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Deacylation Rate Constants of Acylated Human and Porcine Plasmins

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Abstract

Deacylation rate constants, k_3, were measured for the human acyl-plasmins which contain in the acyl portion, RCO, the R groups methyl, cyclohexyl, *p*-nitrophenyl, isobutyl, *trans*-isopropenyl, phenyl, α-naphthyl, anisoyl and *p*-guanidinophenyl. Values of k_3 were also determined for the porcine acyl-plasmins which have R = methyl, cyclohexyl, phenyl, anisoyl and *p*-guanidinophenyl. In general, for both the human and porcine acyl-plasmins, k_3 decreased as the electron-donating ability of the acyl group increased. At 25°C, the human acyl-plasmin with R = methyl has the highest k_3, $1{\cdot}06 \pm 0{\cdot}05 \times 10^{-2}$ s^{-1}, and the porcine acyl-plasmin with R = *p*-guanidinophenyl the lowest k_3, $1{\cdot}4 \pm 0{\cdot}1 \times 10^{-6}$ s^{-1}.

Introduction

The discovery of inverse substrates for trypsin (Tanizawa *et al.* 1977), for which acyl derivatives of *p*-hydroxybenzamidine (1, Fig. 1) are hydrolysed so that the amidine moiety is in the leaving group, enabled the preparation of a wide range of acyl-trypsin derivatives and trypsin-like enzymes. Since then numerous studies have been made of the acylation and deacylation rates of trypsin for a wide range of hydroxybenzamidine derivatives (Tanizawa *et al.* 1980; Fujioka *et al.* 1981). However, only a few studies have been made for the trypsin-like enzymes, plasmin and thrombin (Nozawa *et al.* 1980).

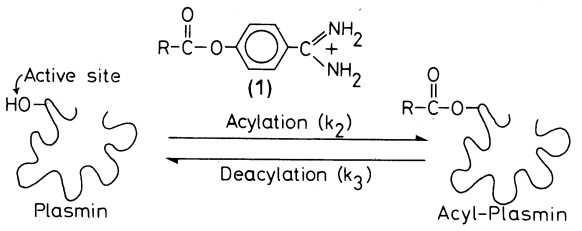

Fig. 1. Acylation and deacylation of plasmin.

Recently two clinical applications of acylated plasmin have been reported. In the field of thrombosis therapy, acylation of plasmin has been utilized for temporarily blocking the proteolytic activity of plasmin without affecting the clot-binding ability (Smith *et al.* 1981).

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This approach has also been applied to the scintigraphic detection of blood clots. Acyl-plasmin derivatives labelled with the gamma-emitting radionuclide, technetium-99m, have been found to provide an improved uptake in blood clots compared with untreated plasmin (Baker *et al.* 1984). In both these applications knowledge of the life-time, i.e. deacylation rate of the acylated plasmin, is the major consideration for the choice of the particular acyl-plasmin to be used.

In this study, the deacylation rate constants, k_3, were measured for the eight human acyl-plasmins which have in the acyl portion the R groups listed in Table 1. Four of these acyl groups were studied with porcine plasmin. Values of k_3 are also reported for the *p*-nitrophenyl guanidinobenzoate (NPGB) derivative (R = *p*-guanidinophenyl) for both porcine and human plasmins. The nomenclature, k_3, used in this study was adopted from the kinetic expressions which have been developed for enzymes which catalyse reactions according to the three-step sequence:

$$E + S \overset{K_s}{\rightleftharpoons} E.S \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P .$$

The enzyme (E) forms an enzyme–substrate (S) complex, which transforms to an acyl-enzyme (ES′) followed by breakdown and regeneration of the enzyme (Bender *et al.* 1969).

Materials and Methods

Four buffer solutions were used in this study—buffer A: 0·05 M Tris, 0·1 M NaCl, 0·001 M lysine, pH 8·0; buffer B: 0·05 M Tris, 0·1 M NaCl, 0·02 M lysine, pH 8·0; buffer C: 0·05 M Tris, 0·02 M $CaCl_2$, pH 8·0; buffer D: 0·1 M potassium phosphate, 0·1 M NaCl, pH 6·0. For each experiment with porcine plasmin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), approximately 1 mg of the dry powder was dissolved initially in 100 µl of HCl, pH 3, diluted with 2 ml of either buffer A or buffer B and then centrifuged. For human plasmin, plasminogen was first prepared from human plasma by affinity chromatography on lysine–Sepharose (Deutsch and Mertz 1970) and was found to be homogeneous, giving only one stained band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fairbanks *et al.* 1971). The plasminogen was then extensively dialysed against buffer B, pH 9, and converted to plasmin with urokinase. Gel electrophoresis confirmed that the conversion was complete. The plasmin was stored at −25°C until required.

