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PATHOPHYSIOLOGICAL INTERPRETATION OF KINETIC CONSTANTS OF PROTEASE INHIBITORS

INTERPRÉTATION PHYSIOPATHOLOGIQUE DES CONSTANTES CINÉTIQUES DES INHIBITEURS DE PROTÉASES

J.G. Bieth *

ABSTRACT : Natural proteinase inhibitors may form reversible or irreversible complexes with proteinases. In the former case, the simplest reaction scheme is : $E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E.I.$ The dissociation constant of the complex is given by $Ki = k_{off}/k_{on}$. In the latter case, the complex formation can only be characterized by k_{on}.

It is frequently possible to determine the above kinetic parameters by very simple enzymatic means. The principle of these determinations is outlined. Practical details are also given.

The *in vivo* significance of these constants is as follows. The association rate constant k_{on} allows the calculation of the time required for a proteinase to be inhibited *in vivo*. The dissociation rate constant k_{off} as well as the equilibrium constant Ki reflect the stability of the proteinase-inhibitor complex. Thus, an inhibitor, although efficient *in vitro*, may be considered as inefficient *in vivo* because of its low k_{on} value or its high k_{off} or Ki values. Temporary inhibition will also be considered.

The above theoretical considerations are illustrated by practical examples. Particular emphasis is made on enzyme inhibitor system important in lung emphysema like the reaction of native and oxidized α_1-proteinase inhibitor with leucocyte elastase.

Kinetic constants ; protease inhibitors.

Many biological systems are controlled by proteinases whose activity is controlled by proteinase inhibitors. Typical examples are blood coagulation or generation of kinins. Degenerative connective tissue diseases like pulmonary emphysema are also produced by proteinases and prevented by proteinase inhibitors.

In recent years a growing number of investigators have been interested in searching for new proteinase inhibitors in biological fluids or tissues as well as

* Laboratoire d'Enzymologie, FRA 47 de l'INSERM, Faculté de Pharmacie, Université Louis Pasteur, 67048 Strasbourg Cedex, France.

in testing known inhibitors on new proteinases. New inhibitors or inhibiting properties of known inhibitors have thus been found *in vitro*.

It is commonly thought that if an inhibitor is active *in vitro* it will also be active *in vivo* (*i.e.* it will play a physiological role). This is not always the case as will be shown. One way to demonstrate the physiological function of an inhibitor is to see whether it forms a complex with proteinases *in vivo*. Another way consists of determining the kinetic parameters of the proteinase/inhibitor interaction and inferring the physiological function from the data obtained *in vitro*.

1. DEFINITION OF KINETIC PARAMETERS

Natural proteinase inhibitors are proteins. Their reaction with proteinases may thus be considered as a special case of protein-protein interaction. Such interactions are usually characterized by a very high affinity between the two reaction partners. A special feature of proteinase/inhibitor interactions is that the proteinase may split one or several bonds within the peptide chain of the inhibitor. This limited proteolysis may either stabilize the enzyme-inhibitor complex or lead to a breakdown of this complex with or without release of active proteinase. In the latter case, the inhibition is said to be « temporary ». Three general reaction schemes may be defined.

1.1. Reversible inhibition

In this case the proteinase (E) reacts with the inhibitor (I) to form a reversible complex (E.I) according to scheme 1 :

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E.I \qquad (1)$$

where k_{on} is the complex formation rate constant, k_{off} the complex dissociation rate constant and Ki the equilibrium constant of the complex which is related to the rate constants by the following relationship :

$$Ki = \frac{k_{off}}{k_{on}} \qquad (2)$$

Scheme 1 is probably an oversimplification of the reaction pathway of reversible proteinase inhibitors. Two examples will illustrate this statement. *Example 1* concerns the reaction between basic pancreatic inhibitor and chymotrypsin. This system behaves according to scheme 1 if the kinetic parameters are determined using low concentrations (*ca* 10^{-7} M to 10^{-8} M) of enzyme and inhibitor [15]. With higher concentrations of reactants (*ca* 10^{-5} M to 10^{-6} M), an intermediate species E.I* could be detected with a stopped-flow apparatus [13]. The postulated mechanism is thus :

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.I^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E.I \qquad (3)$$

Example 2 is concerned with the reaction between soybean trypsin inhibitor and trypsin. Again when the concentration of the reactants is low, this system obeys scheme 1. However detailed kinetic and chemical investigations revealed that a peptide bond of the inhibitor is hydrolyzed by the reaction with trypsin [10]. This bond is, however, resynthesized by the enzyme upon dissociation of the enzyme-inhibitor complex. The association is thus truly reversible. The proposed reaction scheme is the following [10] :

$$T + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L^* \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} T + I^* \qquad (4)$$

where T is trypsin, I the virgin inhibitor, I* the modified inhibitor (peptide bond cleaved), L and L* loose non-covalent complexes, and C the stable complex.

