Specificity of activated human Protein C

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Peptide *p*-nitroanilide substrates and peptidylchloromethane inhibitors were used to examine the specificity of activated human Protein C. Substrates with arginine in the P_1 position had the highest activity. The best substrates and inhibitors, as judged by the second-order rate constant for their interaction with the enzyme, had an apolar residue in the P_2 position. In contrast with thrombin [Kettner & Shaw (1981) *Methods Enzymol.* **80**, 826–842], activated Protein C was able to accommodate large hydrophobic residues such as phenylalanine and leucine in the P_2 position. In the P_3 position, the enzyme preferred an apolar D-amino acid residue. The results of the present study have also indicated a suitable substrate and inhibitor to be used in the assay of functional protein C and of thrombomodulin.

Protein C appears to play an important role in the regulation of blood coagulation (Esmon, 1983). Activated Protein C inhibits coagulation by inactivating Factors V and VIII (Kisiel *et al.*, 1977; Vehar & Davie, 1980; Malar *et al.*, 1982). The importance of Protein C is indicated by the fact that low concentrations of Protein C are associated with recurrent familial thrombosis (Griffin *et al.*, 1981; Bertina *et al.*, 1982).

Protein C circulates in plasma as a two-chain zymogen (Kisiel, 1979) that is slowly activated by thrombin alone, but is much more rapidly activated by thrombin in the presence of an endothelial-cell cofactor called thrombomodulin (Esmon *et al.*, 1982; Salem *et al.*, 1984). Additionally, Factor Va is a less efficient cofactor in the activation reaction (Salem *et al.*, 1983*a,b*).

Despite the proposed importance for Protein C in the regulation of coagulation, very little is known about the substrate specificity of activated human Protein C with respect to its natural or synthetic substrates. In the current study, we have determined the kinetic parameters of commercially available peptide *p*-nitroanilide substrates with human activated Protein C. These experiments yielded information on substrate specificity of the enzyme. The specificity of the enzyme was further examined by determining the kinetics of its inactivation by a number of peptidylchloromethane inhibitors.

Experimental

Materials

Peptide *p*-nitroanilide substrates with the nomenclature 'Chromozym' were obtained from Boehringer Biochemicals, Tutzing, Germany, and those with the 'S-****' nomenclature were from AB Kabi, Molndal, Sweden. Peptidylchloromethanes were generously provided by Dr. E. Shaw. Snake venom from *Oxyuranus scutellatus* was from Sigma Chemical Co., St. Louis, MO, U.S.A., and frozen rabbit lungs were supplied by Pel-Freez, Rogers, AR, U.S.A. Fresh or fresh-frozen human plasma was provided by the Blutspendezentrum, Basel, Switzerland. All other chemicals were of the highest purity available commercially.

Preparation of activated Protein C

Protein C and prothrombin were purified from human plasma by the methods of Suzuki *et al.* (1983) and Miletich *et al.* (1981) respectively. Thrombomodulin was isolated from frozen rabbit lungs (Esmon *et al.*, 1982). Prothrombin was activated to thrombin with the venom from *O. scutellatus* (Owen & Jackson, 1973) and purified on SP- (sulphopropyl-)Sephadex (Lundblad, 1971). Protein C was activated by human thrombin in the presence of rabbit thrombomodulin (Salem *et al.*, 1984) and purified by the method of Kisiel & Davie (1981).

Abbreviations used: (in sequences): <Glu, 5-oxo-pyrrolidine-2-carboxylic acid; Pip, pipecolyl; ΛPro, 3,4-dehydroproline; Suc, 3-carboxypropionyl; Tos, toluene-*p*-sulphonyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; Nan, *p*-nitroanilide.

Assay of activated Protein C

The rate of peptide p-nitroanilide hydrolysis was determined by using a Shimadzu UV240 spectrophotometer to measure the increase in absorbance at 405 nm that resulted from the release of p-nitroaniline. The reactions were performed in polystyrene cuvettes at 37°C in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl, 0.1% poly-(ethylene glycol) (M_r 6000) and 1.0 mM-CaCl$_2$. Activated Protein C was present in the assays at concentrations of 2–200 nM. Under the assay conditions, activated Protein C was stable for at least 10 min, as determined by the progress-curve method of Selwyn (1965). An absorption coefficient of 9920 M^{-1}·cm^{-1} at 405 nm for p-nitroaniline was used in the calculation of the amount of product formed (Lottenberg & Jackson, 1983). The concentration of the peptide p-nitroanilide substrates was determined spectrophotometrically at 342 nm by using an absorption coefficient of 8270 M^{-1}·cm^{-1} (Lottenberg & Jackson, 1983).

