INACTIVATION OF TRYPSINOGEN BY METHANE SULFONYL FLUORIDE

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1. Introduction

It has been recently reported that several zymogens, including pepsinogen [1], procarboxypeptidase A and B [2–5], trypsinogen [6–8] and chymotrypsinogen [8,9] are catalytically active toward substrates of the corresponding enzymes. The zymogens of mammalian serine proteases, trypsinogen and chymotrypsinogen, react stoichiometrically with DFP [7–9] and hydrolyze pseudosubstrates 4 to 6 orders of magnitude more slowly than do the active enzymes. Evidence has been provided that the suppressed catalytic effectiveness of these zymogens is due primarily to a distorted substrate binding site and only secondarily due to an impaired catalytic apparatus [9,10].

In the present study, the relative reactivities of trypsinogen and trypsin toward methane sulfonyl fluoride (MSF) have been compared and found to be more closely related than those of the previously studied pseudosubstrates. Methane sulfonyl fluoride was chosen for the following reasons: a) Like other sulfonyl fluorides, MSF reacts preferentially with the serine residue of the active site of serine proteases [11]; b) Because the reaction is irreversible, measurements of reaction rates are not influenced by the reaction products; c) Since MSF reacts with trypsin and chymotrypsin at nearly the same rates [11, 12] it may be assumed that interactions related to the substrate specificity are minimal and that the relative rates of reactions with zymogen and enzyme are indicative of the intrinsic reactivities of the serine residues of the active site.

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2. Materials and methods

Preliminary to kinetic studies, the spontaneous hydrolysis of MSF (Eastman) in solutions of alkaline pH (pH 7–8) was determined. The pH was maintained constant by adding NaOH (0.1 N–1.0 N) to a closed, jacketed vessel of a pH stat (Radiometer TTT-1) maintained at 25°C. Dimethyl formamide (10–20% v/v) was included to increase the solubility of MSF. Hydrolysis followed pseudo-first order kinetics.

Reactions of bovine trypsin and trypsinogen (Worthington) with MSF were similarly carried out as follows: The proteins were dissolved in 1 mM HCl and allowed to react with MSF at pH 7.5–7.6. The reaction mixture (5.0 ml) contained 2.1–7.0 mg protein per ml, 15–20% (v/v) dimethylformamide, 0.25 M KCl and up to 1.15 M MSF which was added last. At selected time intervals 0.2 ml aliquots were removed and diluted 8-fold with 0.1 M sodium formate (pH 3.5) in order to terminate the reaction. Residual trypsinogen was determined by converting it to trypsin with an acid protease from *Aspergillus oryzae* [13]. Trypsin was assayed with the ester substrate *N*-α-benzoyl-L-arginine ethyl ester as previously described [14,9].

The progress of inactivation of trypsinogen and trypsin by MSF was analyzed in terms of pseudo-first order kinetics using an integrated rate equation which corrects for the spontaneous hydrolysis of the reagent and for progressive dilution during titration in the pH stat [8].

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3. Results

Preliminary experiments on the inactivation of trypsinogen by MSF in the presence of 0.05 M $CaCl_2$ indicated deviations from pseudo-first order kinetics which could be avoided by the omission of calcium. In the absence of calcium, and in the presence of 0.1 M NaF, pseudo-first order kinetics was observed up to 70% inactivation of trypsinogen (fig. 1).

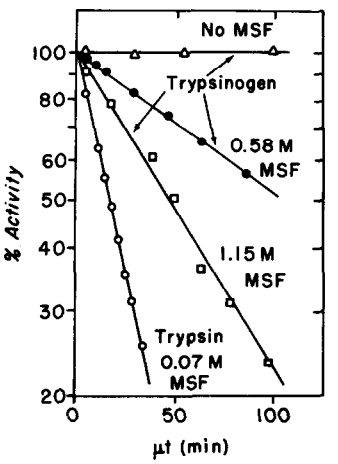

Fig. 1. Pseudo-first order plots of inactivation of trypsin (5.0 mg/ml) and trypsinogen (6.8 mg/ml) by MSF at 25°C in 20% dimethylformamide, 0.1 M NaF, 0.25 M KCl (pH 7.6).