Most reversible proteinase inhibitors will probably interact with proteinases by a multistep mechanism. Therefore the above kinetic constants k_{on}, k_{off} and Ki probably have a complex meaning. Nevertheless, these overall constants are sufficient for inferring the physiological role of the inhibitors.

1.2. Irreversible inhibition

The term « irreversible » is not thermodynamically correct. Irreversible inhibitor is an inhibitor whose complex with a protease does not dissociate within reasonable limits of time. Two cases may exist : 1) the inhibition is truly reversible but k_{off} is extremely slow. A typical example is the trypsin-basic pancreatic inhibitor complex which dissociates with a half-life of 14 weeks [14] ; 2) the inhibitor is broken down during its association with the proteinase but is not resynthesized by complex dissociation. A typical example is human plasma α_1-proteinase inhibitor which associates with trypsin, chymotrypsin or elastase to form an SDS-stable complex and a fragment of a molecular weight of 8,000 daltons [8]. In these cases the only important and measurable kinetic constant is k_{on}.

1.3. Temporary inhibition

The inhibition is said to be temporary if active proteinase can escape spontaneously from the complex after more or less prolonged incubation under physiological conditions [10]. The liberation of proteinase is due to the breakdown of the complexed inhibitor. This breakdown is operated either by the complexed proteinase or by free proteinase which is in excess over the complex.

The most simple and general scheme is as follows :

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E.I \xrightarrow{k} E + \text{inhibitor products} \qquad (5)$$

The hydrolysis constant k is a further valuable parameter to be measured.

A typical example is the reaction between trypsin and the pancreatic secretory (Kazal) inhibitor [10]. The inhibition of porcine pancreatic trypsin by α_1-proteinase inhibitor may also be considered as temporary since active enzyme is liberated from the complex after one week of incubation [12].

2. DETERMINATION OF KINETIC PARAMETERS

It is generally easy to measure kinetic parameters. In this paragraph we shall give some hints which should be helpful to investigators who are not familiar with kinetic measurements.

2.1. Preliminary experiments

Add increasing amounts of inhibitor to constant amounts of proteinase. Let the mixtures incubate for a fixed period of time (say 5 min). Add the substrate (preferably under a small volume) and measure the residual enzyme activities.

The plot of residual enzyme activity *vs* inhibitor concentration (*i.e.* the inhibition curve) may have various shapes : 1) *the inhibition curve is linear up to almost 0 % activity.* This is interpreted to mean first that the 5 min incubation time is sufficient to achieve complete association of proteinase and inhibitor, and second that : either the inhibition is reversible with $Ki \leqslant 0.01\ [E^\circ]$ (*i.e.* if the proteinase concentration $[E^\circ]$ is 10^{-7}M, Ki will be lower or equal to 10^{-9}M), or the inhibition is irreversible. In any case, this experiment yields the equivalence between enzyme and inhibitor, a value which is very important especially if the exact concentrations of active enzyme and inhibitor are unknown. 2) *the inhibition curve is concaved.* This may occur if the 5 min incubation time is not sufficient to achieve complete association of the two partners, or if the association is completed but the inhibition is reversible with a Ki of the same order of magnitude as the total enzyme concentration ($Ki \simeq [E^\circ]$).

To decide between these two alternatives, it is suggested that the enzyme/inhibitor mixtures should be incubated until the residual activity shows no further change with time. If the resulting inhibition curve is then linear, the discussion of « case 1 » applies. If on the other hand, the inhibition curve is still concave, the data may be used to calculate Ki (see section 2.4). Since it is difficult to determine an equivalence point from a concave curve, it is advisable to use a higher enzyme concentration. Theoretical basis for this has been developed elsewhere [3]. For instance, if an inhibitor whose $Ki = 10^{-8}$M is tested with $(E^\circ) = 10^{-8}$M, the inhibition curve is concave, whereas if it is tested with $(E^\circ) = 10^{-6}$M the curve will be linear. This may be done by using a less sensitive substrate.

