A comparison of the catalytic activities of human plasma kallikreins I and II

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Subsites in the S_2 – S_4 region [Schechter & Berger (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162] were identified in human plasma kallikrein II (EC 3.4.21.8). Kinetic constants (k_{cat} , K_m) were determined for a series of seven extended *N*-aminoacyl-L-arginine methyl esters based on the C-terminal sequence of bradykinin (-Pro-Phe-Arg) or (Gly) $_n$ -Arg. With these substrates it was found that deacylation of the enzyme was rate-limiting. It was possible to infer that L-proline at residue P_3 interacted with subsite S_3 of the enzyme and L-phenylalanine at residue P_2 interacts hydrophobically with subsite S_2 in addition to hydrogen-bonded interactions with this subsite region. By comparison with the results of a similar study with human plasma kallikrein I, it is observed that although broadly similar subsite interactions occur between the two enzyme forms, the rate of deacylation of kallikrein II is approx. 35% of that observed for kallikrein I, and the latter form is up to ten times more active (in terms of k_{cat}/K_m) than kallikrein II.

Human plasma kallikrein (EC 3.4.21.8) liberates the nonapeptide vasodilator bradykinin from high- M_r kininogen, in addition to its role in initiation and mediation of the intrinsic blood-coagulation system (Schachter, 1980). Two active forms of human plasma kallikrein, namely kallikrein I (M_r 88 000) and kallikrein II (M_r 85 000) have been isolated (Mandle & Kaplan, 1977; Nagase & Barrett, 1981; Levison & Tomalin, 1982a). It has been shown that both enzymes, and their inactive precursors, are antigenically identical and are not interconvertible (Mandle & Kaplan, 1977). Both active forms comprise heavy and light chains linked by disulphide bonds (Mandle & Kaplan, 1977), the heavy chain being of identical size (M_r 52 000) in both forms. The difference occurs in the light chains of kallikreins I and II (M_r 36 000 and 33 000 respectively), which both contain the active-site serine residues (Mandle & Kaplan, 1977; Nagase & Barrett, 1981). It has not been previously reported whether any difference in catalytic activity exists between the two forms of plasma kallikrein.

In a previous study it was shown that human plasma kallikrein I possesses a marked secondary specificity for the Pro-Phe-Arg sequence of high- M_r kininogen comprising the C-terminal portion of the excised bradykinin moiety (Levison & Tomalin, 1982a). It was shown that interaction of L-phenyl-

alanine (P_2) and L-proline (P_3) with the enzyme subsites S_2 and S_3 (Schechter & Berger, 1967), accounted for this secondary specificity (Levison & Tomalin, 1982a). The interactions at enzyme subsites S_2 and S_3 of human plasma kallikrein II were investigated by using the substrates Ac-Arg-OMe·HCl, Ac-Gly-Arg-OMe·HCl, Ac-Gly-Gly-Arg-OMe·HCl, Ac-Phe-Arg-OMe·HCl, Ac-Gly-Phe-Arg-OMe·HCl, Ac-Pro-Gly-Arg-OMe·HCl and Ac-Pro-Phe-Arg-OMe·HCl for reasons given previously (Levison & Tomalin, 1982a,b).

Materials and methods

Materials

Cohn Fraction IV of human plasma was obtained from Blood Products Laboratory, Elstree, Herts., U.K. Kallikrein II was purified from Cohn Fraction IV as described previously (Levison & Tomalin, 1982a) and stored in 1 mM-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl at 4°C. 4-Methylumbelliferyl *p*-guanidinobenzoate was synthesized by Dr. C. R. Hill, Department of Rheumatology, Victoria University of Manchester (Hill, 1979). Ac-Arg-OMe·HCl, Ac-Phe-Arg-OMe·HCl, Ac-Pro-Phe-Arg-OMe·HCl, Ac-Gly-Arg-OMe·HCl, Ac-Gly-Gly-Arg-OMe·HCl, Ac-Gly-Phe-Arg-OMe·HCl and Ac-Pro-Gly-Arg-OMe·HCl were synthesized from their constituent amino acids (Levison & Tomalin, 1982a).

Abbreviations used: Ac, *N*-acetyl; OMe, methyl ester; ·HCl, hydrochloride.

Methods

Active-site titration. The operational molarity of the stock enzyme solutions was determined by spectrofluorimetric titration with 4-methyl-umbelliferyl *p*-guanidinobenzoate in a Perkin-Elmer/Hitachi model 204 spectrofluorimeter by the method of Jameson *et al.* (1973).