In experiments that examined the activity of activated Protein C with various peptide p-nitroanilide substrates, the substrate was varied over a 9-fold range. Five different initial substrate concentrations were used, and each point was measured in duplicate.

In experiments that examined the inhibitory effects of peptidylchloromethanes, the substrate S-2238 was present at a concentration of 400 μM. The inhibitor was added immediately before the assay was started by the addition of the enzyme. In the absence of inhibitors, the rate of product formation was linear up to a product concentration of greater than 10 μM, which represents 2.5% substrate utilization. In the presence of inhibitor, the reaction was allowed to proceed until the amount of product formed was 10 μM or until the activity of the enzyme was negligible. Ten data points were taken for analysis from each curve at approximately equal increments of product formation.

## Determination of the concentration of activated Protein C

The concentration of activated Protein C was determined by active-site titration with methylumbelliferyl p-guanidinobenzoate by the method of Jameson et al. (1973). This method assumes that the concentration of titrant is sufficient to saturate the enzyme; and, under these conditions, the amplitude of the initial burst is equal to the concentration of the active enzyme. Activated Protein C was not, however, saturated with the concentrations of the titrant routinely used (10 μM). Therefore the amplitude of the initial burst that occurred before a steady-state turnover was measured at a number of titrant concentrations

and extrapolation was made to infinite concentration. The concentration of activated Protein C was taken to be equal to the calculated amplitude of the initial burst at infinite titrant concentration. The concentration of activated Protein C calculated in this manner agreed well with that based on the absorbance of the enzyme solution at 280 nm (Kisiel, 1979).

Data analysis

For initial-velocity experiments in which the velocity was hyperbolically dependent on the substrate concentration, the data were fitted to the Michaelis–Menten equation by weighted nonlinear regression (Duggleby, 1981). The weighting used assumed proportional errors (Cornish-Bowden, 1977), and, in addition, bisquare weighting was included to mitigate the effect of outliers as recommended by Mosteller & Tukey (1977). For some substrates, velocity was directly proportional to substrate concentration, and for these substrates the second-order rate constant V/K_m was determined by weighted linear regression.

The irreversible inhibition of an enzyme in the presence of a substrate can be represented by the following scheme:

$$E+S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_0} E+P$$
$$K_i \Big\updownarrow I$$
$$EI \xrightarrow{k_i} EI^*$$
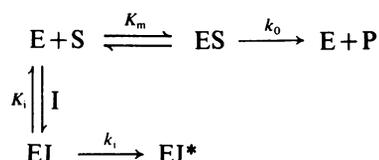
where EI* is the irreversibly inactivated enzyme. If it is assumed that substrate and inhibitor binding are in rapid equilibrium and that the substrate concentration does not change significantly during the course of the assay, the concentration of product, [P], at time t after the initiation of the reaction will be given by eqn. (1) (Walker & Elmore, 1984; Tian & Tsou, 1982):

$$[P] = \frac{v_i}{k_{app.}}\left[1 - \exp(-k_{app.}t)\right] + d \qquad (1)$$

where

$$v_i = \frac{V[S]}{[S] + K_m(1 + [I]/K_i)} \qquad (2)$$

$$k_{app.} = \frac{k_i[I]}{[I] + K_i(1 + [S]/K_m)} \qquad (3)$$

and d is a displacement term to account for the fact that at $t = 0$ the absorbance is not accurately known. Progress curves for the inactivation of activated Protein C by peptidylchloromethanes were fitted by non-linear regression to eqn. (1).

Progress curves were obtained with at least five different concentrations of inhibitor. The values of $k_{app.}$ obtained from these analyses were weighted according to the squared inverse of their standard errors and fitted to eqn. (3). This procedure yielded values for k_i and $K_i(1 + [S]/K_m)$. The value of K_m obtained from initial-velocity studies was used to calculate the value of K_i. The variation of v_i with [I] could also be used to estimate K_i (eqn. 2). As discussed by Duggleby et al. (1982), however, $k_{app.}$ is less subject to systematic error, and consequently eqn. (3) was used for the estimation of K_i. For one inhibitor, $k_{app.}$ was directly proportional to [I], and a value for the second-order rate constant k_i/K_i was obtained by weighted linear regression.