2.2. Association rate constant k_{on}

For an irreversible inhibitor, the rate of association $-\frac{d(E)}{dt}$ is given by :

$$-\frac{d(E)}{dt} = k_{on}\ (E)\ (I) \tag{6}$$

whereas for a reversible inhibitor it is given by :

$$-\frac{d(E)}{dt} = k_{on}\ (E)\ (I) - k_{off}\ (EI) \tag{7}$$

which can be replaced by equation 6 during the initial part of the association.

If equivalent (equimolar) concentrations of proteinase and inhibitor are reacted, the half-life of inhibition $t_{1/2}$ is given by :

$$t_{1/2} = \frac{1}{k_{on}\ (E^{\circ})} \tag{8}$$

where (E°) is the initial enzyme concentration. Since k_{on} values may be as high as $10^7\ M^{-1}\ s^{-1}$, it may be calculated from equation 8 that (E°) should be in the nanomolar range in order to measure the time dependency of fast inhibition. The use of highly sensitive fluorogenic proteinase substrates renders this possible [5].

If the data are obtained with equivalent concentrations of proteinase and inhibitor they may be analysed using the following linear equation :

$$\frac{1}{(E)} = \frac{1}{(E^{\circ})} + k_{on} \cdot t \tag{9}$$

where (E°) is enzyme concentration at time zero, (E) is enzyme concentration at any time, and t is time.

Figure 1 shows an example illustrating the above theoretical considerations. The constant k_{on} may also be measured by competition experiments [14].

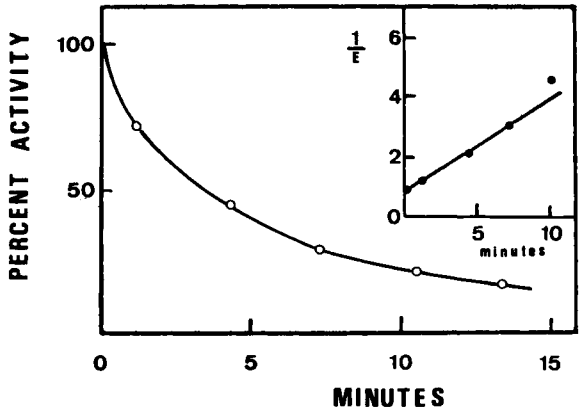

Fig. 1. — Time dependency of the inhibition of porcine pancreatic elastase by human plasma α_1-proteinase inhibitor. The insert is a second order replot in accordance with equation 9. From MEYER *et al.* [11].

2.3. Dissociation rate constant k_{off}

The rate of complex dissociation $-\frac{d(EI)}{dt}$ is given by :

$$-\frac{d(EI)}{dt} = k_{off}\ (EI) - k_{on}\ (E)\ (I) \qquad (10)$$

which may be simplified as :

$$-\frac{d(EI)}{dt} = k_{off}\ (EI) \qquad (11)$$

This equation is valid during the initial stages of the dissociation process only. The integrated form of equation 11 is :

$$\ln \frac{(EI)}{(EI^{\circ})} = -k_{off} \cdot t \qquad (12)$$

In order to follow the dissociation, equilibrium [5] must be shifted towards the left and the appearence of active enzyme has to be measured as a function of time. There are many ways to do this : a) Since there are two parts on the left hand side of the equation, but only one on the right side such an equilibrium may be shifted by simple dilution. b) « Removal » of free enzyme from the equilibrium : this may be achieved by adding a large excess of substrate which complexes with all of the free enzyme. Dissociation is then monitored by recording the substrate hydrolysis as a function of time. At any time, the rate of substrate hydrolysis will be proportional to the concentration of free enzyme. Hence (EI) of eqn 12 will be known. This method has been successfully used