Synthesis of Substrates

The preparation of substrates 1 (Tanizawa *et al.* 1977), 2, 3, 6, 8 (Baker *et al.* 1984) and 9 (Chase and Shaw 1969) have been reported previously.

Substrate 5: p-*Amidinophenyl crotonate toluenesulfonate*

Crotonic acid (2 g) was converted to the carbonyl chloride with $SOCl_2$ (27 g) in the normal manner and the excess $SOCl_2$ removed. *p*-Hydroxybenzamidine toluenesulfonate (1·2 g) was added and the mixture was kept at 100°C for 5 h. Evaporation and addition of ether yielded the crude product which was filtered off and recrystallized from ethanol–ether to give colourless needles (0·55 g, m.p. = 204–207°C). Analysis: %C = 58·19, %H = 6·14, %N = 7·22, %S = 8·21; theoret.: %C = 58·14, %H = 6·16, %N = 7·13, %S = 8·16; i.r.: 1730 cm^{-1} (C = 0).

Substrate 4: p-*Amidinophenyl isobutyrate toluenesulfonate*

This compound was prepared from isobutyric acid by the same method as used for substrate 5. Recrystallization from ethanol–ether gave colourless leaflets, m.p. = 251–252°C. Analysis: %C = 57·15, %H = 5·94, %N = 7·39; theoret.: %C = 57·08, %H = 5·81, %N = 7·40; i.r.: 1730 cm^{-1} (C = 0).

Substrate 7: p-*Amidinophenyl α-naphthoate toluenesulfonate*

α-Naphthoyl chloride (14·2 g) was added dropwise to *p*-hydroxybenzamidine toluenesulfonate (27 g) dissolved in dry pyridine. The mixture was then heated at 65°C for 6 h and the solvent removed to give a yellow oil. This oil was dissolved in 100 ml ethanol and then poured into 1·5 litres of water with rapid stirring. The precipitate was filtered off, washed with ether and recrystallized from

acetonitrile to give colourless crystals (20 g, m.p. = 192–193°C). Analysis: %C = 64·95, %H = 4·62, %N = 6·16, %S = 6·6; theoret.: %C = 64·92, %H = 4·80, %N = 6·06, %S = 6·6; i.r.: 1746 cm^{-1} (C = 0).

Compound 10: p-*Amidinophenyl* N,N-*dimethylcarbamate hydrochloride*

N,N-Dimethylcarbamoyl chloride (12·5 g) was added dropwise to *p*-hydroxybenzamidine hydrochloride dissolved in 70 ml pyridine and the mixture was kept at 80°C for 6 h. The solvent was then removed by vacuum distillation and the resulting oil was left to crystallize. The crude product was recrystallized from ethanol (8·4 g, m.p. = 240–242°C). Analysis: %C = 47·71, %H = 5·65, %N = 16·34, Cl = 13·9; theoret. calc. for $C_{10}H_{13}N_3O_2 . HCl . \frac{1}{2}H_2O$: %C = 47·53, %H = 5·98, %N = 16·63, %Cl = 14·0; i.r.: 1700 cm^{-1} (C = 0).

Absorbance and Kinetic Measurements

All absorbance and kinetic measurements were made on a Hitachi model 200-10 double beam UV-VIS spectrophotometer.

Concentrations of Enzyme Stock Solutions

Concentrations of the enzyme stock solutions were determined by the absorbance change measured at 410 nm for the burst titration with NPGB in buffer A (Chase and Shaw 1969) and the measured extinction coefficient, $1·5 \times 10^4$, for the liberated *p*-nitrophenol.

Measurement of k_3 *values* $> 5 \times 10^{-4}$ s^{-1}

For the concentrations of the stock enzyme solutions used in this study, k_3 values for the substrates 1–5 could be determined directly from the velocity of the substrate–enzyme reaction after subtracting the velocity of the self-hydrolysis of the substrate (Chase and Shaw 1969). Measurements were made at 305 nm where the liberated product of the reaction, *p*-hydroxybenzamidine, has a measured extinction coefficient of $1·48 \times 10^4$. Typically 50 µl of the substrate stock solution (10^{-2} M in dimethylformamide, DMF) was added to a thermostatted cuvette containing 3·0 ml of buffer A and the self-hydrolysis velocity measured. Then 100 µl of the enzyme stock solution ($6·39 \times 10^{-6}$ M for human and $6·50 \times 10^{-6}$ M for porcine) was added, mixed and the velocity determined. Values of k_3 were calculated from the apparent net rate, V, and the cell concentration of the enzyme, E_0 (Bender *et al.* 1969):

$$V = k_3 [E_0]$$

This expression could be used since it was observed for all substrates that $k_2 \gg k_3$.

