Activation of Bovine Factor X (Stuart Factor)—Analogy with Pancreatic Zymogen–Enzyme Systems†

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ABSTRACT: The activation of bovine coagulation factor X has been studied by kinetic and spectrophotometric measurements. The pH dependence of the hydrolysis of specific ester substrates by activated factor X_a can be ascribed to two independently ionizing groups with pK_a values of 6.9 and 8.8, respectively. The rates of reaction of factor X, before and after activation, with the active-site titrant methanesulfonyl fluoride, suggest that the reactivity of the active-site serine residue in factor X is similar to that in trypsinogen and in factor X_a similar to that in trypsin. Analogous comparisons using diisopropyl phosphofluoridate as the titrant suggest that a hydrophobic binding site is absent in both the enzyme and zymogen. This conclusion is consistent with the lack of change in circular dichroism when acyl derivatives of factor X are converted to their acyl enzyme counterparts.

Factor X is the zymogen of a serine protease, factor X_a, which participates in the middle stage of blood coagulation where the intrinsic and extrinsic pathways converge (Davie and Fujikawa, 1975). Factor X can be isolated from bovine plasma in two forms, X_1 and X_2 (Jackson and Hanahan, 1968). Both are glycoproteins with molecular weights of 55 000, each comprising two polypeptide chains (heavy and light) linked by a single disulfide bond (Fujikawa et al., 1972a). The two forms appear identical in terms of amino acid composition after acid hydrolysis but may differ in the number of γ-carboxylglutamyl residues in the amino-terminal portion of the molecules (Neal et al., 1976). Factor X is activated by factor IX_a, factor VIII, calcium, and phospholipid in the intrinsic pathway or by tissue factor and factor VII_a in the extrinsic pathway (Davie and Fujikawa, 1975). It is also activated in vitro by other proteolytic enzymes such as trypsin and a protease from Russell's viper venom (Fujikawa et al., 1972b).

The activation by the protease from Russell's viper venom has been shown to result from cleavage of a glycopeptide from the amino terminus of the heavy chain (Fujikawa et al., 1972b). Other proteolytic enzymes release in addition a smaller glycopeptide from the carboxyl terminus of the heavy chain (Fujikawa et al., 1974). The heavy chain of factor X_a is homologous to the pancreatic serine proteases, showing, for example, 40% sequence identity with bovine trypsin (Titani et al., 1975). The activation peptide is, however, far larger than that of trypsinogen. The light chain of factor X binds Ca^{2+} and phospholipid and has no counterpart in the pancreatic serine proteases.

The discovery of low intrinsic catalytic activity in trypsinogen and chymotrypsinogen (Morgan et al., 1972) has provided a useful tool for the study of the activation of these zymogens. Considerable information regarding the activation process has been obtained from the interaction of trypsinogen and chymotrypsinogen with specific active-site reagents known to react with the corresponding enzymes (Morgan et al., 1972; Gertler et al., 1974; Kerr et al., 1975, 1976). We have therefore extended these studies to factors X and X_a in order to elucidate the molecular rearrangements which take place following cleavage of the activation peptide in this more complex regulatory zymogen–enzyme system.

Materials and Methods

Diisopropyl phosphofluoridate (DFP)[1] was obtained from Pierce Chemicals and [^{14}C]DFP from New England Nuclear Corp. Tosylarginine methyl ester (TosArgOMe) and benzoylarginine ethyl ester (BzArgOEt) were obtained from Cyclo Chemicals.

Bovine factors X_1 and X_2 were isolated from plasma by Dr. K. Fujikawa (Fujikawa et al., 1972a). Both factors X_1 and X_2 were used interchangeably in these studies. In no case was any difference in activity of the enzymes or activatability of the zymogens observed. Both are referred to throughout the work as factor X. A molecular weight of 55 000 and an extinction coefficient of 12.4 were assumed. Factor X_a was prepared by incubation of factor X with a protease from Russell's viper venom (Fujikawa et al., 1972b) and assayed spectrophotometrically by following the hydrolysis of BzArgOEt (0.5 mM) or TosArgOMe (1 mM) in 0.2 M Tris-HCl buffer, pH 8.0, at 253 and 247 nm, respectively (Schwert and Takenaka, 1955).

