The irreversible inhibition of urokinase, kidney-cell plasminogen activator, plasmin and β -trypsin by 1-(*N*-6-amino-*n*-hexyl)carbamoylimidazole

Brian WALKER and Donald T. ELMORE

Department of Biochemistry, Medical Biology Centre, Queen's University, Belfast BT9 7BL, N. Ireland, U.K.

(Received 24 April 1984/Accepted 3 May 1984)

1. 1-(*N*-Amino-*n*-hexyl)carbamoylimidazole hydrochloride was synthesized and shown to be a potent irreversible inhibitor of human urokinase (EC 3.4.21.31), pig kidney-cell plasminogen activator (EC 3.4.21.-), human plasmin (EC 3.4.21.7) and bovine pancreatic β -trypsin (EC 3.4.21.4). 2. The kinetics of inhibition of the enzymes were determined by monitoring the hydrolysis of an appropriate fluorogenic substrate. 3. Bovine thrombin and Factor Xa are hardly affected by the inhibitor.

Groutas *et al.* (1980) have described a series of irreversible inhibitors (I; R = *n*-Pr, *i*-Pr, *n*-Bu) of elastase. Encouraged by the success of Wong *et al.* (1978) in designing an irreversible inhibitor (II) that reacted much faster with urokinase than with plasmin, we have synthesized the compound (I; R = $^+H_3N-[CH_2]_6$) and examined its specificity as an inhibitor towards several trypsin-like proteinases. This kinetic study was achieved by monitoring the progress of inhibition with fluorogenic substrates derived from lysyl- or arginyl-7-amino-4-methylcoumarin (Lys-Amc, Arg-Amc).

Experimental

Materials

Trasylol was purchased from Bayer U.K., Haywards Heath, West Sussex, U.K. Boc-Val-Leu-Lys-Amc (Kato *et al.*, 1980) and Z-Arg-Amc (Zimmerman *et al.*, 1977) were purchased from the Peptide Research Foundation, Osaka, Japan. Z-Gly-Gly-Arg-Amc was purchased from Serva, Heidelberg, West Germany. *N*-*t*-Butyloxycarbonyl-1,6-diaminohexane hydrochloride was purchased from Fluorochem, Glossop, Derbyshire,

U.K. 1,1'-Carbonyldi-imidazole was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. AnalaR-grade chemicals (BDH Chemicals, Poole, Dorset, U.K.) were used for preparation of buffers. 7-Amino-4-methylcoumarin was synthesized as described by Zimmerman *et al.* (1977). Urokinase was purchased from Calbiochem, Bishop's Stortford, Herts., U.K. The contents of each vial (approx. 2000 Ploug units) were dissolved in 50% (w/v) glycerol/50mM-sodium phosphate buffer, pH 8.0, and stored at -15°C . The pig kidney-cell plasminogen activator was a gift from Dr. J. Bobbitt of Eli Lilly Research Laboratories, Indianapolis, IN, U.S.A. The enzyme was stabilized by the presence of albumin, which was not removed from the preparation for the present work. Glu-plasminogen was isolated from outdated human plasma by affinity chromatography on lysine-Sepharose in the presence of Trasylol (Deutsch & Mertz, 1970). It was activated to plasmin in 50% (w/v) glycerol/50mM-sodium phosphate buffer, pH 8.0, at 37°C with the use of about 20 Ploug units of urokinase/mg of plasminogen. Human Factor Xa was supplied by Mr. P. J. Turkington, Department of Haematology, Royal Victoria Hospital, Belfast, N. Ireland, U.K. Human thrombin was purchased from Sigma Chemical Co., Poole, Dorset, U.K. Trypsin (twice recrystallized, salt-free) was purchased from

Worthington Biochemical Corp., Freehold, NJ, U.S.A., and the β -form was isolated by the method of Schroeder & Shaw (1968).

