Studies on the temperature-dependent autoinhibition of human plasma kallikrein I

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(Received 23 March 1982/Accepted 2 June 1982)

At 37°C, human plasma kallikrein I follows Michaelis–Menten behaviour and exhibits a normal linear relationship between the initial velocity of hydrolysis of Ac-Pro-Phe-Arg-OMe.HCl and enzyme concentration in the range 0–150 μ M. At temperatures of 30°C and below substantial deviations from linearity are observed over the same enzyme concentration range. The temperature-dependent autoinhibition of kallikrein I activity is reversible and is not due to low-molecular-weight endogenous inhibitors or cofactors. The kinetic effect is apparently due to aggregation and can be abolished by the addition of sodium deoxycholate.

In most cases where a kinetic investigation is conducted on an enzyme it is found that there is an exact proportionality between the initial velocity of reaction and active enzyme concentration. Such observations are in accordance with simple Michaelis–Menten theory. Cases where deviations from such behaviour are observed have been listed by Dixon & Webb (1979).

Enzyme kinetic measurements are often made at the standard temperature for physicochemical studies (25°C), and this has been the case for studies with the physiological proteolytic enzymes bovine chymotrypsin A₁ (EC 3.4.21.1) (Hill & Tomalin, 1981), bovine β -trypsin (EC 3.4.21.4) (Green & Tomalin, 1976) and pig pancreatic kallikrein (EC 3.4.21.8) (Fiedler & Leysath, 1979). A preliminary study with human plasma kallikrein I (EC 3.4.21.8) has been reported (Levison & Tomalin, 1982*b*) in which a linear relationship between initial velocity of ester hydrolysis and enzyme concentration was observed at the physiological temperature (37°C). However, substantial deviations from linearity were observed over the same range of enzyme concentration at temperatures of 30°C and below. Although such deviations remain unreported elsewhere in the literature, effects that may have been caused by this phenomenon have been observed (F. Fiedler, personal communication). In the present paper these deviations from normal kinetic behaviour were investigated and tentative explanations are proposed.

Materials and methods

Materials

Cohn fraction IV of human plasma was obtained from Blood Products Laboratory, Elstree, Herts.,

U.K. Kallikrein I was purified from Cohn fraction IV as described previously (Levison & Tomalin, 1982*a*) and stored in 1 mM-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl at 4°C. Sodium deoxycholate was from Sigma Chemical Co., Poole, Dorset, U.K. 4-Methylumbelliferyl *p*-guanidino-benzoate was synthesized by Hill (1979). Ac-Pro-Phe-Arg-OMe.HCl was synthesized from its constituent amino acids (Levison & Tomalin, 1982*a*). 18/32 Visking dialysis tubing (molecular-weight cut-off 12000–14000) was from Scientific Instruments Centre, Liverpool, Merseyside, U.K.

Active-site titration

The operational molarity of the stock enzyme solutions was determined by spectrofluorimetric titration with 4-methylumbelliferyl *p*-guanidino-benzoate in a Perkin–Elmer–Hitachi model 204 spectrofluorimeter by the method of Jameson *et al.* (1973).

Kinetic measurements

The progress of ester hydrolysis was followed 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 jacketed reaction vessel was maintained at various temperatures ($\pm 0.1^\circ$ C) by circulation from a Grant LE8 thermostatically controlled water bath. All measurements were performed under an atmosphere of CO₂-free N₂ 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). All kinetic measurements were made with 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl in 0.1 M-NaCl (10 ml), adjusted to pH 8.00 with 0.15 M-NaOH. Samples of kallikrein I solution were 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.

The effect of sodium deoxycholate on the enzyme-catalysed reaction was investigated by adding portions of stock aq. 150 mM-sodium deoxycholate to the reactants, and monitoring the reaction in a similar manner to that described above.

Dialysis studies

Samples of kallikrein I solution were dialysed at 4°C and 37°C (18/32 Visking dialysis tubing, molecular-weight cut-off 12000–14000) against 1 mM-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl, for 3 h. The effect of enzyme concentration on velocity of hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl at 25°C was investigated in a similar manner to that described above.

Results and discussion

Effect of enzyme concentration on velocity of ester hydrolysis

Results for the kallikrein I-catalysed hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl at pH 8.00 are summarized in Fig. 1. At 37°C (K_m 0.01 mM; Levison & Tomalin, 1982a) it was found that linear behaviour was observed within experimental error over a range of enzyme concentrations up to 150 μ M. Attempts to make kinetic measurements at the standard temperature for physicochemical studies (25°C) under similar conditions gave results that showed substantial negative deviations from linear behaviour. Within the same range of enzyme concentration significant deviations from linearity were also observed at temperatures of 30°C and below. The results were reproducible within the limits of experimental error on different batches of kallikrein I obtained from unrelated enzyme purifications.

