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Inactivation of human thrombin by water-soluble carbodiimides

The essential carboxyl has a pK_a of 5.6 and is one other than Asp-189

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Human thrombin is inactivated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide (EAC) by a second order process. A plot of the pseudo-first order rate constant for inactivation by 20 mM EDC at different pH values from 4.2 to 7.7 at 25°C shows that this inactivation is due to the modification of a protonated carboxyl with a pK_a of 5.6. The rate of inactivation by EDC at pH 6.0 is reduced, but not eliminated by saturating levels of the competitive inhibitor dansyl-L-arginine *N*-(3-ethyl-1,5-pentanediyl)amide, suggesting that the essential carboxyl modified is one other than Asp-189 in the substrate specificity pocket of thrombin. Complete inactivation by ^{14}C-EAC correlates with the incorporation of approximately 2.5–3 EACs per thrombin.

Thrombin; Carboxyl, essential; Carbodiimide; EDC; EAC; DAPA

1. INTRODUCTION

Several studies [1,2] have suggested that carboxyl groups play a critical role in the catalytic function of thrombin, a serine protease with trypsin-like specificity [3]. The amino acid sequences of bovine [4] and human [5] thrombins have been determined, and the results show that the enzyme has high sequence homology with trypsin and chymotrypsin. Asp-189 is conserved in thrombin, when compared to trypsin, and this carboxylate likely plays the role of a negatively charged recognition site in the substrate specificity pocket. Furie et al. [6], based on a computer-generated model of thrombin derived from X-ray crystallographic data on trypsin and chymotrypsin, suggest that 3 other carboxyls which have no counterpart in trypsin may also be near the catalytic center.

We have previously shown, using inactivation with ECD, that bovine thrombin has a critical carboxyl group with a pK_a of 5.5 [2]. We report herein similar data for human thrombin, plus stoichiometry of inactivation of the human enzyme by [^{14}C]EAC. In addition, we show that while both human and bovine thrombin show significant protection against inactivation in the presence of DAPA, a potent competitive inhibitor, the DAPA-saturated enzymes are still inactivated by EDC at a significant rate. This suggests that the essential carboxyl group modified by water-soluble carbodiimides may be one other than Asp-189. A preliminary account of some of this work has appeared [7].

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Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EAC, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carboiimide iodide; DAPA, dansyl-L-arginine *N*-(3-ethyl-1,5-pentanediyl)-amide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; S-2238, tosyl-D-phenylalanyl-D-pipecolyl-L-arginine-*p*-nitroanilide; *k'*, the pseudo-first order rate constant for inactivation of thrombin by ECD or EAC under a given set of conditions; K_{50}, the DAPA concentration at which thrombin is half-saturated

Additional footnote: Thrombin residues are numbered using the numbering system for bovine chymotrypsin.

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2. MATERIALS AND METHODS

Human thrombin was a generous gift from Dr F.C. Church. It had been prepared by the method of Church and Whinna [8] and was stored frozen in 20 mM Hepes, 750 mM NaCl, 3 mM NaN_3, 0.1% (w/v) PEG, pH 6.5, until use. [^{14}C]CH_3I (50 mCi/mmol) and Ecolume scintillation fluid were purchased from ICN Radiochemicals, Irvine, CA. EDC and buffer salts were obtained from Sigma Chemical Company, St. Louis, MO, while S-2238, a product of Kabi Vitrum, Stockholm, Sweden, was purchased from Helena Laboratories, Beaumont, TX. DAPA was a gift from Dr S.T. Olson. EAC ([^{14}C]EAC, 2–2.5 mCi/mmol) was synthesized from EDC and CH_3I ([^{14}C]-CH_3I) as described elsewhere [9].

