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HISTIDINE RESIDUES ARE ESSENTIAL FOR THE SURFACE BINDING AND AUTOACTIVATION OF HUMAN COAGULATION FACTOR XII [1,2]

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The role of histidine residue in the surface binding and autoactivation of human factor XII has been investigated by chemical modification with diethyl pyrocarbonate. It is found that low concentrations of diethyl pyrocarbonate have profound inhibitory effects on the surface binding activity of factor XII. At 2.5-fold molar excess of the reagent, six histidines are modified and 80% of the amidolytic activity is lost. Electrophoretic studies show that the modified protein has lost the capacity to bind to the surface, resulting in diminished proteolytic autoactivation. When modification is performed in the presence of the surface, dextran sulfate, two of the six histidines are protected from modification and the amidolytic activity is completely preserved. It is concluded that histidine residues in factor XII play key role in its surface binding activity.

Human blood coagulation factor XII (Hageman factor) is a single chain 80 kDa plasma glycoprotein. Upon cleavage of a single peptide bond, factor XII is converted to a serine protease, factor XIIa, which initiates the intrinsic pathway of blood coagulation. The enzyme plays a role in the activation of the fibrinolytic system, in the production of kinins, in the initiation of cell-mediated inflammatory responses and in the activation of the classical complement pathway (1-6). *In vitro* activation of factor XII occurs when the zymogen becomes

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The Abbreviations used are: HMWK, High Molecular Weight Kininogen; DEP, ethoxyformic anhydride (diethyl pyrocarbonate); CD, circular dichroism; DS5, dextran sulfate, Mr= 5,000; DS500, dextran sulfate, Mr= 500,000; S-2302, H-D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide dihydrochloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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bound to negatively charged surfaces, such as kaolin, glass, dextran sulfate and sulfatides (7-10). *In vivo*, contact of plasma with anionic components of the subendothelial basement membrane or cell surface may be responsible for the activation of factor XII (11, 12) but the specific component(s) have not been identified. When factor XII is bound to the surface, it acquires enzymatic activity towards its protein substrates, prekallikrein and factor XI (5, 13), which are complexed *in vivo*, with the contact activation procofactor high molecular weight kininogen (HMWK) (14). Plasma kallikrein produced by the activation of prekallikrein by factor XIIa, in turn, cleaves more factor XII to factor XIIa. This reciprocal activation accounts for the rapid and amplified activation of the intrinsic pathway.

In HMWK, a histidine-rich region has been implicated for its surface binding activity (15, 16). Since factor XII also exhibits this activity, chemical modification of the histidine residue was undertaken in order to determine its functional significance and to gain information regarding the mechanism of surface activation of factor XII.

MATERIALS AND METHODS

The chromogenic substrate, S-2302 was purchased from Helena Laboratories (Beaumont, TX). Dextran sulfates (DS5 and DS500), DEP, 2,4,6-trinitrobenzenesulfonic acid (TNBS), N-acetyl-histidine ethyl ester and sigmacote were purchased from Sigma Chemicals (St. Louis, MO). *p*-Hydroxy phenylglyoxal was from Pierce (Rockford, IL). Pure factor XII was purchased from Enzyme Research Laboratories (South Bend, IN). Prior to use, the purity and activity of the purchased factor XII were verified by gel electrophoresis and amidolytic assay and was found to be single band on reduced SDS-PAGE with a specific activity of 60-80 U/mg. Stock factor XII was stored at -70°C. Factor XII concentration was calculated from absorption measurements using $\epsilon_{1\%} = 14.2$ at 280 nm (17). β-Factor XIIa was kindly provided as a gift by Dr. R.Pixley in Dr. R.W.Colman's laboratory. All studies were carried out in acid-cleaned (9:1, H_2SO_4:HNO_3) quartz cuvettes which have been coated with siliconizing agent, sigmacote. Plasticware (pipet tips, micro centrifuge tubes etc.) were coated with 1% solution of polyethylene glycol (PEG 8000). The presence of low concentration of PEG, which might be expected in coated tubes, does not interfere with the factor XII activity. All modification studies were carried out at room temperature in 50 mM NaCl, 2 mM phosphate, pH 7.4 unless otherwise specified. Buffers and salts of analytical grade were from Fisher Scientific (Pittsburgh, PA).