Results and discussion

Substrate specificity of human activated Protein C

The values for the maximum velocity (V) and Michaelis constant (K_m) obtained with a number of peptide p-nitroanilide substrates are given in Table 1. The second-order rate constant V/K_m is the simplest single parameter for assessing the substrate specificity of an enzyme (Fersht, 1977), and values of this parameter for thrombin are given for comparison in Table 1. With activated Protein C, values obtained for V/K_m varied by four orders of magnitude, from $3.3 \times 10^5 M^{-1} \cdot s^{-1}$ for D-Ile-Pro-Arg-Nan to less than $10 M^{-1} \cdot s^{-1}$ for <Glu-

Pro-Val-Nan and MeOSuc-Arg-Pro-Lys-Nan. The preference of the enzyme for an arginine residue in the P_1 position is clear from the data of Table 1. Under the assay conditions used <Glu-Pro-Val-Nan was not cleaved and substrates with lysine in the P_1 position were in general cleaved poorly. There were two substrate pairs that differed only in their P_1 residue: Tos-Gly-Pro-Arg/Lys-Nan (Chromozym TH and PL) and D-Val-Leu-Arg/Lys-Nan (S-2266 and S-2251). For these two substrate pairs, the value of V/K_m for the arginine derivatives were 17-fold and 316-fold higher respectively. It may be noted that the substitution of lysine for arginine in Tos-Gly-Pro-Arg-Nan caused a decrease in K_m as well as a decrease in V. Activated Protein C appears to prefer a bulky non-polar amino acid residue in the P_2 position. For thrombin, the best p-nitroanilide substrates have a proline in this position (Lottenberg et al., 1981, 1983). Such substrates were also among the best for activated Protein C, but it was also able to accommodate much larger residues, such as phenylalanine and leucine, in the P_2 position (Table 1). Cho et al. (1984) have also shown that phenylalanine is preferred to glycine in the P_2 position of tripeptide p-nitroanilide substrates by activated human Protein C. In the same study, phenylalanine was a preferred residue in the P_2 position of dipeptide thioester substrates. In its preference for phenylalanine in the P_2 position, activated

Table 1. *Kinetic parameters for the cleavage of peptide p-nitroanilide substrates by human activated Protein C*
Values of V and K_m were determined by weighted non-linear regression and are given together with their standard errors.

| p-Nitroanilide derivative of peptide | Activated human Protein C | | | Human thrombin |
	V (s^{-1})	K_m (μM)	$10^{-4} \times V/K_m$ $(M^{-1} \cdot s^{-1})$	$10^{-4} \times V/K_m$ $(M^{-1} \cdot s^{-1})$
Tos-Gly-Pro-Arg (Chromozym TH)	87 ± 7	762 ± 95	11.7	3100*
D-Phe-Pip-Arg (S-2238)	53 ± 1	373 ± 37	14.2	6000*
D-Ile-Pro-Arg (S-2288)	200 ± 8	606 ± 36	33.0	4000†
D-Pro-Phe-Arg (S2302)	31 ± 1	505 ± 37	6.1	10‡
D-Val-Leu-Arg (S-2266)	35 ± 1	345 ± 18	10.1	5.2*
D-Val-cyclohexylAla-Arg (Chromozym GK)	1.9 ± 0.1	259 ± 28	0.73	—
Tos-Gly-Pro-Lys (Chromozym PL)	0.81 ± 0.03	116 ± 11	0.69	8.7*
Cbz-Val-Gly-Arg (Chromozym TRY)	0.38 ± 0.04	528 ± 90	0.07	2.5*
<Glu-Gly-Arg‖ (S-2244)	—	—	0.24 ± 0.01	0.36*
Cbz-Ile-Glu-Gly-Arg‖ (S-2222)	—	—	0.038 ± 0.002	1.7*
D-Val-Leu-Lys‖ (S-2251)	—	—	0.032 ± 0.001	0.086*
Bz-Pro-Phe-Arg‖ (Chromozym PK)	—	—	0.067 ± 0.001	0.033*
<Glu-Pro-Val§ (S-2484)	—	—	—	—
MeOSuc-Arg-Pro-Lys§ (S-2586)	—	—	—	—

* Lottenberg et al. (1981).
† AB Kabi, manufacturer's literature.
‡ Value for bovine thrombin (Lottenberg et al., 1983).
§ No activity was observed with $400 \mu M$ substrate and 200 nM enzyme; V/K_m can be assumed to be less than $10 M^{-1} \cdot s^{-1}$.
‖ The K_m was greater than $2000 \mu M$ and the velocity appeared to be directly proportional to substrate concentration.

Protein C is similar to kallikreins (Lottenberg et al., 1981). Thrombin, in contrast, cleaves substrates with phenylalanine in the P_2 position relatively poorly (Table 1).