Calcium ions had no untoward effect on the kinetics of reaction of trypsin with MSF. Control experiments in the absence of MSF demonstrated that trypsinogen was not spontaneously activated and that the zymogen was stable under conditions of inactivation. MSF reacts approximately 40–50 times faster with trypsin than with trypsinogen (table 1). In the presence of 2 M urea, the rate of inactivation of trypsinogen by MSF is retarded by a factor of three (table 1).

4. Discussion

The principal conclusion to be derived from the present investigation is that MSF reacts with trypsinogen approximately 50 times slower than with trypsin. In contrast, DFP inactivates trypsinogen over 7000 times slower than trypsin [8], and the rate of acylation of trypsinogen by *p*-nitrophenyl-*p'*-guanidinobenzoate is 7.7×10^6 times slower than that of trypsin [10]. On the other hand, the relative rates of reaction of trypsinogen and trypsin with MSF are comparable to the relative rates of deacylation (70:1) of the *p*-guanidinobenzoyl derivatives of chymotrypsinogen and chymotrypsin [10]. The most obvious explanation of these results is a lack of specificity of binding of the methane sulfonyl group to either the zymogen or the enzyme. The relative rates of inactivation (and of deacylation of acyl

Table 1
Second order rate constants for reaction of methane sulfonyl fluoride with trypsinogen and trypsin*

	k_2 M^{-1}, min^{-1}		Rate Ratio
Conditions	Trypsin	Trypsinogen	Trypsin/Trypsinogen
pH 7.6	0.70	0.017	41
pH 7.6, 0.1 M NaF	0.58	0.012	48
pH 7.5, 0.05 M $CaCl_2$	0.64	(0.041)**	–
pH 7.2, 0.015 M $CaCl_2$	0.75***	nd	–
pH 7.6, 2 M urea	nd	0.0061	–

* 2.1–7.0 mg protein/ml, 0.02–1.15 M MSF, 15–20% dimethylformamide and 0.25 M KCl.
** Steady state rate. This was preceded by an initial phase (about 1 hr) of relatively slower zymogen inactivation.
*** Fahrney and Gold [11].

chymotrypsinogen and acyl chymotrypsin) thus express the relative reactivities of the serine residues of the active site (Ser 195). The much greater difference in rates of reaction of DFP with chymotrypsin and chymotrypsinogen arises in part from the contribution of the diisopropyl groups to the binding, by interacting with hydrophobic portions of the binding site of the enzyme. These conclusions are consistent with the known propensity of the substituted sulfonyl halides to react with the serine residue of the active site of serine proteases [15–18] and with electron density maps of chymotrypsinogen [19], chymotrypsin [20] and *p*-toluenesulfonyl-chymotrypsin [17] which demonstrate that the site of tosylation of chymotrypsin is Ser 195 and that the 'tosyl pocket' is imperfectly developed in the zymogen.

The reason for the anomalous rate behavior of the reaction of trypsinogen with MSF in the presence of calcium ions is unknown. A product of the hydrolysis of MSF is the fluoride ion which is known to associate tightly with calcium ions. Trypsinogen binds one more calcium ion per molecule than does trypsin [21], and it is possible that these two phenomena are interrelated. In any event, the kinetic anomalies could be avoided by omitting calcium ions (table 1). The retardation of the reaction of trypsinogen with MSF by 2 M urea is consistent with the observation of Radhakrishnan et al. [22] that 2 M urea markedly inhibits the activation of trypsinogen, probably due to a perturbation of the native protein structure. It is likely that the same explanation applies to the present findings since the unique reactivity of Ser 195 is dependent on the structural integrity of the active site.

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