Synthesis of 1-(N-6-amino-n-hexyl)carbamoyl-imidazole (I; R = $^+H_3N-[CH_2]_6$)

N-t-Butyloxycarbonyl-1,6-diaminohexane hydrochloride (100mmol) was suspended in dry tetrahydrofuran (100ml) containing *N*-methylmorpholine (100mmol). 1,1-Carbonyldi-imidazole (100mmol) was added in one portion, and the mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure, and the residue was distributed between ethyl acetate and water. The organic layer was separated and dried (over $MgSO_4$). Evaporation of the solvent yielded an oil, which was homogeneous by t.l.c. The i.r. spectrum showed strong bands at 3370cm^{-1} (ν -NH), 1750cm^{-1} (ν -C=O, carbamoyl) and 1700cm^{-1} (ν -C=O, urethane). The oil was treated with a mixture of trifluoroacetic acid (25ml) and dichloromethane (25ml) at 0°C for 30min, after which analysis by t.l.c. revealed the absence of starting material. Solvent was removed under reduced pressure, and the oily residue was treated with ethereal HCl and then triturated with ethanol/diethyl ether (1:3, v/v), affording a white crystalline solid (yield 75%). The product was washed with diethyl ether and recrystallized from tetrahydrofuran/diethyl ether. It had m.p. $92\text{--}94^\circ\text{C}$ (decomp.) (Found: C, 48.7; H, 7.7; N, 22.4. $C_{10}H_{19}ClN_4O$ requires C, 48.7; H, 7.8; N, 22.7%).

Kinetic techniques

(a) *Discontinuous assay.* A solution of inhibitor (I; R = $^+H_3N-[CH_2]_6$) in *N*-methylpyrrolid-2-one was added to a solution of enzyme ($1\text{--}10\mu\text{M}$) in 50mM-sodium phosphate buffer, pH 8.0, at 37°C such that the final concentration of inhibitor was $0.055\text{--}1.4\text{mM}$. With thrombin and Factor Xa, the inhibitor concentration was $3.44\text{--}9.0\text{mM}$. Samples were removed at intervals and assayed for residual enzyme activity with an appropriate substrate (Z-Arg-Amc for trypsin, Boc-Val-Leu-Lys-Amc for plasmin and Z-Gly-Gly-Arg-Amc for urokinase and kidney-cell plasminogen activator). The dilution involved in setting up the assay effectively stopped further reaction with inhibitor. The rate of hydrolysis of substrate was determined by measuring the rate of increase of fluorescence at 455nm ($\lambda_{\text{exc.}}$ 383nm) in a Perkin-Elmer MPF 44B spectrofluorimeter.

(b) *Continuous assay.* To a solution (1ml) of enzyme ($3\text{--}9\text{nM}$) in 50mM-sodium phosphate buffer, pH 8.0, in a cuvette housed in the sample

compartment of a Perkin-Elmer MFP 44B spectrofluorimeter, was added simultaneously solutions (1ml each) of inhibitor ($0.15\text{--}1.5\text{mM}$) and substrate ($0.05\text{--}0.15\text{mM}$) in *N*-methylpyrrolid-2-one. The addition was achieved by mixing the solutions of substrate and inhibitor by using two syringes linked to a single barrel that was introduced to the sample cuvette containing the enzyme solution via the air inlet in the cell housing. Mixing was complete in less than 1s.

(c) *Determination of operational molarity of enzyme solutions.* The operational molarity of all enzymes was determined by spectrofluorimetric titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson *et al.*, 1973). The normal method of titration was used with plasmin, thrombin and the kidney-cell plasminogen activator, since there is turnover of titrant with these enzymes. The concentrated incubation method was used with the other enzymes, since turnover was negligible in the time required for acylation.

Analysis of kinetic data

Initial velocities were computed by the method of Elmore *et al.* (1963). K_m and k_{cat} values were computed by applying the least-squares method directly to the Michaelis-Menten equation (Roberts, 1977). The amplitude, C , and apparent first-order constant, k_{app} , for inhibition of enzymes were computed by applying the least-squares method directly to eqn. (4).

Theory

In the experiments in which residual active enzyme was assayed discontinuously after exposure to the inhibitor for various times, the kinetics are pseudo-first-order. The situation is slightly more complex, however, in the experiments in which irreversible inhibition and hydrolysis of substrate proceeded concurrently. Assuming that the acylation step is rate-determining for the hydrolysis of an amide, the reaction scheme can be simplified to the following:

where EI^* is irreversibly inhibited enzyme. The concentrations of substrate, $[\text{S}]$, and inhibitor, $[\text{I}]$, were chosen so that $\leq 1\%$ of substrate was hydrolysed before the enzyme was completely inhibited. Consequently, in the treatment below $[\text{S}]$ is effectively constant.