With activated human Protein C the best substrates yielded a V/K_m value of about $10^5 M^{-1} \cdot s^{-1}$, whereas with thrombin the best values were in excess of $10^7 M^{-1} \cdot s^{-1}$ (Table 1). Thus it should be possible to obtain better synthetic substrates for activated Protein C. Any improvement in the value of V/K_m for substrates of activated Protein C is likely to be achieved by decreasing the value of K_m rather than by increasing V. In comparison with the best substrates for thrombin, the best substrates for activated Protein C yielded similar values for V, but the values for K_m were two orders of magnitude larger (Lottenberg et al., 1981).

Protein C circulates in plasma as a zymogen (Esmon, 1983), and in order to measure the concentration of functional Protein C it must first be activated by thrombin (Sala et al., 1984; Comp et al., 1984). Thrombin must subsequently be inactivated before activated Protein C is measured. The assay of the endothelial-cell cofactor thrombomodulin involves the same procedure (Esmon et al., 1982; Salem et al., 1984). An ideal substrate for activated Protein C in these assays would have a much higher activity with activated Protein C than with thrombin at concentrations of both that are present in the assay. With such a substrate, incomplete inactivation of thrombin would not produce significant error. If the difference in activities were large enough, the thrombin-inactivation step could be omitted in the assay of functional Protein C. From the data presented in Table 1, D-Val-Leu-Arg-Nan (S-2266) would appear to be the best available substrate for this purpose. Protein C and thrombomodulin assays usually contain thrombin at a concentration of about 1% of that of Protein C. At these concentrations, hydrolysis of S-2266 by thrombin is negligible in comparison with that of activated Protein C at all but the lowest extents of Protein C conversion. In fact, if S-2266 is used in the Protein C assay, only a slight increase in background absorbance is observed if the thrombin inactivation step is omitted.

Inactivation of human activated Protein C by peptidylchloromethanes

Twenty peptidylchloromethanes were tested as inhibitors by using a continuous assay. The data from a typical experiment are shown in Fig. 1, and the results obtained for all the inhibitors tested are given in Table 2. The inhibitors tested could be divided into three major classes: 1, inhibitors with a proline in the P_2 position, which had been
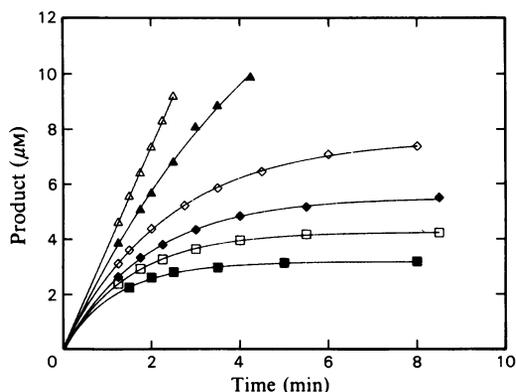

Fig. 1. *Inactivation of human activated Protein C by D-Phe-Pro-Arg-CH₂Cl*

Assays were performed in the presence of the substrate S-2238 as described in the Experimental section. The Figure shows progress curves obtained for the inhibition of the enzyme by D-Phe-Pro-Arg-CH₂Cl at the following concentrations: $0 \mu M$ ($\triangle$), $2.6 \mu M$ ($\blacktriangle$), $5.1 \mu M$ ($\diamond$), $7.7 \mu M$ ($\blacklozenge$), $10.3 \mu M$ ($\square$) and $12.3 \mu M$ ($\blacksquare$). Additional assays were performed at 0.6, 3.9, 6.4, 9.0 and 11.6 μM inhibitor. The data were fitted to eqn. (2), and the lines in the Figure represent the fit to this equation. These analyses provided values for $k_{app.}$ that were weighted and fitted to eqn. (3) to yield the values of k_i and K_i given in Table 2. For the sake of clarity, data obtained at times less than 1.0 min are not included in the Figure.

designed as inhibitors of thrombin; 2, inhibitors with a phenylalanine in the P_2 position, which had been designed as inhibitors of kallikreins; 3, inhibitors with glycine in the P_2 position, which had been designed as inhibitors of Factor Xa. In addition, several other compounds were tested. Inhibitors with glycine in the P_2 position failed to inactivate the enzyme. With the slightly larger residue alanine in the P_2 position inhibition was observed (Table 2). Thrombin inhibitors with a proline in the P_2 position were among the best inhibitors of activated Protein C. Similar results have been obtained by Lijen et al. (1984). Unlike thrombin, however, activated Protein C seems to be able to accommodate the more bulky phenylalanine residue in the P_2 position. Thus, for activated Protein C, D-Phe-Pro-Arg-CH₂Cl and D-Phe-Phe-Arg-CH₂Cl yielded the same apparent second-order rate constant for the inactivation ($k_i/K_i = 3.7 \times 10^3 M^{-1} \cdot s^{-1}$). In contrast, for bovine thrombin D-Phe-Pro-Arg-CH₂Cl yielded a rate constant 1500-fold larger (Kettner & Shaw, 1981). A compound with leucine in the P_2 position is also a satisfactory inhibitor of activated Protein C (Table 2). The importance of a D-amino acid in the P_3 position is seen by comparing the values of k_i/K_i

Table 2. *Parameters for the inactivation of human activated Protein C by peptidylchloromethanes*
Inactivation assays were performed in the presence of the substrate S-2238, and the inactivation parameters k_i and K_i were determined by non-linear regression as described in the Experimental section. The determined values for k_i and K_i are given together with their standard errors.