Kinetic studies on the reversibility of autoinhibition

The reversibility of the inhibitory phenomenon observed at 25°C was tested by measurement of the velocity of reaction at high enzyme concentration, followed by measurement of the velocity after rapid 50% dilution of the reaction mixture with 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl (pH 8.00). The results of such a study are summarized in Table 1. Before dilution all velocities were of similar magnitude, being

Fig. 1. Effect of human plasma kallikrein I concentration on the initial velocity of hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl with temperature (0.1 M-NaCl, pH 8.00). For full experimental details see the text. $\square$, 37°C; $\blacktriangle$, 30.0°C; $\bullet$, 25.0°C.

in the plateau region at high enzyme concentration (>130 μ M) indicated in Fig. 1. In all cases the velocity was found to increase after dilution, giving a value similar to that summarized in Fig. 1, within the limits of experimental error. It is concluded that the enzyme concentration-dependent inhibition of human plasma kallikrein I activity is reversible at 25°C. It seems reasonable to assume this to be the case for all temperatures where the autoinhibition effect is detectable, up to 37°C, over the same enzyme concentration range.

Active-site titration

Active-site titration of the enzyme involves the rapid formation of *p*-guanidinobenzoyl-kallikrein, a covalent species that undergoes a very slow deacylation process. The effect of enzyme concentration on the experimentally determined active-site concentration was investigated at 21°C. The results are summarized in Table 2. It is clear that there is a linear relationship between the active-site titre and concentration of the enzyme, diluted from stock enzyme, within experimental error, up to 500 μ M-enzyme. It is concluded that covalent modification of the active site of human plasma kallikrein I at 21°C decreases the autoinhibition effect to an insignificant level. It seems reasonable to conclude this to be the case at all temperatures where the autoinhibition effect is detectable, up to 37°C, over the same concentration range.

Table 1. *Effect of dilution of kallikrein I on the velocity of hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe,HCl at 25°C*
For full experimental details see the text.

Initial concn. of enzyme (pM)	Initial velocity (nmol/min)			Difference <i>b</i> - <i>c</i> (%)
	(a) Before dilution	(b) After 50% dilution	(c) Estimated value from Fig. 1	
133	0.098	0.272	0.243	+11.1
160	0.111	0.216	0.235	-8.6
173	0.095	0.229	0.218	+2.7

Table 2. *Effect of increasing human plasma kallikrein I concentration on the active-site concentration experimentally determined by using 4-methylumbelliferyl p-guanidinobenzoate at 21 ± 0.5°C*
For full experimental details see the text.

Vol. of kallikrein assayed (μl)	Measured active sites/assay (pmol)	Apparent concn. of active sites/assay (pM)	Apparent concn. of active sites of stock kallikrein (pM)
100	0.72	231	7200
150	1.14	360	7600
200	1.59	494	7950
		Mean ...	7580 ± 370

Thermodynamic activation constants for the catalytic process

Under favourable conditions it is possible to obtain the standard enthalpy ($\Delta H^\ddagger$), entropy ($\Delta S^\ddagger$) and free energy ($\Delta G^\ddagger$) of activation for the rate-limiting step of an enzyme-catalysed reaction from the Arrhenius equation (Dixon & Webb, 1979). The data in Fig. 1 are presented in the form of Arrhenius plots in Fig. 2. It can be seen from Fig. 1 that in the 0–50 pM-enzyme concentration range the velocity of ester hydrolysis is directly proportional to enzyme concentration for all temperatures in the range 25–37°C. Although there are only four data points, the resulting Arrhenius plot (Fig. 2) is linear within experimental error (s.d. = ±9%). On the other hand the Arrhenius plot of 150 pM-enzyme concentration exhibits a large negative deviation from linearity at the lowest temperatures. Arrhenius plots for enzyme-catalysed reactions that are non-linear or exhibit discontinuities are discussed by Dixon & Webb (1979). Such observations involve changes in enthalpies of activation by factors of up to about 2 and are consistent with either a change in the rate-limiting step of the enzymic process or the enzyme existing in at least two active forms with differing enthalpies of activation for the enzymic process or changes in catalytic mechanism. It has been shown that the rate-limiting step for the human plasma kallikrein I-catalysed hydrolysis of methyl ester substrates is deacylation at 37°C (Levison & Tomalin, 1982a). Values of the activation constants

for the deacylation process were thus calculated for the range 0–50 pM-enzyme between 25°C and 37°C. These data are presented in Table 3. It is seen that the values are similar to those obtained for methyl ester hydrolysis catalysed by bovine chymotrypsin A₁ (Hill & Tomalin, 1981) and bovine β-trypsin (Green & Tomalin, 1976) where deacylation is rate-limiting in both cases. These data confirm that kallikrein is behaving in a consistent manner in this concentration range. The Arrhenius plots for higher enzyme concentrations exhibit considerable deviations from linearity at lower temperatures, and it is estimated from the plot for 150 pM-enzyme that the activation constants must attain values of at least $\Delta H^\ddagger = 400 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S^\ddagger = +1100 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. Such values are much greater than values that could possibly be expected for a change in rate-limiting step for the enzyme-catalysed reaction or change in mechanism.

