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INACTIVATION OF HUMAN BLOOD COAGULATION FACTOR X BY CHEMICAL MODIFICATION OF GAMMA-CARBOXYGLUTAMIC ACID RESIDUES

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The inactivation of human factor X by incubation with a reagent known to chemically modify γ-carboxyglutamic acid to γ-methylene glutamic acid was studied. Incubation of factor X at pH 5.0 with a preincubated formaldehyde/morpholine mixture (0.9 M/1.0 M) resulted in a progressive decrease in factor X coagulant activity. In the presence of calcium (20 mM) the rate of factor X inactivation was decreased ~3-fold. By using [14]C-formaldehyde, modified-factor X (<5% residual activity) was found to contain 7 mols of [14]C per mol of protein. Modified-factor X was not activated by Russell's viper venom in the presence of calcium, suggesting that the loss of coagulant activity was related to the inability of modified-factor X to be activated.

Human blood coagulation factor X (M_r= 59000) is a vitamin K-dependent zymogen which consists of two disulfide linked chains; "heavy chain" (M_r= 42000) and "light chain" (M_r= 17000)(1). The amino terminal region of the light chain contains eleven glutamic acid residues which have been post-translationally modified to γ-carboxyglutamic acid (Gla) residues (1). Factor X is activated during blood coagulation by factor IXa or by factor VIIa/tissue factor (2), by cleavage of the arg51-ile52 peptide bond in the heavy chain of the molecule (3). Factor X is also activated by a protease present in Russell's viper venom (RVV-X)(4,5). In each case, the rate of factor X activation is calcium-dependent which has been related to a calcium-induced conformational change in the protein which accompanies the binding of calcium to the Gla residues (2).

Several methods have been used to investigate the role of Gla residues in calcium binding to vitamin K-dependent blood coagulation factors. Bajaj has

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used a method involving the thermal decarboxylation of lyophilized-prothrombin (6). Morita and Jackson have removed the Gla domain of bovine factor X by limited digestion of the protein with chymotrypsin (7). Recently, a method for the chemical modification of Gla residues to γ-methylene glutamic acid was reported (8). We have applied this approach to the modification of Gla residues in human factor IXa (9). While factor IXa inactivation was associated with the modification of the Gla residues, the protein was found to be otherwise stable under the reaction conditions used (9).

The present investigation was undertaken to determine the effects of the Gla-modification procedure (8) on factor X activity. We were particularly interested in determining if modification of the Gla residues of factor X would prevent activation of the zymogen or primarily affect the activity of activated-factor X (factor Xa). Our results indicate that the loss of factor X coagulant activity associated with chemical modification is due to the inability of factor X to be activated.

EXPERIMENTAL PROCEDURES

Human plasma was purchased from the American Red Cross, Carolinas Region, Charlotte, NC. Factor X deficient-plasma was a gift from the Blood Coagulation Laboratory at North Carolina Memorial Hospital. Coagent^{R} activated-partial thromboplastin time (APTT) reagent was obtained from Lancer. Russell's viper venom (RVV) and morpholine were purchased from Sigma. Formaldehyde (37% w/w) and Handifluor^{R} were purchased from Mallinkrodt. [14]C-Formaldehyde (40-60 mCi/mmole) was purchased from New England Nuclear. N^{α}-p-Tosyl-glycyl-L-prolyl-L-arginine-p-nitroanilide (Chromozyme TH^{R}) was purchased from Boehringer Mannheim. Human factor X was isolated from human plasma essentially as described previously (10,11). Factor X (130 units/mg) appeared homogeneous as judged by SDS-polyacrylamide gel electrophoresis (12). Protein concentrations were determined spectrophotometrically by absorbance at 280 nm using an extinction coefficient value of 11.6 for a 1% solution of factor X (10).