Kinetic measurements. The progress of ester hydrolysis was monitored on a Radiometer pH-stat comprising a pH-meter (PHM 62), titration unit (TTT 60c), automatic burette (ABU 13), titration assembly (TTA 60) and recorder (REC 61/REA 160). The reaction vessel, which was surrounded by an insulating jacket, was maintained at $37 \pm 0.1^\circ\text{C}$ by circulation from a Grant LE8 thermostatically controlled water bath. All measurements were performed under an atmosphere of CO_2 -free N_2 saturated with water. The pH electrode was calibrated against two standard buffers (Perrin & Dempsey, 1974). Alkali solutions were prepared by diluting a measured volume of carbonate-free 25 M-NaOH into previously boiled-out distilled water (Vogel, 1961). The absolute molarities of titrant solutions were determined by titration against potassium hydrogen phthalate solution (Vogel, 1961). Stock substrate solutions and subsequent dilutions were made with 0.1 M-NaCl to give a final volume of 10 ml. Final substrate concentrations were in the range 0.01–1.0 mM throughout. Adjustment to pH 8.00 was made with 0.15 M-NaOH. Kallikrein II solution (100 μl) was added and the uptake of alkali at pH 8.00 was recorded as a function of time. After correction for any non-enzymic hydrolysis, initial velocities were calculated and values of k_{cat} and K_m were computed by the method of Wilkinson (1961). Product-partitioning studies were conducted by using Ac-Pro-Phe-Arg-OMe·HCl in the presence of 2 M-methanol (Seydoux & Yon, 1967, 1971) in a similar manner to that described above.

Results and discussion

Kinetics of human plasma kallikrein II

The mechanism of action of serine proteinases is known to involve acylation and deacylation of an active-site serine residue by the acyl portion of the substrate in accordance with the following scheme:

Here ES' is the acyl enzyme, in this case an *N*-aminoacyl-L-arginyl-kallikrein. In order to determine whether the rate-limiting step is acylation or deacylation, for hydrolysis of methyl esters, product-partitioning studies were conducted in the presence and the absence of methanol. This nucleophile competes with water in the deacylation process. If deacylation is the rate-limiting step (i.e. $k_{+2} \gg k_{+3}$), it can be shown that the observed k_{cat} value for the formation of free acid (P_2) will be independent of nucleophile concentration, whereas the observed value of K_m will increase with nucleophile concentration (Seydoux & Yon, 1967). Table 1 gives the results of such a study with Ac-Pro-Phe-Arg-OMe·HCl as substrate and 2 M-methanol as nucleophile. Since k_{cat} was found to be unaltered within the limits of experimental error, it is concluded that deacylation is rate-limiting for human plasma kallikrein II. It seems reasonable to draw the same conclusions for the other substrates with this enzyme. It is therefore possible to relate the observed values of k_{cat} and K_m to the rate constants in the above scheme as follows:

$$k_{\text{cat}} = k_{+3} \quad (1)$$

$$K_m = [(k_{-1} + k_{+2})/k_{+1}](k_{+3}/k_{+2}) \quad (2)$$

Kinetic results for all seven substrates are given in Table 2. It can be seen that the k_{cat} and K_m values for the extended substrates differ markedly from those for Ac-Arg-OMe·HCl, indicating that the P_2 and P_3 residues are interacting with subsites in the enzyme. The values of k_{cat} for the extended substrates remain fairly constant at 14 s^{-1} , indicating that the rate of deacylation is remarkably insensitive to the substitution of the amino acid residues at P_2 and P_3 . It is clear that the enhanced catalytic efficiency for the extended substrates as reflected in k_{cat}/K_m values is a result of subsite interactions at the enzyme surface. Eqn. (2) shows that the steady-state constant $[(k_{-1} + k_{+2})/k_{+1}]$ is greater than the observed Michaelis constant (K_m) by a factor of k_{+2}/k_{+3} . Despite the fact that the measured values of k_{+3} are fairly constant, it is still not possible to conclude whether the measured K_m values predominantly reflect changes in the enzyme-substrate binding affinity (k_{-1}/k_{+1}), the rate of enzyme acylation (k_{+2}) or a combination of both effects.

Extension of the basic Ac-Arg-OMe·HCl substrate to Ac-Gly-Arg-OMe·HCl causes a decrease

Table 1. Kinetic constants for the hydrolysis of Ac-Pro-Phe-Arg-OMe·HCl in the presence and absence of 2 M-methanol, catalysed by human plasma kallikrein II at 37°C , pH 8.00, in 0.1 M-NaCl

[Methanol] (M)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	No. of runs
0	14.3 ± 0.5	0.044 ± 0.004	330	19
2	13.5 ± 0.4	0.075 ± 0.005	180	19

Table 2. Kinetic constants for the hydrolysis of L-arginine methyl ester substrates catalysed by human plasma kallikrein II at 37°C, pH 8.00, in 0.1 M-NaCl