Since:

$$[E]_0 = [E] + [ES] + [EI] + [EI^*]$$

$$K_s = \frac{[E][S]}{[ES]}$$

and

$$K_i = \frac{[E][I]}{[EI]} \quad (1)$$

then:

$$[ES] = A([E]_0 - [EI^*])$$

where

$$A = 1 / \left(\frac{K_s}{[S]} + 1 + \frac{K_s[I]}{K_i[S]} \right)$$

Now:

$$\frac{d[P]}{dt} = k_0[ES] \quad (2)$$

$$\frac{d[EI^*]}{dt} = k_i[EI]$$

and, by differentiation of eqn. (2)

$$\frac{d^2[P]}{dt^2} = k_0 \frac{d[ES]}{dt} \quad (3)$$

Differentiation of eqn. (1) gives:

$$\begin{aligned} \frac{d[ES]}{dt} &= -A \frac{d[EI^*]}{dt} = -A \cdot k_i[EI] \\ &= -A \frac{k_i K_s [I]}{K_i [S]} [ES] = -A \frac{k_i K_s [I]}{k_0 K_i [S]} \cdot \frac{d[P]}{dt} \end{aligned}$$

Substitution in eqn. (3) gives:

$$\frac{d^2[P]}{dt^2} + A \frac{k_i K_s [I]}{K_i [S]} \cdot \frac{d[P]}{dt} = 0$$

Under the boundary conditions that $[P] = 0$ and $[ES] = A[E]_0$ when $t = 0$, this equation has the solution:

$$[P] = \frac{k_0 K_i [S] [E]_0}{k_i K_s [I]} \{1 - \exp(-A \cdot k_i K_s [I] t / K_i [S])\} \quad (4)$$

This represents the kinetics of a first-order process with amplitude:

$$C = \frac{k_0 K_i [S] [E]_0}{k_i K_s [I]} \quad (5)$$

and apparent first-order rate constant:

$$k_{app.} = A \cdot k_i K_s [I] / K_i [S] \quad (6)$$

After evaluating C and $k_{app.}$ by non-linear least-squares fitting of the experimental data, K_i can be obtained from the equation:

$$C \cdot k_{app.} = A \cdot k_0 [E]_0 = k_0 [E]_0 / \left(\frac{K_s}{[S]} + 1 + \frac{K_s [I]}{[S] K_i} \right)$$

Since k_0 and K_s are known from steady-state kinetic studies in the absence of inhibitor and $[E]_0$ is determined by active-site titration, k_i can be obtained from eqns. (5) and (6) by substitution.

Results and discussion

A typical set of results obtained by using the discontinuous assay is shown in Fig. 1. The resistance of thrombin and Factor Xa to the inhibitor contrasts with the sensitivity of plasmin, urokinase and kidney-cell plasminogen activator. Fig. 2 is a typical example of the use of the continuous-assay technique, and shows the effect of inhibitor concentration on the rate of hydrolysis of a fluorogenic substrate by plasmin. The kinetic constants for the inhibition of urokinase, β -trypsin, kidney-cell plasminogen activator and plasmin by the inhibitor are given in Table 1. It will be seen

Fig. 1. Comparison of the time-dependent inhibition of trypsin-like enzymes by 1-(N-6-amino-n-hexyl)carbamoyl-imidazole hydrochloride

The inhibition studies were carried out at 37°C in 50mM-phosphate buffer, pH 8.0. The concentrations of enzyme and inhibitor used in each study were as follows: $\blacktriangle$, Factor Xa, 4.5 μ M, and inhibitor, 3.44 mM; $\bullet$, thrombin, 3.6 μ M, and inhibitor, 9.00 mM; $\square$, plasmin, 1.4 μ M, and inhibitor, 0.72 mM; $\triangle$, urokinase, 1.76 μ M, and inhibitor, 0.55 mM; $\circ$, pig kidney-cell plasminogen activator, 0.79 μ M, and inhibitor, 0.60 mM.