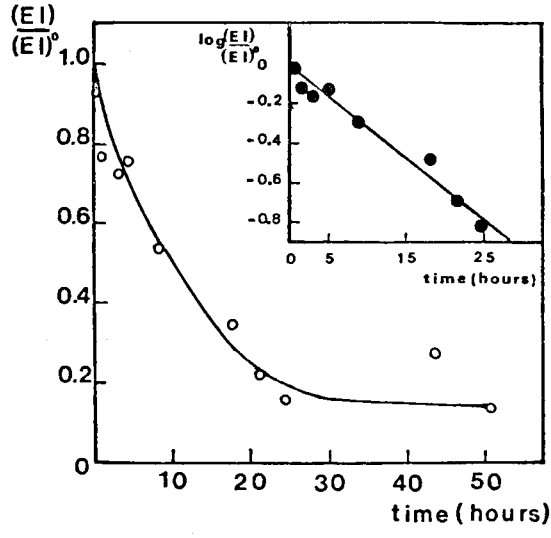

Fig. 2. — Dissociation of the trypsin/inter-α-inhibitor complex by α_2-macroglobulin. The insert is a first order replot in accordance with equation 12. From AUBRY and BIETH [1].

by VINCENT and LAZDUNSKI [14]. Another way to remove the enzyme consists of adding human plasma α_2-macroglobulin [1]. This protein forms stable complexes with the proteinases. These complexes are almost fully active on synthetic substrates : dissociation can thus be easily monitored. Figure 2 illustrates the dissociation of inter-α-inhibitor/trypsin complex by α_2-macroglobulin. A third way is to add an active site titrant of the protease. For instance, p-nitrophenol-p-guanidinobenzoate (NPGB) inhibits trypsin irreversibly and this reaction is accompanied by the release of stoichiometric amounts of p-nitrophenol whose concentration may be determined spectrophotometrically. Since inactive proteinases do not usually bind to natural inhibitors, active site titrants may be considered as dissociating agents. NPGB has been used successfully by ZAHNLEY *et al.* [16]. c) « Removal » of free inhibitor from the equilibrium : this may be achieved by adding another proteinase which will bind to the inhibitor and release the free enzyme. Care must be taken that the added proteinase does not destroy the complex by proteolytic cleavage.

2.4. Dissociation constant Ki

This determination rests on the assumption that the proteinase-inhibitor association is truly reversible. Reversibility may be demonstrated by one of the methods outlined in section 2.3. A concaved inhibition curve may also be taken as evidence for reversibility, provided that the concavity is not due to incomplete association of proteinase and inhibitor nor to release of active enzyme from the complex (= temporary inhibition).

The dissociation constant Ki is determined by measuring the enzymic activity of mixtures formed by increasing the amount of inhibitor and keeping constant the amount of proteinase. The incubation period must be sufficient. The data cannot be plotted using the familiar Lineweaver-Burk or Dixon plots since the equation underlying these plots assume that the complexed inhibitor concentration is negligible with respect to the total inhibitor concentration. This assumption is no longer valid if a substantial degree of inhibition is obtained with inhibitor concentrations which are of the same order of magnitude as those of the enzyme concentration. If the inhibition curve is sufficiently concaved (*e.g.* fig. 3), the EASSON-STEDMAN plot [6] may be used. This plot is based on the following equation :

$$\frac{(I^\circ)}{1 - a} = \frac{Ki}{a} + (E^\circ) \qquad (13)$$

where a is the fractional activity, *i.e.* the ratio between the enzyme activity in the presence and absence of the inhibitor. The plot of $\frac{(I^\circ)}{1 - a}$ *vs* $\frac{1}{a}$ yields a straight line whose slope is Ki (see insert of fig. 3).

Most natural proteinase inhibitors are unable to bind to proteinases whose active center is blocked by an inhibitor or a substrate. If the inhibition is reversible, one may therefore expect competition between substrate and inhibitor for the binding of the enzyme. Conversely, the substrate is expected to dissociate part of the enzyme-inhibitor complexes when added to the enzyme + inhi-

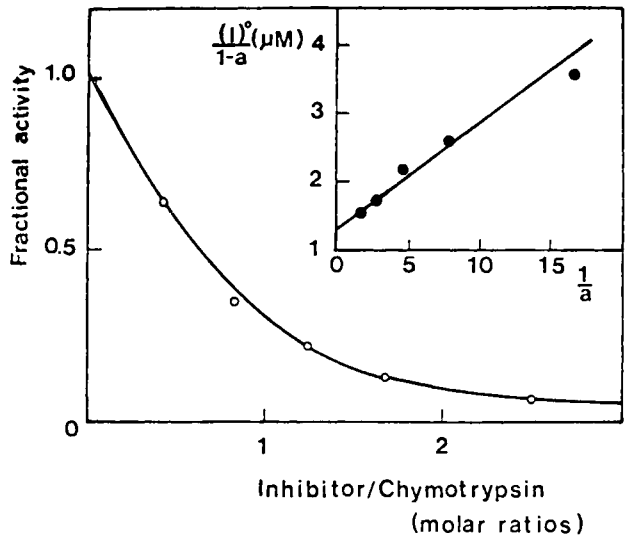

Fig. 3. — Determination of the dissociation constant Ki of the chymotrypsin/inter-α-inhibitor complex. The insert is a replot of the data in accordance with equation 13 (vi/vo = a). From AUBRY and BIETH [1].