Thrombin activity was measured as previously described [2], except that the final concentration of S-2238 in the 1.00 ml assay solution was 50 μM instead of 100 μM. The concentration of human thrombin was determined at neutral pH from A_{280} = 1.75 for a 1 mg/ml solution and a molecular mass of 36 600 Da [10]. Chemical modifications of thrombin, where the activity was followed as a function of time, were carried out at 25°C as described in the figure legends. The activity is expressed as V, the activity of the modified enzyme, divided by V_c, the activity of the appropriate control, multiplied by 100. The activities of the controls were stable for the duration of the experiments at pH 5.9 and above, but were found to decrease with time at lower pH values.

For the correlation of inactivation of human thrombin with incorporation of [^{14}C]EAC, the enzyme (0.6 mg/ml) was incubated at 25°C with 4 mM [^{14}C]EAC, in 75 mM Mes, 25 mM Hepes, pH 6.0, for 25, 45, or 85 min before a 1.0 ml aliquot was passed over a Biogel P-6 column (0.9 × 38 cm) equilibrated with 25 mM sodium phosphate, 100 mM NaCl, pH 6.0, to separate the modified thrombin from the free [^{14}C]EAC. A control without carbodiimide was subjected to the same procedure and used for subsequent analyses. The enzyme solutions from the column were immediately diluted and assayed six times for enzyme activity, twice each for three separate dilutions, and triplicate 200 μl aliquots of each modified thrombin solution were added to 5 ml Ecolume scintillation fluid and counted on a Beckman LS-100C scintillation counter. Finally, the UV-visible spectrum of each thrombin solution was run on a Perkin-Elmer Lambda 6 spectrophotometer to determine the enzyme concentration.

3. RESULTS AND DISCUSSION

It has previously been shown [2] that bovine thrombin is inactivated in a second order process by both EDC and EAC. Human thrombin shows similar inactivation kinetics. In 50 mM MES, pH 6.0, at 25°C, inactivation of the human enzyme by 5, 10, 20, or 40 mM EDC is characterized by half-times for inactivation of 69, 36, 23, and 9.2 min, respectively (data not shown). When modification was carried out with 5, 10, 20, or 40 mM EAC under otherwise identical conditions, the half-times for inactivation were found to be 25, 13, 5.1, and 3.6 min, respectively (data not shown).

Fig.1 shows the effect of pH on k', the pseudo-first-order rate constant for inactivation of human thrombin by 20 mM ECD. The plot strongly suggests that inactivation is critically dependent on a protonated carboxyl group with a pK_a of 5.6, in excellent agreement with earlier data [2] which suggest that the essential carboxyl in bovine thrombin modified by EDC has a pK_a of 5.5.

DAPA, a blocked arginine derivative, is a potent competitive inhibitor of thrombin. At pH 8, it has a kinetic K_i of approximately 0.1 μM [11], although it may have a lower affinity for thrombin under more acidic conditions (R.L. Lundblad, personal communication). Since DAPA is a simple arginine derivative, it likely binds to thrombin with its positively charged guanidinium moiety extending into the substrate specificity pocket where it electrostatically interacts with Asp-189.

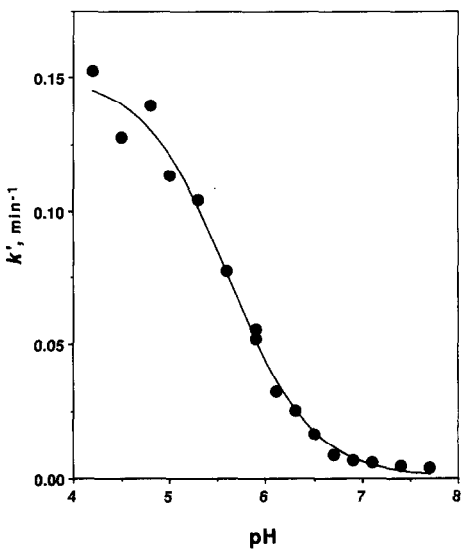

Fig.1. Effect of pH on the rate of inactivation of human thrombin by EDC. Thrombin (60 μg/ml) was treated at 25°C with 20 mM EDC in 75 mM Mes, 25 mM Hepes, at the pH indicated. At various times aliquots were withdrawn and assayed for residual thrombin activity as described in the text. A plot of the logarithm of the residual activity vs time was linear at each pH, and this plot was used to determine k', the pseudo-first-order rate constant for inactivation. The figure shows the relationship between k' and pH. The data points are given in closed circles, while the solid line represents the results of a least-square analysis. The best fit to the data is given by a pK_a of 5.6, k'_{min} = 0 min^{-1}, and k'_{max} = 0.151 min^{-1}.