Chemical modification of factor X. Factor X modification was carried out esssentially as described previously (8). Prior to modification, a solution containing 8.0 uM factor X was dialyzed for 12 h against 0.05 M sodium acetate (pH 5.0), 0.5 M NaCl. A formaldehyde (0.9 M), morpholine (1.0 M) mixture was adjusted to pH 5.0 and incubated for 1 hour at 37 °C. An aliquot (89 ul) of the mixture was then added to 1.0 ml of the dialyzed factor X solution and the modification reaction was followed at 25 °C. To determine the extent of factor X modification, [14]C-formaldehyde was added to the formaldehyde/morpholine mixture to obtain a final specific activity of 1 mCi/mmol of formaldehyde. Incorporation of [14]C was determined after desalting using a PD-10 gel filtration column (Pharmacia) equilibrated with 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl. Samples (0.1 ml) from the void volume of the column were mixed with 5 ml of Handifluor^{R} and [14]C quantitated using a Packard scintillation counter.

Measurement of factor X activity. Factor X coagulant activity was measured by determining the activated partial thromboplastin time of samples

removed from the modification reaction solution. Samples (5 ul) were removed at timed intervals and added to 1.0 ml of a solution containing 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl. Seventy-five microliters of the diluted factor X were then added to a fibrometer cup containing factor X deficient plasma (75 ul) and Coagent (75 ul). After incubation at 37 °C for 2 min, 75 ul of 25 mM calcium chloride solution was added and the fibrometer (Fibrosystem) started. The clotting times were converted into % clotting activities from a standard curve relating clotting times to factor X concentration.

Factor X activity was also measured by determining the extent of synthetic substrate (Chromozyme TH) hydrolysis after incubation of samples with unfractionated-RVV. Samples (10 ul) were added to 1.0 ml of a solution containing 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl, 6.25 mM calcium chloride and 3.0 ug/ml RVV. After incubation at 25 ° for either 15 min or 5 hours, samples (0.2 ml) were removed and added to 0.2 ml of 0.3 mM Chromozyme TH. Substrate hydrolysis was terminated after 5 min by the addition of 0.8 ml of 50% acetic acid. The extent of synthetic substrate hydrolysis was determined by measuring the absorbance of the solutions at 400 nm using a Beckman Acta CIII spectrophotometer.

RESULTS

Incubation of factor X with a 10,000-fold molar excess of the formaldehyde/morpholine reagent resulted in a time-dependent loss of factor X activity. The loss of factor X coagulant activity paralleled the loss of factor Xa synthetic substrate activity determined after incubation of factor X with RVV. These results are shown in Fig. 1. Under the conditions described in Experimental Procedures, complete activation of unmodified-factor X by RVV required incubation for one hour. Incubation of samples with RVV for 5 hours resulted in a small increase in the amount of factor Xa activity suggesting that the modified-factor X might be activated at a slower rate than native factor X.

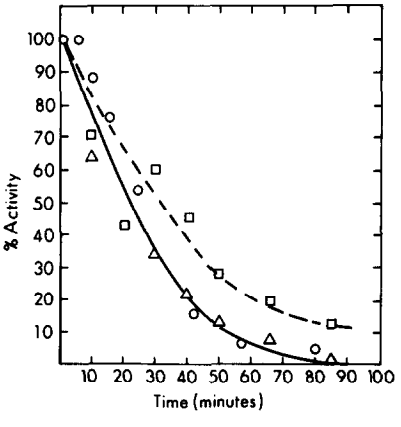
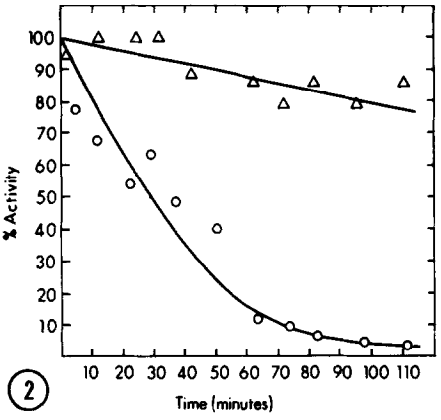


Figure 1. Factor X inactivation by incubation with formaldehyde/morpholine. Factor X was incubated with the formaldehyde/morpholine reagent prepared as described in Expermental Procedures. The reagent:factor X molar ratio was 10,000:1. Factor X coagulant activity (○), factor X amidolytic activity after incubation with RVV/calcium for 15 min (△) and 5 hours (□).