Human plasma kallikrein ...		II*				I†			
Substrate	$k_{\text{cat.}}$ (s ⁻¹)	K_{m} (mM)	$k_{\text{cat.}}/K_{\text{m}}$ (s ⁻¹ ·mM ⁻¹)	No. of runs	$k_{\text{cat.}}$ (s ⁻¹)	K_{m} (mM)	$k_{\text{cat.}}/K_{\text{m}}$ (s ⁻¹ ·mM ⁻¹)		
Ac-Arg-OMe·HCl	71 ± 5	2.23 ± 0.18	32	19	81 ± 18	1.22 ± 0.34	66		
Ac-Gly-Arg-OMe·HCl	14.6 ± 0.7	0.491 ± 0.047	30	20	40.0 ± 1.2	0.560 ± 0.025	72		
Ac-Gly-Gly-Arg-OMe·HCl	14.5 ± 0.9	0.466 ± 0.047	31	20	40.0 ± 0.7	0.180 ± 0.007	220		
Ac-Phe-Arg-OMe·HCl	14.4 ± 0.6	0.335 ± 0.026	43	20	33.9 ± 1.4	0.160 ± 0.014	210		
Ac-Gly-Phe-Arg-OMe·HCl	13.6 ± 0.5	0.247 ± 0.014	55	20	34.1 ± 0.6	0.150 ± 0.006	220		
Ac-Pro-Gly-Arg-OMe·HCl	14.4 ± 0.4	0.102 ± 0.006	140	19	39.4 ± 0.4	0.027 ± 0.001	1460		
Ac-Pro-Phe-Arg-OMe·HCl	14.3 ± 0.5	0.044 ± 0.004	330	19	42.4 ± 0.7	0.014 ± 0.001	2900		

* The present work.

† Levison & Tomalin (1982a).

in the observed K_m value from 2.23 mM to 0.491 mM. It is not possible to conclude whether this is a result of the decreased value of k_{+3} for the extended substrate or a true increase in substrate binding strength. In either case it is clear that the effect is caused by hydrogen-bonded interactions of the Ac-Gly moiety with the enzyme in the S_2 - S_3 subsite region. Extension of Ac-Gly-Arg-OMe·HCl to Ac-Gly-Gly-Arg-OMe·HCl has no effect on the deacylation rate or K_m , indicating that there are negligible hydrogen-bonded interactions occurring between the Ac-Gly moiety and subsites S_3 - S_4 . It is noteworthy that neither of these extended substrates is catalytically more efficient, as reflected in $k_{\text{cat.}}/K_m$ values, than the basic Ac-Arg-OMe·HCl substrate.

Substitution of L-phenylalanine for glycine at P_2 in Ac-Gly-Arg-OMe·HCl to give Ac-Phe-Arg-OMe·HCl decreases K_m from 0.491 mM to 0.335 mM. This is clearly the result of a hydrophobic interaction between L-phenylalanine at residue P_2 and subsite S_2 , in addition to the possibility of hydrogen-bonded interactions. The same effect is observed when a similar substitution is made in Ac-Gly-Gly-Arg-OMe·HCl to give Ac-Gly-Phe-Arg-OMe·HCl.

Insertion of the L-proline residue at P_3 in Ac-Pro-Gly-Arg-OMe·HCl causes a larger decrease in K_m from 0.466 mM for Ac-Gly-Gly-Arg-OMe·HCl to 0.102 mM. The L-proline interaction at residue P_3 has a much larger effect on the selectivity of human plasma kallikrein II than the interaction of L-phenylalanine at residue P_2 . Combination of these two effects in Ac-Pro-Phe-Arg-OMe·HCl leads to a much lower value of K_m (0.044 mM) and an enhanced catalytic efficiency. It is possible that the locked configuration of the Ac-Pro moiety when bound to the enzyme at subsites S_3 - S_4 causes the L-phenylalanine residue bound at subsite S_2 to adopt a more favourable conformation for enhancing catalytic efficiency of the enzyme.

Comparison of human plasma kallikreins I and II

It has been shown for human plasma kallikrein I-catalysed ester hydrolysis, that deacylation of the enzyme is rate-limiting (Levison & Tomalin, 1982a). This is in agreement with the results obtained for kallikrein II.

A direct comparison of the kinetic constants obtained for the hydrolysis of the seven extended N-aminoacyl-L-arginine methyl ester substrates is given in Table 2. A number of striking features are apparent. Although the value of $k_{\text{cat.}}$ for the basic substrate Ac-Arg-OMe·HCl is the same, within experimental error, for both enzymes, the values of $k_{\text{cat.}}$ for kallikrein I (34 – 42 s^{-1}) are significantly larger than those for kallikrein II (14 s^{-1}) with the extended substrates. Furthermore the values of K_m are generally larger for kallikrein II than for kallikrein I, with each substrate. The overall effect in terms of catalytic efficiency ($k_{\text{cat.}}/K_m$) is that kallikrein I is up to ten times more efficient than kallikrein II.

By comparing individual substrates, it can be seen that the overall interactions between L-phenylalanine at residue P_2 and subsite S_2 , and L-proline at residue P_3 and subsite S_3 , are similar to each enzyme. The major difference is the lack of a hydrogen-bonded interaction between the P_3 - P_4 region of the substrate and the S_3 - S_4 subsite region of kallikrein II, which has been invoked for kallikrein I.

Human plasma kallikrein I differs from human plasma kallikrein II in having an additional moiety of approx. 3000 M_r attached to the light chain (Mandle & Kaplan, 1977; Nagase & Barrett, 1981). In the light of our findings, we tentatively propose that the additional region of the kallikrein I light chain gives rise to the differences in catalytic activity with ester substrates demonstrated in this study.

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