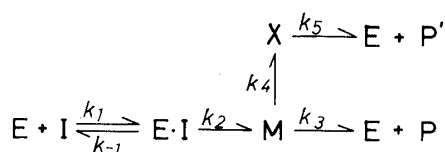
Fig. 7. Alternative Possible Pathway for Inactivation of Type Ib Penicillinase by Imipenem and Thienamycin

P' represents the product obtained on direct decomposition of X.

50% equilibrium point. Complete inactivation can be achieved if k_2 is greater than k_3. The k_2 value for thienamycin is almost the same as the k_3 value, and this is probably the reason for the 80% inactivation by the inhibitor.

An alternative pathway (Fig. 7) in which the transiently inactivated species, X, directly decomposes, i.e., not via the acyl-intermediate M, is also consistent with the experimental results obtained in this study. These two pathways (Figs. 3 and 7) are kinetically indistinguishable from each other unless product P' is detected. In the case of the latter pathway (Fig. 7), the decomposition rate (k_5) for the X-intermediate must be the same as the k_{-4} value of the former pathway under the condition of $k_{-4} \ll k_3$, because:

$$k_{\text{react}} = k_5 = k_3 k_4/(k_3 + k_{-4}) = k_{-4}$$

The two schemes proposed by us are similar to those for related carbapenems, olivanic acid derivatives, reported by Charnas and Knowles[19] in the case of TEM-penicillinase. They also referred to imipenem and thienamycin in their discussion, and claimed that these compounds follow the branched pathway. However, they did not compare the experimental values with the calculated values based on the proposed pathway.

The degradation of intermediate M to the native enzyme, E, was fast, as shown by the higher k_3 value. It could therefore be presumed that the major part of the M fraction is detected as the active enzyme with the dilution assay method. However, our computer-assisted simulation of the enzyme activity well fitted the experimental values only when intermediate M was regarded as the inactive enzyme fraction, F. This disagreement may be due to the structural deformation of the inactive enzyme being stably retained for some time even after the enzyme is released from the inhibitor. A similar observation was reported by Citri et al.[21] for the combination of TEM-1 penicillinase and cefoxitin. With the dilution assay method, the remaining activity was fully expressed about one minute after the dilution. The short lag time may reflect degradation of the Michaelis complex, E·I.

The work described here demonstrates, in addition to the characteristics of the interaction between the penicillinase and the thienamycins, the usefulness of computer-assisted simulation for understanding the interactions between β-lactamases and progressive inhibitors. A detailed explanation of the computer-assisted simulation is given in the Appendix.

Appendix

The derivation of Eqs. 9 and 11 in the text was performed as follows.
Eq. 9

$$d[E]/dt = d[E \cdot I]/dt = d[M]/dt = d[M]/dt = 0 \tag{1'}$$

Substituting the set of differential Eqs. 1—5 in the text into Eq. 1' then solving for [X], [E·I] and [E], we obtain:

$$[X] = k_4[M]/k_{-4} \tag{2'}$$

$$[E \cdot I] = k_3[M]/k_2 \tag{3'}$$

$$[E] = (k_{-1} + k_2)k_3[M]/k_1 k_2[I] \tag{4'}$$

Total enzyme [E_0] in the reaction mixture is given by:

$$[E_0] = E + [E \cdot I] + [M] + [X] \tag{5'}$$

Substituting Eqs. 2′—4′ into Eq. 5′, we obtain:

$$[M]/[E_0] = k_1 k_2 k_{-4}[I]/\{k_3 k_{-4}(k_{-1}+k_2) + k_1(k_2 k_{-4} + k_2 k_4 + k_3 k_{-4})[I]\} \qquad (6')$$

Substituting Eq. 6′ into Eq. 2′, we obtain:

$$[X]/[E_0] = k_1 k_2 k_4[I]/\{k_3 k_{-4}(k_{-1}+k_2) + k_1(k_2 k_{-4} + k_2 k_4 + k_3 k_{-4})[I]\} \qquad (7')$$

Using Eqs. 6′ and 7′ the inactive fraction at the steady state in the presence of excess [I] can be expressed as follows:

$$F = \lim_{I \to \infty} ([X] + [M])/[E_0]$$

$$= \lim_{I \to \infty} [k_1 k_2 k_4/\{k_3 k_{-4}(k_{-1}+k_{-2})/[I] + k_1(k_2 k_{-4} + k_2 k_4 + k_3 k_{-4})\}$$

$$+ k_1 k_2 k_{-4}/\{k_3 k_4(k_{-1}+k_4)/[I] + k_1(k_2 k_{-4} + k_2 k_4 + k_3 k_{-4})\}]$$

$$= 1/\{1 + k_3 k_{-4}/k_2(k_4 + k_{-4})\} \qquad (8')$$

On the assumption of $k_4 \gg k_{-4}$:

$$I/F = 1 + k_3 k_{-4}/k_2 k_4 \qquad (9')$$

Which gives Eq. 9

Eq. 11

At the end of the initial inactivation phase, the following conditions are reached:

$$d[E \cdot I]/dt = d[M]/dt = 0 \qquad (10')$$

$$[X] = 0 \qquad (11')$$

Substituting the set of differential rate equations in the text into Eq. 10′, and solving for [E·I] and [M], considering Eq. 11′:

$$[E \cdot I] = k_1[E][I]/(k_{-1} + k_2) \qquad (12')$$

$$[M] = k_2[E \cdot I]/(k_3 + k_4) = k_1 k_2[E][I]/(k_3 + k_4)(k_{-1} + k_2) \qquad (13')$$

Under the conditions of Eq. 11′, [E_0] is expressed as follows:

$$[E_0] = [E] + [E \cdot I] + [M] \qquad (14')$$

Eq. 14′ can be rewritten by substituting Eqs. 12′ and 13′:

$$[M]/[E_0] = k_1 k_2[I]/\{(k_{-1} + k_2)(k_3 + k_4) + k_1(k_2 + k_3 + k_4)[I]\} \qquad (15')$$

$$\lim_{I \to \infty} [M]/[E_0] = k_2/(k_2 + k_3 + k_4) \qquad (16')$$

Therefore, $d[X]/dt$ at the pre-steady state is expressed by:

$$d[X]/dt = k_4[M] = k_2 k_4[E_0]/(k_2 + k_3) \qquad (17')$$

Comparing Eq. 17′ and Eq. 10 in the text, we can obtain:

$$k_{\text{inact}} = k_2 k_4/(k_2 + k_3) \qquad (18')$$

This is identical with Eq. 11 in the text.

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