bitor mixture. Whether this dissociation occurs in reality depends on the value of the halfe-life of the complex (*i.e.* $0.693/k_{off}$) with respect to the time required for the assay of the enzyme activity. Three cases may exist : 1) dissociation occurs during the time of mixing the substrate, 2) dissociation occurs during the enzymatic assay, and 3) no dissociation occurs. In case 2, only the dissociation process is evident (provided of course that substrate hydrolysis is recorded against time !). It is easily recognized by the fact that the substrate hydrolysis is not linear but exponential with respect to time. Recording should be continued until linearity (*i.e.* end of partial dissociation) is observed.

It is possible to decide between case 1 and case 3 by measuring the fractional activity a of a given enzyme + inhibitor mixture with two widely different substrate concentrations (say 0.5 Km and 3 Km). For case 1 the fractional activity is higher for the lower substrate concentration than for the higher one. For case 3, a does not depend on the substrate concentration.

If substrate-induced dissociation has been clearly established, the slope of the EASSON-STEDMAN plot yields Ki (app) from which Ki may be calculated :

$$Ki = \frac{Ki\ (app)}{1 + \frac{(S^\circ)}{Km}} \qquad (14)$$

If the inhibition curve is linear, it is not possible to determine Ki by the EASSON-STEDMAN plot. In that case, Ki may be calculated using the values of k_{on} and k_{off} (equation 2).

2.5. Hydrolysis constant of temporary inhibitors

Samples from a proteinase + inhibitor mixture are withdrawn at given time intervals and tested for enzyme activity. The release of free proteinase is expected to be of the first order so that a logarithmic plot of the data will yield the hydrolysis constant k.

3. IN VIVO SIGNIFICANCE OF THE KINETIC PARAMETERS

3.1. Association rate constant k_{on}

The association rate constant allows the calculation of the delay time of inhibition, *i.e.* the time required for complete inhibition of a proteinase *in vivo*. A rough estimation of $t_{1/2}$, the half-life time *in vivo*, is given by :

$$t_{1/2} \simeq \frac{1}{k_{on}\,(I^{\circ})} \tag{15}$$

On the other hand, it is assumed that after $5 \times t_{1/2}$ the inhibition will be about complete. Therefore :

$$\text{delay time} \simeq \frac{5}{k_{on}\,(I^{\circ})} \tag{16}$$

The delay time of inhibition may thus be calculated knowing k_{on} and (I°), the inhibitor concentration *in vivo*. If the delay time is too long, the inhibitor cannot play a physiological role.

3.2. Dissociation rate constant k_{off}

The dissociation rate constant allows the calculation of the time during which a reversible proteinase/inhibitor complex remains undissociated *in vivo* in the presence of a dissociating agent (*e.g.* a biological substrate). Since complex dissociation is a first-order process, $t_{1/2}$, the half-life time of dissociation, depends only upon k_{off} [(I°) may be ignored in this case] :

$$t_{1/2} = \frac{0.693}{k_{off}} \tag{17}$$

It may be assumed that the time during which the complex remains undissociated is one fifth of $t_{1/2}$. If this time is too short, the inhibitor cannot play a physiological role.

3.3. Dissociation constant Ki

To be efficient *in vivo*, an inhibitor must have a sufficiently high affinity (*i.e.* 1/Ki) for the proteinase so that the totality of the enzyme is in an inhibited

state. The overall efficiency of the inhibitor depends not only upon its Ki value but also upon its concentration (I^o). The important factor is the ratio (I^o)/Ki [3]. Roughly the inhibitor is efficient only if (I^o)/Ki > 10.

3.4. Hydrolysis constant of temporary inhibitor

The significance of this constant is obvious. It allows one to calculate the time during which the proteinase remains in the inactive state *in vivo*. This time may be roughly equal to one fifth of the half-life time of the enzyme, *i.e.* 0.14/k.