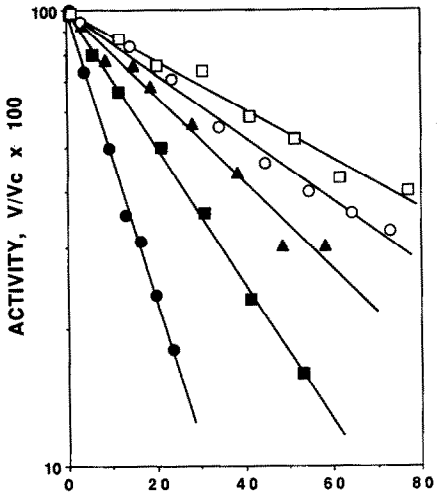

Fig.2. Effect of DAPA on the rate of inactivation of human thrombin by EDC. Thrombin (60 μg/ml) was treated at 25°C with 40 mM EDC in 75 mM Mes, 25 mM Hepes, pH 6.0, in the presence of the following concentrations of DAPA: (□) 44 μM; (○) 15 μM; (▲) 4.4 μM; (■) 1.5 μM; or (●) none. At various times aliquots were assayed for residual thrombin activity as described in the text, and the values of k', the pseudo-first-order rate constant for inactivation, were determined from these data. Separate controls were run for each modification to compensate for the potent inhibitory effect of even low concentrations of DAPA in the thrombin assay.

The rate of inactivation of human thrombin by 40 mM EDC at pH 6.0 is reduced in the presence of DAPA (fig.2), with increasing protection as the concentration of inhibitor is increased. However, it appears that even when the enzyme approaches saturation with DAPA, inactivation proceeds at a significant rate. For example, the pseudo-first order rate constant for inactivation by 40 mM EDC at pH 6.0 and 25°C in the presence of 1.5 μM DAPA is only 47% of that in the absence of DAPA, but this value is decreased only to 17% of the control value when [DAPA] is increased 30-fold.

Fig.3A shows a Dixon plot ($1/k'_{obs}$ vs [DAPA]) of the data derived from the experiment shown in fig.2. If the binding of DAPA provided complete protection against inactivation, the plot in fig.3A would be linear. It is obvious that thrombin saturated with DAPA is still inactivated at a significant rate. When the data shown in fig.3A are corrected for k'_{lim}, the rate of inactivation when the enzyme is saturated with DAPA, the data shown in