Measurement of k_3 *values* $< 5 \times 10^{-4}$ s^{-1}

The study of these slower rates necessitated firstly the preparation and isolation of the acyl-plasmin derivatives of substrates 6–8 and NPGB. For the acylation step, 0·4 ml of the enzyme stock solution was mixed with 1·6 ml of buffer A, 50 µl of the substrate stock solution (10^{-2} M in DMF) was then added and the mixture incubated at 25°C for 15 min. Measurements of the residual enzyme activity showed that 15 min was sufficient to provide complete acylation for the substrates studied. The acyl-plasmin was separated by column chromatography with Sephadex G25 and elution with HCl, pH 3. All separations were carried out in a cold room (4°C). The acyl-plasmin fraction, detected by absorbance measurements at 280 nm, was lyophilized and stored at −25°C. For the study of the deacylation, the lyophilized powder was dissolved in 100 µl of HCl, pH 3, 2 ml of buffer B was added and the solution incubated at 25°C.

At various time intervals, aliquots of the incubated solution were removed and the regenerated enzyme assayed with *N*-α-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester (ZLysONP, Cyclo Chemical, Los Angeles, U.S.A.) in 3 ml of buffer D at 340 nm (Wohl *et al.* 1977). Typically 50 µl of the ZLysONP (5×10^{-3} M in 95% CH_3CN) was added to the thermostatted cuvette and the self-hydrolysis velocity determined. Then 50 µl of the enzyme solution was added and the enzyme-substrate velocity was determined. Values of the k_3 were calculated as the negative slope of a plot of $\log [v_\infty/(v_\infty - v_t)]$ versus time where v_t and v_∞ are the net velocities at time t and $t = \infty$ (Chase and Shaw 1969). For each deacylation study, the measured absorbance at 280 nm for the incubated solution was used to calculate v_∞ from the known absorbance and net velocity of a standard plasmin solution.

Results and Discussion

For the substrates 1–9, the respective reactions with either human or porcine plasmin, monitored by absorbance changes at 305 nm, all produced an initial burst due to a rapid acylation followed by a slow deacylation; i.e. $k_2 \gg k_3$.

Studies were made with compound 10,R = $(CH_3)_2N$, but this substrate was not hydrolysed by human plasmin. Two methods were employed to demonstrate unequivocally a lack of hydrolysis. An attempted acylation, for which the mixture was incubated for 30 min and the plasmin fraction separated, showed upon assay with ZLysONP complete activity. Furthermore, the addition of the compound (dissolved in DMF) to a mixture of plasmin and ZLysONP had no effect on the reaction velocity. It thus appears that the extra stability of the ester group gained from the strong electron donation of the amino group is sufficient to prevent enzymic hydrolysis. The self-hydrolysis of compound 10 is also very slow.

Table 1. Deacylation rate constants of acylated human and porcine plasmins
Measurements were made at 25°C except where indicated; n.d., not determined

R in acyl group, RCO	Buffer solution	k_3, human (s^{-1})	k_3, porcine (s^{-1})
1. Methyl	A	$1{\cdot}06 \pm 0{\cdot}05 \times 10^{-2}$	$6{\cdot}90 \pm 0{\cdot}20 \times 10^{-3}$
2. Methyl	C	$1{\cdot}20 \pm 0{\cdot}05 \times 10^{-2}$	n.d.
3. Cyclohexyl	A	$8{\cdot}80 \pm 0{\cdot}40 \times 10^{-3}$	$1{\cdot}10 \pm 0{\cdot}05 \times 10^{-2}$
3. *p*-Nitrophenyl	A	$3{\cdot}80 \pm 0{\cdot}40 \times 10^{-3}$	n.d.
4. Isobutyl	A	$2{\cdot}80 \pm 0{\cdot}10 \times 10^{-3}$	n.d.
5. trans-Isopropenyl	A	$9{\cdot}40 \pm 0{\cdot}60 \times 10^{-4}$	n.d.
6. Phenyl	A	$1{\cdot}34 \pm 0{\cdot}05 \times 10^{-4}$	n.d.
6. Phenyl	B	$1{\cdot}00 \pm 0{\cdot}05 \times 10^{-4}$	$2{\cdot}70 \pm 0{\cdot}10 \times 10^{-5}$
7. α-Naphthyl	B	$1{\cdot}12 \pm 0{\cdot}03 \times 10^{-5}$	n.d.
8. *p*-Anisoyl	B	$1{\cdot}30 \pm 0{\cdot}05 \times 10^{-5}$	$1{\cdot}40 \pm 0{\cdot}10 \times 10^{-5}$
9. *p*-Guanidinophenyl	B	$4{\cdot}30 \pm 0{\cdot}40 \times 10^{-6}$	$1{\cdot}40 \pm 0{\cdot}10 \times 10^{-6}$
9. *p*-Guanidinophenyl	B	$1{\cdot}16 \pm 0{\cdot}08 \times 10^{-5}$ (37°C)	n.d.