Inactivation of factors X and X_a by DFP and MSF were carried out essentially as described by Morgan et al. (1972, 1974), except that calcium was omitted from the incubation since calcium fluoride, a product of the reaction, precipitated and appeared to remove proteins from solution. Factor X or X_a (1.5 mg in 3.0 mL, 2 mM Tris-HCl buffer, pH 7.8) was treated with [^{14}C]DFP (5–50 mM) in a jacketed vessel at 25 °C, using a Radiometer TTT-1 autotitrator to maintain the pH at 7.8 by the addition of 0.1 N NaOH. Factor X or X_a was exposed to MSF in a similar manner, except that the pH was maintained by the addition of 1.0 N NaOH, and 10% (v/v) dimethylformamide was included to increase the solubility of MSF. In all cases, aliquots (0.2 mL) were removed at selected

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[1] Abbreviations used are: TosArgOMe, tosylarginine methyl ester; BzArgOEt, benzoylarginine ethyl ester; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl phosphofluoridate; DIP, diisopropylphosphoryl; MSF, methanesulfonyl fluoride; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; GB, *p*-guanidinobenzoyl; MUGB, methylumbelliferyl-*p'*-guanidinobenzoate.

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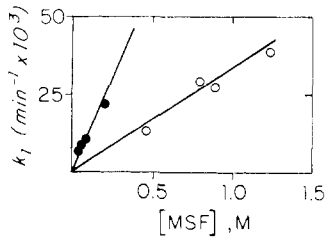

FIGURE 1: Effect of MSF concentration on first-order rates of reaction with factor X (○) and factor X_a (●) at pH 7.8, 25 °C.

time intervals, and excess reagent was removed by gel filtration on columns of Sephadex G-25 equilibrated with 0.01 M Tris-HCl at pH 8.0.

The progress of inactivation by DFP and MSF was analyzed in terms of first-order kinetics using an integrated rate equation which corrects for the spontaneous hydrolysis of the reagent and for the progressive dilution during titration in the pH stat (Morgan et al., 1976).

The reaction of factor X_a with NPGB and MUGB was monitored as described previously for chymotrypsin and chymotrypsinogen using 0.1 M Pipes buffer, pH 7.4 (Gertler et al., 1974). Guanidinobenzoyl-factor X_a was prepared by a method analogous to that for guanidinobenzoyl-trypsin (Kerr et al., 1975), except that the Sephadex G-25 column used for removing excess reagent was equilibrated with 0.01 M Tris-HCl, pH 8.0. Circular dichroic spectra were obtained using a Cary 60 spectropolarimeter with a circular dichroism attachment. Sequenator analysis of DIP-factor X was carried out by the method of Hermodson et al. (1972) and cyanogen bromide digestion as described by Titani et al. (1972). Na-DodSO$_4$ gel electrophoresis was performed as described by Laemmli (1970).

Results

Inactivation of Factors X and X_a by Site-Specific Reagents

Methanesulfonyl Fluoride (MSF). Factor X_a, like trypsin, is inhibited by phenylmethanesulfonyl fluoride (Fujikawa et al., 1972b). Methanesulfonyl fluoride is an analogue which lacks the aromatic ring and, therefore, much of the potential for interaction with the hydrophobic binding pocket of the pancreatic serine proteases. The rate of reaction of serine proteases with this reagent has been suggested to give an indication of the reactivity (nucleophilicity) of the active-site serine residues (Morgan et al., 1974).

Methanesulfonyl fluoride inactivates factor X_a and inhibits the potential activity of factor X. In both cases, first-order kinetics were observed after correction for the hydrolysis of the reagent and its dilution during incubation in the pH stat. The relative rates of inhibition of the zymogen and enzyme are shown in Figure 1. Second-order rate constants of 0.03 and 0.19 min^{-1} M^{-1} were obtained for zymogen and enzyme (Table I) which compare with values of 0.02 and 0.70 min^{-1} M^{-1} for trypsinogen and trypsin under similar conditions (Morgan et al., 1974).