Amidolytic Assay: Amidolytic activity was measured on a 14DS spectrophotometer (AVIV Associates) using the initial rate of hydrolysis of chromogenic substrate S-2302 as previously described (18). In all cases, the increase in absorbance was linear over time.

Gel Electrophoresis: Native-PAGE was performed on a PhastSystem electrophoresis unit (Pharmacia LKB) using precast 12.5% homogeneous polyacrylamide gel (4.5% stacking gel). Samples were applied as described in the PhastSystem Guide (Technique File No. 120) using the eight-toothed, 1 μl sample applicator comb. The "band shift" assay to demonstrate the surface binding activity of factor XII is based on the fact that when factor XII alone is electrophoresed under native condition (Native-PAGE), it migrates in the separation gel with an R_f value of 0.34 ± 0.05. When bound to a negative surface such as DS500, the protein band migrates only a short distance within the stacking gel and does not enter the separation gel. This is due to increase in molecular size because of complex formation (factor XII-DS500). A detailed description of this new technique is published elsewhere (18, 19). Electrophoresis on

reduced SDS gel was performed similarly but on a precast 10-15% gradient polyacrylamide gel (Technique File No. 110) using SDS buffer strips. The samples for the SDS gels were prepared by heating on a boiling water bath for 2 minutes in a sample buffer containing 2% SDS and 2% mercaptoethanol. The gels were silver stained using the automated development unit (Technique File No. 210). The reduced SDS-PAGE analysis monitors the fragmentation of the uncleaved 80 kDa factor XII to the 52 kDa and 28 kDa fragments which correlates with the proteolytic autoactivation process.

Histidine Modification: Modification of the histidine residues by DEP was carried out as follows: Stock solution of factor XII is diluted to 1.25 μM with phosphate buffer, pH 7.4, in a polypropylene microcentrifuge tube. Ninety five μl aliquots of the solution are pipetted into the ultramicro quartz cuvettes in the reference and in the sample compartments of the double beam spectrometer. After scanning from 350 nm to 230 nm to obtain the baseline, 5 μl of absolute ethanol is added to the cuvette in the reference compartment and 5 μl of DEP dissolved in absolute ethanol to the cuvette in the sample compartment. DEP solutions of various concentrations are made so that the desired molar excess over histidine residues is obtained by adding 5 μl to the final mixture. After 30 minutes when excess DEP is decomposed to ethanol and carbon dioxide, 10 μl aliquots are taken from each cuvette for amidolytic assay and the difference spectrum is scanned from 350 to 230 nm. The extent of histidine modification is determined from the difference absorbance at 242 nm using $\Delta\epsilon = 3{,}600\ M^{-1}\ cm^{-1}$ (20). Alternatively, a kinetic scan of the modification reaction is carried out by monitoring the increase in absorbance at 242 nm until the curve reaches a plateau indicating the completion of the reaction. When modification is performed in the presence of dextran sulfate, factor XII is incubated for one minute in the presence of 25 μg/ml dextran sulfate before the addition of DEP. In one minute, the level of factor XII autoactivation is less than 2% so that factor XII is essentially in the zymogen form. All the chemicals and conditions used for the modification experiments were first tested with a model compound, N-acetyl-histidine ethyl ester.

During DEP modification, the possibility of modifying tyrosine residues is excluded by the absence of UV difference spectrum between 290 nm and 250 nm when the ethoxyformylated factor XII is scanned against the unmodified control (data not shown). Quantitation of the number of free amino groups according to the method of George and Borders (21) gave the same number of modifiable amino groups for both control and histidine-modified factor XII (data not shown). This indicates that no amino groups are modified under the conditions used for the modification of histidines. Similarly it was found that no arginine residues were modified by the DEP treatment when arginines were quantitated by reaction with *p*-hydroxy phenylglyoxal according to published procedure (22). There are no modifiable free sulfhydryl groups in factor XII (23).

Circular Dichroism: The CD measurements were conducted on a Jasco J500C Spectropolarimeter using the ADALAB-PC hardware and "ADAPT" computer software (Interactive Microware, Inc., State College, PA). The machine was routinely calibrated using ammonium-d-camphor-10-sulphonate. The far ultraviolet spectra were measured from 190 nm to 250 nm with a sensitivity of 2 mdeg/cm, a time constant of 4 seconds, a scan speed of 2 cm/min and a wavelength expansion of 5 nm/cm. Sodium chloride was replaced by sodium fluoride in the solvent in order to improve the sensitivity and signal to noise ratio at lower wavelengths. The use of sodium fluoride did not affect the measured functional activities of factor XII.