Although there is still the possibility that there could be a change in rate-limiting step at lower temperatures in the high enzyme concentration range, the effect on the activation constants would be undetectable alongside the large effect produced by the autoinhibition process. Results from partitioning studies with methanol as nucleophile (Levison & Tomalin, 1982a) at lower temperatures would clearly be impossible to interpret unambiguously, since methanol may affect the aggregation equilibrium in addition to its role as a competing nucleophile in the rate-limiting process. It is con-

Fig. 2. Effect of human plasma kallikrein I concentration on Arrhenius plots for the hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl (0.1 M-NaCl, pH 8.00)

For full experimental details see the text. ●, 0–50 pM; ○, 75 pM; ▲, 100 pM; □, 125 pM; ■, 140 pM.

cluded that the observed values are not open to interpretation without recourse to at least a simple model for the autoinhibition phenomenon.

The above effects can possibly be partially explained by either of two hypotheses. Firstly, there may be an endogenous reversible low-molecular-weight inhibitor or cofactor present in the enzyme preparation. The inhibition constant for this would be very low and is estimated to be in the region of 100 pM. The reaction would necessarily have a very high enthalpy of reaction. Secondly, the enzyme may exist in a number of different forms, possibly a monomer–multimer relationship, giving a range of different species with markedly different catalytic activities. The equilibrium process again would necessarily be very temperature-dependent. Cases of such behaviour and the resulting Arrhenius plots are discussed by Dixon & Webb (1979).

Effect of endogenous inhibitor or cofactor

After purification of human plasma kallikrein I, a single band of M_r 91 000 $\pm$ 2000 was obtained on

sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Levison & Tomalin, 1982a). This result is in good agreement with the value of M_r 88 000 previously reported (Mandle & Kaplan, 1977). If an endogenous inhibitor or cofactor were present in the enzyme preparation, it would thus necessarily be of low molecular weight, in the region of M_r 3000. As shown above, any endogenous inhibitor or cofactor would be readily dissociable from the enzyme at temperatures above 30°C. To test this hypothesis it is necessary to consider the results of the dialysis studies performed at 37°C. Since the dialysis membrane has a porosity with a molecular-weight cut-off 12 000–14 000, a low-molecular-weight inhibitor or cofactor of the type described above would be diffusible at 37°C but tightly bound at 25°C. The effect of enzyme concentration after dialysis at 37°C, compared with dialysis at 4°C, on the initial velocity of hydrolysis of the assay substrate at 25°C is shown in Fig. 3. It is apparent that the two curves are identical, within experimental error, and therefore dialysis at 37°C has had no significant effect on enzymic activity compared with dialysis at 4°C. It is therefore concluded that the enzyme preparation does not contain any low-molecular-weight diffusible inhibitors or cofactors.

Effect of aggregation

Sodium deoxycholate is a detergent capable of dissociating hydrophobically associated protein aggregates without significant denaturation of the constituent protein (Helenius & Simons, 1975). The effect of kallikrein I concentration on velocity of ester hydrolysis at 25°C was hence determined in the presence and in the absence of 75 μ M-sodium deoxycholate (Fig. 4). At kallikrein concentrations above 50 pM it is evident that sodium deoxycholate causes the enzyme activity to be increased. In a similar experiment at 37°C, 75 μ M-sodium deoxycholate had no significant effect on enzyme activity over the same enzyme concentration range. From this result it is concluded that human plasma kallikrein I undergoes aggregation at high enzyme concentrations and that the aggregates dissociate in the presence of sodium deoxycholate. Furthermore it is clear that the aggregates formed at high enzyme concentrations have lower catalytic activities than has the monomer, which predominates at low enzyme concentration.