Diisopropyl Phosphofluoridate (DFP). The inhibition of the esterase activity of factor X_a by DFP follows first-order kinetics with respect to enzyme (Figure 2). There was a direct correlation between inhibition and incorporation of [^{14}C]DFP into the protein. The enzymatic activity of the zymogen (factor X) toward BzArgOEt is also inhibited by DFP. The reaction again follows first-order kinetics, but higher concentrations of DFP and the longer incubation times required for inhibition result in the slow incorporation of [^{14}C]DFP into additional

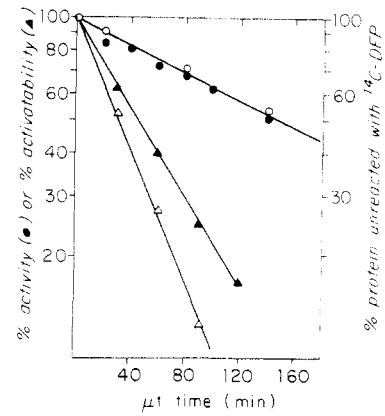

FIGURE 2: The inhibition of esterase activity of factor X_a (●) and incorporation of ^{14}C (○) on incubation with [^{14}C]DFP (3.3. mM) at pH 7.8, 25 °C. The decrease in activatability (▲) and incorporation of ^{14}C (△) upon incubation with [^{14}C]DFP (54.2 mM) at pH 7.8, 25 °C. The abscissa is expressed in minutes but includes a correction factor (μ); see Materials and Methods.

parts of the molecule. This is probably due to the alkyl phosphorylation of tyrosyl residues (Morgan et al., 1972). Incorporation of up to 1.2 molecules of [^{14}C]DIP per molecule were found in completely inhibited factor X.

Analysis of the fully inhibited zymogen by NaDodSO$_4$ gel electrophoresis showed two bands which corresponded to the light chain and the intact heavy chain. No evidence of the smaller heavy chain of factor X_a was seen. Sequenator analysis confirmed the presence of the intact chains of the zymogen with no evidence of the Ile-Val-Gly-Gly- sequence of the enzyme. Chromatography of a cyanogen bromide digest of S-pyridylethylated [^{14}C]DIP-factor X showed most of the radioactivity (80%) to be associated with the peptide which contains the active-site serine residue (peptide IV of Figure 1 of Titani et al., 1972).

The second-order rate constants for the inhibition of factors X and X_a by DFP at pH 7.8 were 1.10 min^{-1} M^{-1} for the enzyme and 0.130 min^{-1} M^{-1} for the zymogen. Morgan et al. (1972) obtained values of 300 and 0.041 min^{-1} M^{-1} for the inhibition of trypsin and trypsinogen by DFP at pH 7.0. If these reactions show a similar pH dependence to that of chymotrypsinogen with DFP, then the rate constants would be approximately doubled at pH 7.8 (Gertler et al., 1974). The rates for the two zymogens are thus very similar and may reflect a similar reactivity of the serine residues at the active sites of both.

p-Nitrophenylguanidinobenzoate (NPGB) and Methylumbelliferylguanidinobenzoate (MUGB). *p*-Nitrophenylguanidinobenzoate is a colorimetric active-site titrant for trypsin (Chase and Shaw, 1969), and MUGB is a spectrofluorometric titrant (Jameson et al., 1973). Both react with factor X_a (Smith, 1973; Jameson et al., 1973), the reaction proceeding via the formation of an acyl-enzyme, guanidinobenzoyl-factor X_a (GBX_a):

$$NPGB + X_a \overset{K_s}{\rightleftharpoons} X_a \cdot NPGB \xrightarrow{k_2} GBX_a \xrightarrow{k_3} GBOH + X_a$$

Reaction of both titrants with factor X_a is rapid. The second-order acylation rate constant $k_{11}(k_2/K_s)$ for the reaction of factor X_a with MUGB was determined to be 1×10^4 min^{-1} M^{-1} which compares with that for the reaction of factor X_a with NPGB of 3×10^3 min^{-1} M^{-1} (Smith, 1973). Both rates

TABLE I: Reaction of Serine Proteases and Their Zymogens with Active-Site-Specific Reagents.