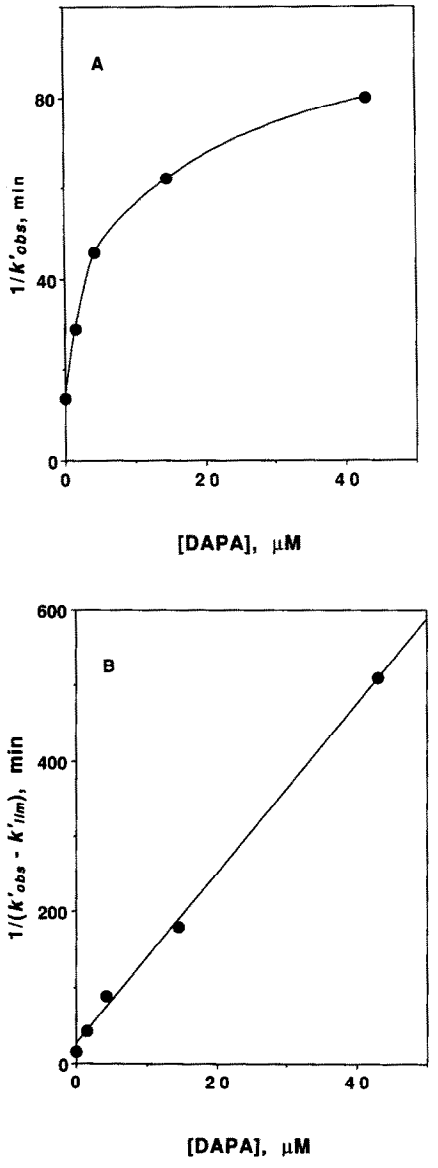

Fig.3. Dixon plots of the data derived from the experiment shown in fig.2. (A) The plot of $1/k'_{obs}$ vs [DAPA] is nonlinear, suggesting that even when thrombin is saturated with DAPA, inactivation by EDC proceeds at a significant rate. (B) The k'_{obs} at each concentration of DAPA was corrected for k'_{lim}, the pseudo-first-order rate constant for inactivation by EDC when thrombin is saturated with DAPA. k'_{lim} was determined by least-square analysis to give the best linear fit to the modified Dixon plot $[1/(k'_{obs}-k'_{lim})$ vs [DAPA]]. The best fit straight line, given for $k'_{lim} = 0.0105$ min^{-1}, was determined to be: $y = 11.3x + 24.7$, from which K_{50} for DAPA was calculated to be 2.19 μM.

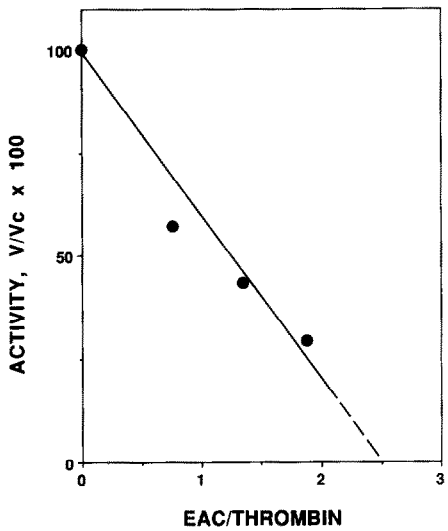

Fig.4. Correlation of inactivation of human thrombin with incorporation of [^{14}C]EAC. See text for details.

fig.3B are obtained. The best value of k'_{lim} was determined to be 0.0105 min^{-1}, or 21% of the estimated k' for the free enzyme in the absence of this competitive inhibitor. K_{50} for DAPA was estimated to be 2.2 μM from the data in fig.3B. Re-examination of data previously reported for the inactivation of bovine thrombin by EDC in the presence of DAPA (fig.4 in [2]) shows that this enzyme behaves in a very similar fashion. The k'_{lim} for the bovine enzyme was estimated to be 18% of estimated k' in the absence of DAPA, while K_{50} was calculated to be 3.0 μM (data not shown). A possible explanation of these data is that if indeed DAPA binds to thrombin to shield Asp-189, the carboxyl group modified by water-soluble carbodiimides is one other than Asp-189. Likely candidates might be Glu-192, Glu-97, and Asp-221, which are not carboxyl residues in trypsin and which have been proposed to form a cluster of 3 negative charges around the catalytic triad of thrombin [10]. Other interpretations of the data shown in figs 2 and 3 are possible.

In the absence of added amine nucleophiles, the most likely end product of carboxyl modification by a carbodiimide is an *N*-acylurea [9,12,13]. Indeed it has been found that amine nucleophiles are not necessary for the inactivation of bovine thrombin by EDC, and in fact significantly reduce the rate of inactivation [2]. When the loss of human thrombin activity is correlated with the stoichiometry of [^{14}C]EAC incorporation, full inactivation corresponds to the modification of approximately 2.5–3 carboxyls per enzyme molecule (fig.4). Human α-thrombin has 35 carboxyl groups per molecule [5,14], so the selectivity of carboxyl modification by EAC is quite high. Further work needs to be done to determine which carboxyl groups are modified by EAC to decide the role of the essential carboxyl group(s) in thrombin catalytic activity.

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