Peptidylchloromethane inhibitor	K_i (μM)	$10^{-2} \times k_i$ (s^{-1})	$10^{-2} \times k_i/K_i$ (M$^{-1} \cdot$s^{-1})
D-Phe-Pro-Arg-CH$_2$Cl	8.4 ± 1.0	3.08 ± 0.27	37
D-Tyr-Pro-Arg-CH$_2$Cl	5.8 ± 1.6	2.10 ± 0.40	37
D-Phe-ΛPro-Arg-CH$_2$Cl	7.5 ± 3.9	0.92 ± 0.25	12
I$_2$-D-Phe-Pro-Arg-CH$_2$Cl	1.5 ± 0.1	0.57 ± 0.01	38
p-NH$_2$-D-Phe-Pro-Arg-CH$_2$Cl	5.7 ± 1.5	1.93 ± 0.37	33
ε-Bz-D-Lys-Pro-Arg-CH$_2$Cl	—	—	318 ± 17*
Bz-Ala-D-Phe-Pro-Arg-CH$_2$Cl	—	—	<0.2†
Ile-Pro-Arg-CH$_2$Cl	210 ± 20	2.82 ± 0.18	0.38
D-Phe-Phe-Arg-CH$_2$Cl	4.9 ± 0.4	1.18 ± 0.07	37
Phe-Phe-Arg-CH$_2$Cl	87 ± 6	1.03 ± 0.03	1.18
Tyr-Phe-Arg-CH$_2$Cl	127 ± 30	1.23 ± 0.17	0.97
Ala-Phe-Arg-CH$_2$Cl	135 ± 48	1.55 ± 0.40	1.15
Pro-Phe-Arg-CH$_2$Cl	—	—	<0.2†
Ac-Pro-Phe-Arg-CH$_2$Cl	—	—	<0.2†
Ile-Glu-Gly-Arg-CH$_2$Cl	—	—	<0.2†
Ac-Gly-Gly-Arg-CH$_2$Cl	—	—	<0.2†
Glu-Gly-Arg-CH$_2$Cl	—	—	<0.2†
Val-Val-Arg-CH$_2$Cl	135 ± 35	1.60 ± 0.28	1.28
Phe-Ala-Arg-CH$_2$Cl	157 ± 33	0.97 ± 0.10	0.62
Ile-Leu-Arg-CH$_2$Cl	105 ± 55	2.28 ± 0.92	2.18

* The value of $k_{app.}$ varied linearly with inhibitor concentration.
† No inhibition was observed with a concentration of 100 μM inhibitor within a period of 5 min, and therefore the second-order rate constant k_i/K_i was assumed to be less than 20 M$^{-1} \cdot$s^{-1}.

for the two isomers of Phe-Phe-Arg-CH$_2$Cl; the D-isomer is 31-fold more active than the L-isomer, and the increase in the second-order rate constant with the D-isomer is due entirely to a decrease in the binding constant (Table 2). The best substrates for activated Protein C also have D-amino acid residues in the P$_3$ position (Table 1). Similar observations have been made with thrombin (Kettner & Shaw, 1981; Lottenberg et al., 1983).

The compound Bz-Ala-D-Phe-Pro-Arg-CH$_2$Cl is interesting from the point of view of the assay of Protein C and thrombomodulin. This compound is a very effective inhibitor of human thrombin, yielding a value of 3.8×10^5 M$^{-1} \cdot$s^{-1} for k_i/K_i (Walker et al., 1985). In contrast, the value of k_i/K_i for this compound with human activated Protein C was less than 20 M$^{-1} \cdot$s^{-1}. As discussed above, the assay for Protein C and thrombomodulin requires that thrombin be inactivated after the first stage. Bz-Ala-D-Phe-Pro-Arg-CH$_2$Cl would be a most suitable compound for this purpose. At a concentration of 10 μM thrombin would be inactivated within seconds, whereas the half-life for inactivation of activated Protein C would be greater than 100 min.

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