Further experiments on the effect of sodium deoxycholate concentration on enzymic activity were performed at 25°C with 150 pM-enzyme. The results are summarized in Fig. 5. Here the apparent enzymic activity is increased to a limiting value of 7.2 s⁻¹ by sodium deoxycholate concentrations above 1.5 mM. This limiting value agrees, within experimental error, with that expected by extrapolation of the linear portion of the curve of velocity

Table 3. Activation constants estimated for ester hydrolysis catalysed by human plasma kallikrein I and other mammalian serine proteinases

Enzyme	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (J·K ⁻¹ ·mol ⁻¹)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)
Kallikrein I (0–50 pM)	38.6	–100.2	69.0
Chymotrypsin A _α *	22.3	–130.3	61.1
β-Trypsin†	32.7	–94.5	60.9

* With Ac-Gly-Gly-Phe-OMe (Hill & Tomalin, 1981).

† With Ac-Lys-OMe (Green & Tomalin, 1976).

Fig. 3. Effect of human plasma kallikrein I concentration, after dialysis at 4°C or 37°C, on the initial velocity of hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl at 25°C (0.1 M-NaCl, pH 8.00)

For full experimental details see the text. O, Dialysis at 4°C; ●, dialysis at 37°C.

Fig. 5. Effect of sodium deoxycholate concentration on the initial velocity of hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl catalysed by 150 pM-human plasma kallikrein I at 25°C (0.1 M-NaCl, pH 8.00)

For full experimental details see the text.

Fig. 4. Effect of human plasma kallikrein I concentration on the initial velocity of hydrolysis of 0.1 mM-Ac-Pro-Phe-Arg-OMe, HCl in the presence and in the absence of 75 μM-sodium deoxycholate at 25°C (0.1 M-NaCl, pH 8.00)

For full experimental details see the text. ♦, Sodium deoxycholate absent; ◇, sodium deoxycholate present.

plotted versus enzyme concentration at 25°C (Fig. 1). It is concluded that it is possible to break down the aggregates into fully active monomers by using sodium deoxycholate at concentrations in excess of 1.5 mM.

It has been observed in earlier studies that related bile salts affect the esterolytic activity of thrombin (EC 3.4.21.5) (Curragh & Elmore, 1964; Exner & Koppel, 1973) and trypsin (Calvert & Legg, 1977).

For thrombin such effects have been proposed to result from both conformational changes occurring in the enzyme or enzyme-substrate complex and the binding of solvent molecules to the enzyme (Curragh & Elmore, 1964). For trypsin it has been suggested that the bile salts affect the ester substrate rather than the enzyme itself (Calvert & Legg, 1977). It has been demonstrated that the esterase activity of rat urinary kallikrein increases by a factor of up to 4-fold in the presence of sodium deoxycholate (0.48–2.4 mM) at room temperature (Chao, 1978). Although this effect was tentatively explained in terms of a conformational change in the enzyme molecule, it could also result from an aggregatory effect, similar to the one observed in the present work, occurring at temperatures below the physiological temperature (37°C).

Conclusions

It is concluded that human plasma kallikrein I undergoes temperature-dependent autoinhibition resulting from aggregate formation. The aggregate is of lower specific activity than the monomer, and aggregation is reversible by either (i) raising the temperature above 30°C or (ii) dilution or (iii) covalent modification of the active site or (iv) addition of non-denaturing detergents. It must be stressed that the mechanism of this autoinhibition

remains unclear, and attempts to elucidate a kinetic scheme based on aggregation of the enzyme so far have been unsuccessful. At this stage it must be concluded that the aggregation phenomenon is a very complex one.

The existence of a high-molecular-weight form of human plasma kallikrein has been reported (M_r 165000; Colman *et al.*, 1969; Heber *et al.*, 1978). From measurements made by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis this form has been shown to be a dimer of kallikrein I (Levison, 1982). A similar autoinhibition effect to that of kallikrein I was observed with the high-molecular-weight kallikrein (P. R. Levison & G. Tomalin, unpublished work), suggesting the existence of even larger aggregates.

The results from the present study suggest that, before extensive kinetic measurements on newly isolated proteolytic enzymes are made, preliminary measurements should be made to ensure that suitable conditions (i.e. temperature, range of enzyme concentrations) for exact proportionality between initial velocity and enzyme concentration are identified. In a recent study on the substrate-specificity of bovine plasma kallikrein (McRae *et al.*, 1981) kinetic measurements were made at 30°C with an enzyme concentration of 110 pM. Since strong similarities exist between bovine and human plasma kallikreins (Heimark & Davie, 1979), it is suggested that the quantitative results be treated with some caution until such time as the type of phenomenon reported in the present paper is either eliminated or taken into account for the bovine enzyme. Under the experimental conditions used for bovine plasma kallikrein, the values of k_{cat} and K_m obtained for human plasma kallikrein I would have been complicated by the autoinhibition phenomenon and would not be the true values.

We thank the Medical Research Council for a research studentship (to P. R. L.) and a grant for equipment (to G. T.), and Blood Products Laboratory for plasma fractions.

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