4. EXAMPLES

4.1. Plasma α_1-proteinase inhibitor

We have measured the rate constants for the association of human plasma α_1-proteinase inhibitor with a series of proteinases [2]. The results for the most important enzymes are shown in table I. It can be seen that α_1-proteinase inhibitor reacts extremely fast with leucocyte elastase, so that if this proteinase is liberated in blood it will be caught immediately. Of the two trypsins present in human pancreas which may be massively liberated into blood during acute pancreatitis, only the anionic one is inhibited fast enough. Cationic trypsin may reach targets like prothrombin or fibrinogen before being inhibited. α_1-proteinase inhibitor is not a physiological inhibitor of the important blood coagulation proteinases plasmin or thrombin, contrary to general opinion.

TABLE I

Physiological function of human plasma α_1-proteinase inhibitor as inferred from the measurement of its association rate constant (k_{on}) with various proteinases

Proteinases	k_{on} ($M^{-1} s^{-1}$) *	Delay time **	Physiological function
Leucocyte elastase	6×10^7	2 ms	yes
Pancreatic anionic trypsin	7×10^4	2 s	yes
Pancreatic cationic trypsin	1×10^4	12 s	no
Plasmin	190	12 min	no
Thrombin	48	42 min	no

* Data from BEATTY *et al.* [2]; ** according to equation 16.

Oxidation of the methionine residue of the active center of α_1-proteinase inhibitor leads to a partial loss of the inhibitory activity of this protein [9]. The oxidative inactivation may also be brought about by cigarette smoke conden-

sates [4]. From this, it has been concluded that pulmonary emphysema is more frequent in smokers than in non-smokers because alveolar α_1-proteinase inhibitor of smokers is unable to prevent proteolytic lung damage. When we examined the reaction of oxidized α_1-proteinase inhibitor with various proteinases, we have found that the modified protein inhibits all proteinases tested except porcine pancreatic elastase [2]. The reaction rates were however much slower. For instance, leukocyte elastase is inhibited 2,000 times slower by the oxidized inhibitor than by the native one. Oxidized α_1-proteinase inhibitor does not therefore play a physiological function at the lung alveolar level (table II).

TABLE II

Reaction of human leucocyte elastase with native and oxidized α_1-proteinase inhibitor at the alveolar epithelium

	k_{on} ($M^{-1} s^{-1}$) *	Delay time **	Physiological function
Native α_1-proteinase inhibitor	6×10^7	0.2 s	yes
Oxidized α_1-proteinase inhibitor	3×10^4	7 min	no

* Data from BEATTY *et al.* [2] ; ** calculated assuming the α_1-proteinase inhibitor concentration is 1/100 of that of plasma.

4.2. Plasma inter-α-inhibitor

This protein forms reversible complexes with bovine and human pancreatic trypsins and chymotrypsins [1]. Table III shows that human trypsin is inhibited too slowly and that the trypsin/inhibitor complex is not very stable. On the other hand, for chymotrypsin, the $(I^\circ)/Ki$ ratio is not very high so that part of the enzyme which may be liberated into blood will exist in free form. Inter-α-inhibitor therefore cannot be considered as a physiological inhibitor of pancreatic trypsin or chymotrypsin.

TABLE III

Physiological function of human plasma inter-α-inhibitor as inferred from the kinetic parameters of its reaction with human pancreatic cationic trypsin and chymotrypsin II

Proteinase	k_{on} ($M^{-1} s^{-1}$) *	Delay time **	k_{off} (s^{-1}) *	Stability time ***	Ki (M) *	$\frac{(I^\circ)}{Ki}$	Physiol. function
Trypsin	1.4×10^6	11 s	1.7×10^{-3}	80 s	1.2×10^{-8}	260	no
Chymotrypsin					1.3×10^{-7}	24	no

* Data from AUBRY and BIETH [1] ; ** according to equation 16 ; *** see paragraph 3.2.

5. CONCLUDING REMARKS

Scientific papers describing new proteinase inhibitors frequently lack information on kinetic parameters of these inhibitors. The only information usually found is... « percent inhibition... » under certain assay conditions. We hope to have demonstrated clearly in the present paper that such qualitative data are insufficient to enable conclusions to be drawn concerning the physiological function of new inhibitors. We hope also that the theoretical and practical points we have outlined will help those who are interested in new proteinase inhibitors.