RESULTS AND DISCUSSION

The histidine residues in factor XII were chemically modified by DEP and it is found that factor XII activity towards the chromogenic substrate, S2302, is very sensitive to this reagent. The results in Figure 1 show that in the presence of 90 μM DEP, 80% of the amidolytic activity

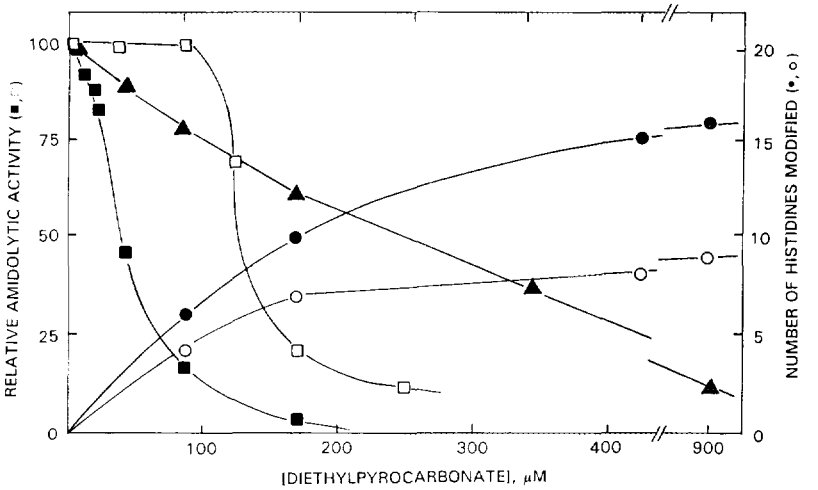

FIGURE 1. Inactivation of Factor XII and β-Factor XIIa by DEP and Quantitation of the Degree of Histidine Modification. The experiments were carried out as a function of DEP concentration in the absence (■,•) and in the presence (□, ○) of DS500. The factor XII and DS500 concentrations used were 1.25 μM and 25 μg/ml, respectively. (▲) is the relative amidolytic activity of β-factor XIIa modified with various concentrations of DEP at a concentration of 1.25 μM.

of factor XII is lost and 6 ± 0.5 histidine residues per mole of the protein are ethoxyformylated. Four additional residues are modified at 180 μM DEP which is accompanied by 96% loss of activity. The reactivity levels off between 300 μM to 900 μM indicating that of the 27 histidine residues in factor XII (23), a maximum of only 15 to 16 residues are reactive to the reagent under the experimental conditions used. Although the loss of activity is observed at all DEP concentration tested, the minimum concentration that exhibited the largest effect on the activity of factor XII is obtained at 90 μM DEP, which is about 2.5-fold molar excess of the reagent.

A comparison of the circular dichroism spectra of unmodified factor XII and factor XII with 15 modified histidine residues shows no detectable differences suggesting that the inactivation is not caused by changes in secondary structures (data not shown). However, conformational changes in the region of the random structure cannot be ruled out because random to random structural changes are not detectable in the CD spectra. This is particularly true, in the case of factor XII, because the protein is largely random in structure with very little organized secondary structures such as α-helix and β-sheets (18,24).

When factor XII is modified in the presence of a negatively charged surface, DS500, there is a significant reduction in the degree of modification. In the presence DS500 (open circles), 90 μM DEP modifies only 4.4 ± 0.5 histidines and 180 μM DEP modifies 7.0 ± 0.7 histidines. At high DEP concentration (900 μM), a maximum of 9.0 ± 1.0 histidines are modified. This means that at 90 μM DEP, 1.6 to 1.8 less histidines are modified when the

modification is carried out in presence of DS500. Separate experiments shows that DEP does not react with DS500. The decrease in modification,therefore, is not due to a reduction in the effective concentration of DEP by interaction with DS500. Therefore, the binding of DS500 to factor XII appears to protect two histidine residues from modification at low concentration of the reagent.