	Second-order rate constants (min^{-1} M^{-1}) 25 °C				
	DFP		MSF		NPGB
	Enzyme	Zymogen	Enzyme	Zymogen	Enzyme
Factor X	1.1[a]	0.13[a]	0.2[a]	0.03[a]	3×10^{3}[b]
Chymotrypsin	2700[c]	0.15[c]	1.3[d]	Nd	$>10^{4}$[e]
Trypsin	300[c]	0.04[c]	0.7[f]	0.02[f]	2×10^{8}[e]

[a] pH 7.8; This work. [b] pH 7.4; Smith (1973). [c] pH 7.0; Morgan et al. (1972). [d] pH 7.0; Fahrney and Gold (1963). [e] pH 7.4; Kerr et al. (1976). [f] pH 7.6; Morgan et al. (1974).

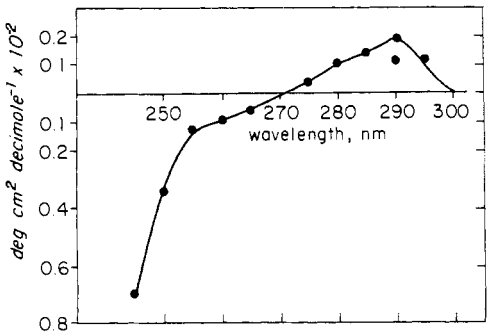

FIGURE 3: Circular dichroic spectra of factors X (solid line) and X_a (●), 1.0 mg/mL in 0.1 M Tris-HCl, pH 8.0. The spectrum of the acyl derivative guanidinobenzoyl-factor X_a was identical with that shown.

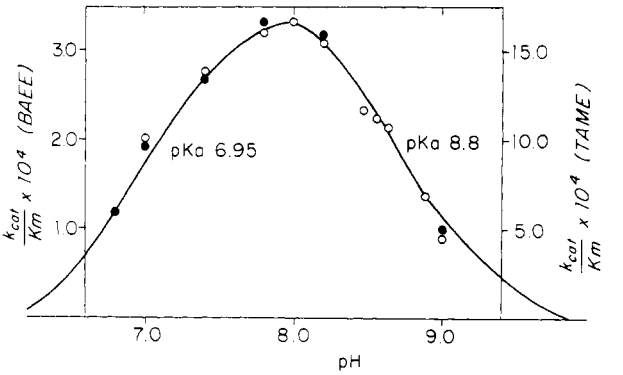

FIGURE 4: The effect of pH on esterase activity of factor X_a toward TosArgOMe (○) and BzArgOEt (●). The solid line is the theoretical curve for two independently ionizing groups with pK_a 6.95 and 8.8.

are, however, considerably slower than those for the trypsin, $>10^6$ min^{-1} M^{-1} (Kerr et al., 1975). The deacylation rates (k_3) of guanidinobenzoyl-factor X_a and guanidinobenzoyltrypsin at pH 7.4, 25 °C, are comparable, viz., 2.6×10^{-3} (Smith, 1973) and 2.0×10^{-3} min^{-1} (Kerr et al., 1975). Because of this slow deacylation rate, the acyl-enzyme intermediates could be isolated.

It has previously been shown (Kerr et al., 1975) that the circular dichroic spectrum of guanidinobenzoyltrypsin exhibited a large negative ellipticity due to the environment of the guanidinobenzoyl moiety (approximately 220 deg·cm^2·$dmol^{-1}$). No such contribution from the acyl group in GB-factor X_a was observed, the circular dichroic spectra of factor X, factor X_a, and GB-factor X_a being identical (Figure 3).

Hydrolysis of Specific Ester Substrates by Factor X_a—Effect of pH

Factor X_a has been shown to hydrolyze esters of arginine and more slowly those of lysine (Elmore, 1973). The zymogen showed no detectable activity toward these substrates. The effect of pH on the hydrolysis of BzArgOEt and TosArgOMe by factor X_a is shown in Figure 4. The bell-shaped curve is interpreted as evidence of the involvement of two ionizing groups in the catalytic activity of the enzyme. The pK_a calculated from the acidic limb is about 6.9 and that from the basic limb 8.8. The values of k_{cat}/K_m were derived from double-reciprocal plots and, although the adsorption of the substrates precluded accurate determination of the individual components k_{cat} and K_m from the spectrophotometric assay, it was apparent that the increase in activity up to pH 8.0 was due mainly to an increase in k_{cat}, whereas the decrease at higher pH was due to an increase in K_m.