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RÉSUMÉ

Les inhibiteurs naturels de protéases peuvent former des complexes réversibles ou irréversibles avec les protéases. Dans le premier cas, le schéma réactionnel le plus simple est le suivant : $E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E.I$. La constante de dissociation du complexe est donnée par la relation : $Ki = k_{off}/k_{on}$. Dans le dernier cas, la formation du complexe ne peut être caractérisée que par k_{on}.

L'interprétation *in vivo* de ces constantes est la suivante. La constante de vitesse d'association k_{on} permet de calculer le temps nécessaire à l'inhibition d'une protéase *in vivo*. La constante de vitesse de dissociation k_{off} ainsi que la constante d'équilibre Ki reflètent la stabilité du complexe protéase-inhibiteur. Un inhibiteur actif *in vitro* peut être considéré comme inefficace *in vivo* s'il possède un k_{on} trop bas ou un k_{off} ou un Ki trop élevé. L'inhibition temporaire est également analysée.

Les considérations théoriques ci-dessus sont illustrées à l'aide d'exemples. Une place particulière est accordée aux systèmes enzyme-inhibiteurs importants dans l'emphysème pulmonaire tel que la réaction de l'α_1-inhibiteur de protéases natif ou oxydé avec l'élastase leucocytaire.

Discussion

CRYSTAL (USA). — What is the concentration of alpha$_1$-antitrypsin at the alveolar level ? We know that relative to albumin, which is about the same molecular weight, the ratio in lavage fluid is similar to plasma. But, we do not know what the concentration actually is in the epithelial fluid. For your calculations, do you make the assumption that it was of a similar concentration to plasma ?

BIETH (France). — If I assume that the concentration is the same as in the plasma, then the oxidized inhibitor is a physiological inhibitor !

CRYSTAL. — A physiologic inhibitor at the same rate ?

BIETH. — It reacts sufficiently fast, so that it can inhibit efficiently human leukocyte elastase, although it is oxidized. The only enzyme which is not inhibited at all by oxidized alpha$_1$ is porcine elastase ; I have shown this in TRAVIS' laboratory.

ROBERT (France). — I wanted to ask exactly the same question as Dr CRYSTAL and to widen it a little bit : what is known at all on the local concentration of inhibitors in the alveolar wall ?

BIETH. — I really do not know. I have asked a number of people, they were unable to tell me.

ROBERT. — What is the role of the dissociation rate constant ? You have shown that the only thing that really determines the physiological role of an inhibitor is the rate constant for association, but don't you think that the rate constant of dissociation is also an important parameter ?

BIETH. — Yes, it is. Even if they associate very fast, if they also dissociate very fast the inhibitors are not good ; this is then reflected in Ki. Moreover, most of the plasma inhibitors are irreversible inhibitors ; so there is no reversibility.

TRAVIS (USA). — Just a comment regarding dissociation rate constant. It is true that the oxidized inhibitor will inhibit elastase, but the association constant is obviously very fast because most of the inhibitor complexes that we find with alpha$_1$-antiproteinases are very stable. In the case of the oxidized inhibitor plus leukocyte elastase, the complex is not stable ; it dissociated very rapidly, so that, although you have complex formation with the oxidized inhibitor, you also have complex dissociation, and it is obvious that the elastase that is not removed from the lung at this particular time can dissociate again and presumably bind to elastin. So dissociation is more important there than with the native inhibitor.

Calling this protein antitrypsin always bothers me. There are a lot of enzymes which are inhibited by alpha$_1$-antitrypsin. So I prefer to call it « alpha$_1$-proteinase inhibitor ». The real function of this particular protein is to inhibit leukocyte elastase ; it has no real function to inhibit trypsin, so that it is why it is a bad name. If you continue to call this protein antitrypsin you are going to continue to fill the literature with evidence that trypsin is responsible for lung disease, and we all know that it is elastase ; so why continue to call it antitrypsin when it is not antitrypsin ?

KRAMPS (The Netherlands). — Have you any data about kinetics of the low molecular weight inhibitor, as described by HOCHSTRASSER and OHLSSON ?

BIETH. — Dr OHLSSON gave us a sample of low molecular weight inhibitor. We have no quantitative data, because it reacts very fast, about three times faster than alpha$_1$-proteinase inhibitor.