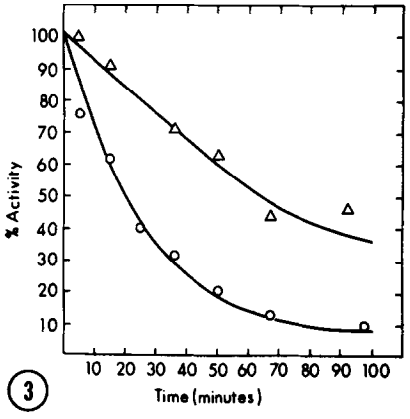


Figure 2. Factor X inactivation by incubation with formaldehyde/morpholine. Factor X was incubated with the formaldehyde/morpholine reagent prepared as described in Experimental Procedures and the % coagulant activity determined as a function of incubation time. The reagent:factor X molar ratio were 10,000:1 (○) and 1,000:1 (△).

Figure 3. Effect of calcium on factor X inactivation. Factor X was incubated with the formaldehyde/morpholine reagent in the absence (○) and presence of 20 mM calium (△) and the % coagulant activity determined as a function of incubation time. The reagent:factor X molar ratio was 10,000:1.

Under otherwise identical conditions, when either formaldehyde or morpholine was substituted for the formaldehyde/morpholine reagent, factor X did not lose activity (not shown). In addition, when the formaldehyde/morpholine concentration was decreased 10-fold the rate of factor X inactivation was also decreased 10-fold as shown in Fig. 2. Finally, the rate of factor X inactivation was decreased approximately 3-fold in the presence of 20 mM calcium as shown in Fig. 3. These observations provided reasonable evidence that the loss of factor X activity was due to the preferential modification of the Gla residues and not to the denaturation of the protein under modification reaction conditions used. Modified-factor X (<5% residual activity) was found to contain 7 mols of [14]-C per mol of protein indicating that over half of Gla residues had been modified.

DISCUSSION

The present study has shown that the incubation of factor X with a reagent previously shown to chemically decarboxylate the Gla residues of vitamin K-dependent proteins (8,9) results in a loss of factor X activity. Our results indicate that the loss of factor X activity is associated with the modification

of 7 Gla residues per mol of protein. Recent studies in our laboratory have suggested that partial decarboxylation of 11 of the 12 Gla residues of factor IXa results in complete inactivation of the enzyme even though a total of only 2 to 3 mols of Gla were modified per mol of factor IXa (9). It seems likely, therefore, that partial modification of the 11 Gla residues of factor X, rather than complete modification of 7 specific residues, is responsible for the loss of factor X activity observed in the present study.

While factor X activation by RVV does not appear to require calcium, the kinetic parameter values for the activation reaction are changed considerably when calcium is present (25-fold increase in k_{cat}, 25-fold decrease in Km) (13). The kinetic parameter values for the activation of Gla-domainless factor X by RVV are not significantly affected by calcium (13), which provides reasonable evidence that the interactions between calcium and the Gla-domain of the protein are linked to the effects of calcium on the kinetics of factor X activation by RVV. The kinetic parameter values for Gla-domainless factor X activation by RVV are significantly different from those observed for the activation of native factor X in the absence of calcium (13). This suggests that the conformation of Gla-domainless factor X, at least in terms of the structural requirements for binding and cleavage by RVV, differs from that of native factor X. If true, the interaction of calcium with the high affinity, Gla-independent site of factor X (14) could also be different in Gla-domainless factor X. Unfortunately, in the preparation of Gla-domainless factor X the only tryptophan residue in the light chain of the protein is removed and the conformational changes associated with calcium binding to the high affinity site, measured by fluorescence quenching, cannot be compared with native factor X.

The present study has shown that chemically decarboxylated-factor X is not activated at a significant rate by incubation with RVV and calcium. This suggests that the Gla-dependent calcium induced conformational changes do not occur in the modified protein. Since the chemical decarboxylation of factor X leaves the protein otherwise intact, further investigation of the effects of calcium on the kinetics of RVV activation of the modified protein and on the

fluorescence quenching associated with calcium binding, may provide additional insight into the relative roles of Gla-dependent and Gla-independent calcium binding sites as they relate to factor X structure and function.

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