Fig. 2. Representation of the course of hydrolysis of Boc-Val-Leu-Lys-Amc by plasmin in the presence of 1-(N-6-amino-n-hexyl)carbamoylimidazole hydrochloride. The incubation mixture contained the following in 50 mM-phosphate buffer, pH 8.0, at 37°C: plasmin, 5.04 nM; Boc-Val-Leu-Lys-Amc, 55.3 μ M; inhibitor, 0.972 mM (curve A) or 1.458 mM (curve B).

Table 1. Binding and rate constants for the inhibition of various trypsin-like proteinases by 1-(N-6-amino-n-hexyl)-carbamoylimidazole hydrochloride

Values are given as means $\pm$ S.E.M. for four determinations.

Enzyme	K_i (mM)	k_i (min^{-1})	k_i/K_i ($\text{M}^{-1} \cdot \text{min}^{-1}$)
Urokinase	0.81 ± 0.06	2.66 ± 0.21	3300 ± 370
Plasminogen activator	0.47 ± 0.04	0.79 ± 0.06	1700 ± 190
Plasmin	0.60 ± 0.05	0.25 ± 0.02	420 ± 50
β -Trypsin	0.72 ± 0.06	1.79 ± 0.19	2500 ± 280

that K_i values differ by a factor of less than 2-fold whereas the k_i for urokinase and plasmin differ by an order of magnitude.

Groutas *et al.*, (1980) have postulated a 'suicide' mechanism for the inhibition of elastase by inhibitor (I; R = n-Pr, i-Pr or n-Bu) in which the

imidazole N atom of His-57 abstracts a proton from the R-NH-CO- moiety of the inhibitor, causing the breakdown of (I) to the alkyl isocyanate. They cite as supporting evidence (a) the ready dissociation of compounds of type (I) into isocyanates and imidazole (Staab & Benz, 1961) and (b) the reaction of the hydroxy group of Ser-195 in serine proteinases with alkyl isothiocyanates (Brown & Wold, 1973a,b). Since, however, aryl carbazates are able to acylate Ser-195 in trypsin and chymotrypsin directly (Smyth & Elmore, 1968), and *p*-nitrophenyl esters of *N*-(ω -aminoalkyl)carbamate acids acylate Ser-195 of trypsin (Scofield *et al.*, 1977), it is possible that the inhibition of trypsin-like enzymes by the inhibitor (I; R = $^+ \text{H}_3\text{N}-(\text{CH}_2)_6$) described in the present paper operates by direct acylation.

We are indebted to Dr. J. Bobbitt and Mr. P. Turkington for the gifts of pig kidney-cell plasminogen activator and human Factor Xa respectively. This work was supported by a grant from the Cancer Research Campaign.

References

- Brown, W. E. & Wold, F. (1973) *Biochemistry* **12**, 828-834
- Brown, W. E. & Wold, F. (1973) *Biochemistry* **12**, 835-840
- Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095-1096
- Elmore, D. T., Kingston, A. E. & Shields, D. B. (1963) *J. Chem. Soc.* 2070-2080
- Groutas, W. C., Badger, R. C., Ocain, T. D., Felker, D., Frankson, J. & Theodorakis, M. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1890-1894
- Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A. & Elmore, D. T. (1973) *Biochem. J.* **131**, 101-117
- Kato, H., Adachi, N., Ohno, Y., Iwanaga, S., Takada, K. & Sakakibara, S. (1980) *J. Biochem. (Tokyo)* **88**, 183-190
- Roberts, D. V. (1977) *Enzyme Kinetics*, pp. 299-306, Cambridge University Press, Cambridge
- Schroeder, D. D. & Shaw, E. (1968) *J. Biol. Chem.* **243**, 2943-2949
- Scofield, R. E., Werner, R. P. & Wold, F. (1977) *Biochemistry* **16**, 2492-2496
- Smyth, J. J. & Elmore, D. T. (1968) *Biochem. J.* **107**, 103-107
- Staab, H. A. & Benz, W. (1961) *Chem. Ber.* **648**, 72-80
- Wong, S. C., Green, G. D. & Shaw, E. (1978) *J. Med. Chem.* **21**, 456-462
- Zimmerman, M., Ashe, B., Yurewicz, E. C. & Patel, G. (1977) *Anal. Biochem.* **78**, 47-51