MITTMAN (USA). — I am sorry Dr OHLSSON is not here ! Perhaps somebody else could comment. If I recall correctly, Dr OHLSSON had postulated that alpha$_1$ was acting more or less in a cascade fashion as the initial recipient of the proteinase, and then passing it onto the alpha$_2$-macroglobulin inhibitor. If this is the case, then the characteristics you described for the oxidized form would continue to be, I would suspect, ideal as an effective inhibitor, as it has a rapid association and dissociation rate. Is my recollection correct and is the theory still valid ?

BIETH. — The rapid transfer of trypsin from alpha$_1$ to alpha$_2$ which you mentioned has been observed only in the dog. We have tried it with human alpha$_1$-AT and alpha$_2$-M : it is considerably slower. In Dr TRAVIS laboratory, I tried to see if there was any transfer of leukocyte elastase from alpha$_1$-AT to alpha$_2$-M : there was none.

JANOFF (USA). — In addition to what Dr BIETH just told us, OHLSSON's proposed transfer mechanism does not hold in the case of the lung. Transfer was demonstrated in circulating blood where the concentration of alpha$_2$-macroglobulin is high. However, in the alveolar interstitial lymph or in the air space there is very little alpha$_2$-macroglobulin. It is too big to enter transudates under normal circumstances.

TRAVIS. — Dr BIETH was in my laboratory two summers ago, and we tried to demonstrate the transfer of enzymes from alpha$_1$ into alpha$_2$ as you described. What

we could find was that the rate of dissociation of $alpha_1$ complexes and the rate of formation of $alpha_2$ complexes, in the presence of the $alpha_1$ and the enzyme, were identical. In other words, there was no acceleration of the transfer : $alpha_2$ had no positive effect at all, it only waited for the $alpha_1$ complexes to dissociate. We have done the experiments with elastase ; the only way we can observe any evidence for much transfer from $alpha_1$ to $alpha_2$ is to raise the temperature of these complexes very high, so they dissociate very quickly. I do not think this is important in the lung ; as Dr JANOFF said, the $alpha_2$-macroglobulin is a very big molecule, it is not likely to have much function in tissue ; only if the $alpha_1$-API complexes can find their way back to the circulation, you are going to have a situation where any type of transfer can occur ; and with the oxidized inhibitor plus the enzyme if dissociation occurs very rapidly, as we think is occurring, then I would say that the oxidized inhibitor is not a physiological inhibitor, as Dr BIETH says, but rather association and dissociation take place so quickly that elastase activity on parenchyma lung elastin is probably going to be very high.

KIMBEL (USA). — Is there any evidence that the other constituents of the alveolar lining layer, mainly the lipid fraction, have any effect on the behaviour of $alpha_1$-antiproteinase at that level ?

BIETH. — No evidence for that.

GEE (USA) : What is known on the level of $alpha_2$-macroglobulin in acute inflammatory reactions such as in pneumonias ?

TRAVIS. — I think there is very little data. We will hear some data later on this morning about $alpha_2$-macroglobulin in lavage fluid.

BIETH. — I have no data on that.

STOCKLEY (UK). — I could perhaps partly answer that question. Certainly during pneumonia the concentration of $alpha_2$-macroglobulin in sputum goes up, so that it is more readily detectable, but its concentration related to $alpha_1$-antitrypsin is still low, dependent upon its size.

TRAVIS. — The regeneration of trypsin activity from an $alpha_1$-trypsin complex is a function of time. In a work done by Dr BIETH and Dr BEATTY in my laboratory, trypsin activity regenerates as these complexes break down. Now if you put $alpha_2$-macroglobulin in, which will also bind the trypsin, there is not much increase ; the $alpha_2$-macroglobulin does not have a very important effect in accelerating the transfer, only a matter of about 20-25 %, so we do not think $alpha_2$-macroglobulin has any very important role in terms of this so-called transfer, and as Dr LAURELL has told me, unfortunately Dr OHLSSON is not here to reinforce this statement up, he does not talk about a transfer any longer. It is a matter of dissociation/re-association. Not transfer.

KUHN (USA). — Some years ago GANROT *et al.* (*Acta physiol. scand.*, 1970, **79**, 280) reported that the concentration of $alpha_2$-M was quite high in lung lymph, and so it may still be relevant. I also think it is worth pointing out that perhaps the inhibitors of the interstitial fluid compartment are really the important ones, although not as readily studied as those obtained by lavage.