The values of k_3 determined in this study for both human and porcine plasmins are listed in Table 1. For the measurement of k_3 values $<5 \times 10^{-4}$ s^{-1}, the use of 0·02 M lysine in the buffer medium was found to be necessary to stabilize the regenerated plasmin for the usual 6 h over which measurements were made. A lower lysine concentration of 10^{-3} M had to be used for the direct measurements of k_3 values $>5 \times 10^{-4}$ s^{-1} because higher lysine concentrations produced a marked increase in the velocity of the self-hydrolysis of the substrates and consequently made the measurements difficult. However, the concentration of lysine has only a small, if any, effect on k_3 as shown by the studies with R = methyl in buffer solutions containing 10^{-3} M and no lysine, and also by the studies with R = phenyl in 10^{-3} and 0·02 M lysine-containing buffers (Table 1).

It is evident from the data in Table 1 that, as shown for acyl-trypsins (Tanizawa *et al.* 1980; Fujioka *et al.* 1981) k_3 for both human and porcine plasmins is markedly dependent on the nature of R. There is a more than 10^4 range between the highest k_3 (R = methyl) and the lowest (R = *p*-guanidinophenyl). The values of k_3 generally parallel the electron-donating ability of the R group, so that lower k_3 values are obtained as the R group more readily donates electrons to the carbonyl group. For example, R = phenyl has a lower rate than R = *trans*-isopropenyl which in turn is lower than R = methyl. The lower k_3 of R = naphthyl compared with R = phenyl is, however, probably due to a steric effect since it has been observed that a steric effect is responsible for the lower rates of hydrolysis of 1-naphthoate esters than would be predicted from the pK_a of the naphthoic acids (Price *et al.* 1954).

In a previous study of the deacylation rates of a series of *p*-substituted benzoyl-trypsin and chymotrypsins (Wang and Shaw 1972), a good correlation was observed between k_3 and the sigma values of the acyl groups. Plots of k_3 versus sigma yielded slopes of 2·68 and 3·24, respectively. Similar plots in this study for human and porcine plasmins with the R groups *p*-nitrophenyl, phenyl and *p*-anisoyl gave the smaller slopes of 2·2 and 1·1, respectively, which indicate that plasmin, in particular porcine plasmin, is less sensitive to electronic effects than is trypsin or chymotrypsin. The exceptional stability of the *p*-guanidinobenzoyl-trypsin (NPGB derivative), considering the sigma value (−0·02, which is most similar to that of 0·00 for phenyl) was observed previously and is also displayed by both human and porcine plasmins. A previously measured k_3, $5·25 \pm 0·05 \times 10^{-6}$ s^{-1} (0·1 M sodium veronal, 0·02 M $CaCl_2$, pH 8·3, 25°C) for the NPGB derivative of human plasmin (Chase and Shaw 1969), is in good agreement with the value measured in this study ($4·3 \pm 0·4 \times 10^{-6}$ s^{-1}), considering the differences in the pH and composition of the reaction buffer solutions used for the two studies.

Compared with the k_3 values for human plasmin, k_3 for porcine plasmin tends to be lower for most of the acyl groups (Table 1). The ratio of k_3(human)/k_3(porcine) is 3·0, 3·7 and 1·5 for *p*-guanidinophenyl, phenyl and methyl. However, for R = *p*-anisoyl the rates are the same, within experimental error, for both human and porcine, and for R = cyclohexyl, k_3 for porcine is slightly higher than for human plasmin.

For the R = *p*-guanidinophenyl derivative of human plasmin, k_3 was measured at both 25 and 37°C. From these two values, the activation energy, E, and log A of the usual Arrhenius equation are calculated as 64 ± 10 kJ and $5·84 \pm 0·04$, respectively.

The relationships between R and k_3 observed in this study are useful for the design of acyl-plasmin derivatives which have specific half-lives. With regard to the two applications of acyl-plasmin described previously, half-lives of only 1–2 h are required for use in thrombosis therapy, whereas the scintigraphic detection of thrombosis requires much longer half-lives, of the order of 30 h.

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