Discussion

The activation of the zymogens of pancreatic serine proteases has been the subject of extensive study by structural, crystallographic, optical, and kinetic techniques. The results can account in part for the difference in catalytic efficiency between zymogens and enzymes (Neurath and Walsh, 1976; Kerr et al., 1976). The present kinetic data suggest strongly that factor X_a functions in a manner analogous to that of the pancreatic serine proteases and that despite the greater molecular complexity of the zymogen the activation process is also analogous.

The pH dependence of ester hydrolysis by factor X_a (with a pK_a of 6.9) is similar to that observed for chymotrypsin and trypsin (Himoe et al., 1967; Bender et al., 1964). This result suggests that a charge-relay system analogous to that in chymotrypsin (Blow et al., 1969) is operative in factor X_a, and structural analyses have located the appropriate three residues in the corresponding loci of the heavy chain (His-93, Asp-138, Ser-233) (Titani et al., 1975).

The rate of reaction of factor X with the specific active-site titrant, MSF (Table I), is comparable with that of trypsinogen, indicating similar reactivities of the serine residues in their charge-relay systems. In neither case does activation of the zymogen result in a large increase in that reactivity. Similar conclusions can be drawn from the rates of reaction of DFP with the zymogens. The rates for the enzymes, however, differ considerably, which may reflect differing reactivities of the serine residues but, more likely, different affinities of the enzymes for DFP. The ninefold greater rate of inhibition of chymotrypsin by DFP, compared with trypsin, has been proposed to be the result of the interaction of the isopropyl groups with the more hydrophobic regions of the binding pocket of chymotrypsin (Morgan et al., 1974). In the present case it is suggested that the substrate-binding site of factor X_a is less hydrophobic than that of either trypsin or chymotrypsin.

The integrity of the substrate-binding site of chymotrypsin is maintained by a salt bridge from the amino-terminal isoleucine residue (Ile-16) to Asp-194 (Matthews et al., 1967). The basic limb of the pH–activity curve of this enzyme is due to deprotonation of the amino group of this isoleucine, dis-

ruption of the salt bridge, and decreased affinity for substrate. The decrease in factor X_a esterase activity at high pH may also be due to a decrease in affinity for the ester substrate. It may therefore be proposed that activation of factor X results from the formation of a substrate-binding site upon generation of the amino-terminal isoleucine residue by peptide bond cleavage.

There is, however, evidence that the substrate-binding site of factor X_a is not completely analogous to that of the pancreatic serine proteases—the so-called tosyl pocket (see Blow, 1971). Differences between the substrate binding sites of trypsin and factor X_a have previously been observed (Lonsdale-Eccles, 1974). Though both preferentially hydrolyze esters and amides of basic amino acids, factor X_a shows greater preference for arginyl bonds as compared with lysyl bonds (Elmore, 1973). In addition, factor X_a shows much greater affinity for acetylarginine esters compared with the more bulky hydrophobic tosyl or benzoyl esters (Adams and Elmore, 1971). The present results extend these data. The similar rates of reaction of DFP with factors X and X_a suggest that if hydrophobic binding sites do exist around the active-site serine residue they are not greatly improved upon zymogen activation. Analogous data with MSF also indicate little change in the catalytic apparatus.

The differences in specificity, and in particular the apparent lack of hydrophobic binding sites in factor X_a, are perhaps surprising in view of its similarity in amino acid sequence to those surrounding the corresponding substrate binding sites of trypsin and chymotrypsin (Titani et al., 1975).

Despite these differences, it is apparent that activation of factor X, like that of trypsinogen, must involve reshaping of the substrate-binding site, and that the catalytic apparatus, i.e., charge-relay system, is largely preformed in the zymogen.

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