The presence of DS500 during DEP modification also protects the activity of factor XII. As shown in Figure 1, at 90 μM DEP about 80% of the activity is lost in the absence of DS500 (closed squares). However, 100% of this activity is preserved when DS500 is present during modification (open squares). In this experiment, factor XII is incubated with DS500 for 60 seconds before the addition of DEP. Our earlier studies (18) showed that within 60 seconds, the amount of activated factor XII (factor XIIa) formed is negligibly small ($<2\%$). Therefore under these experimental conditions, DEP is modifying mostly the zymogen, factor XII, even though DS500 is present. These results suggest that the reactive histidine residues which are protected by bound DS500 are essential for the expression of the surface-mediated activation of factor XII.

It is known that DS500 induces aggregation of factor XII in the DS500 chain (18). Thus, the protection of histidine residues by DS500 could be either due to histidines located at the aggregation site or the surface binding site. In order to distinguish between these two possibilities, factor XII is modified in the presence of the same amount of DS5 because DS5 does not cause aggregation (18). It is found that the degrees of modification and protection in the presence of DS5 are the same as in the case of DS500 (Data not shown). This suggests that the protected histidines must be located at the surface binding site.

The expression of factor XII activity occurs in two steps. First, activation occurs when the zymogen is bound to a negatively charged surface. Once activated, factor XIIa can cleave more factor XII and initiates the autoactivation process. As discussed under METHODS, these two steps can be analyzed separately by native-PAGE and reduced SDS-PAGE analysis to determine which step is critically affected by the modification of histidines. Figure 2A shows the native-PAGE analysis of factor XII and the histidine modified factor XII. It can be seen that one of the effects of histidine modification is increased electrophoretic mobility in the native gels relative to the unmodified protein (Lanes 1 and 2). This is consistent with the increase in negative charge due to the incorporation of ethoxyformyl groups. When the experiment is carried out in the presence of DS500, the unmodified factor XII band remains in the stacking gel due to the formation of factor XII-DS500 complex (Lane 3). Under the same conditions, the histidine-modified factor XII band moves to the separation gel like free factor XII indicating that it bound weakly or not at all to DS500 (Lane 4).

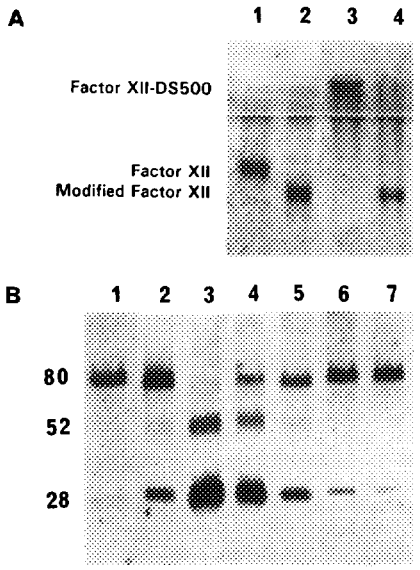

FIGURE 2. Electrophoretic Analysis of the Surface Binding and Proteolytic Activities of Histidine-Modified Factor XII. (A) A "band shift" assay using native-PAGE to visualize factor XII-DS500 binding. Lane 1, factor XII; Lane 2, histidine-modified factor XII; Lane 3, factor XII + DS500; and Lane 4, histidine-modified factor XII + DS500. (B)Reduced SDS-PAGE to demonstrate the effect of increasing histidine modification on the proteolytic fragmentation of factor XII in the presence of DS500. Lane 1 is the factor XII preparation used for this study prior to incubation; Lane 2 is a control in which factor XII is incubated for 60 minutes at 25°C (30 minutes to simulate the incubation time used in the DEP reaction and an additional 30 minutes to simulate the activation time in the presence of DS500). Lane 3 is another control factor XII which is incubated for 30 minutes and an additional 30 minutes in the presence of DS500. Lanes 4-7 are factor XII incubated for 30 minute with 2.5, 5.0, 10, and 25-fold molar excess of DEP, respectively, and then incubated for 30 minutes in the presence of DS500.

Figure 2B shows the reduced SDS-PAGE analysis of the effect of DEP modification on the DS500-induced activation of factor XII. The appearance of 28 kDa fragment in lane 2 represents the degree of proteolytic activation which occur in the absence of DS500[4]. Lane 3 represents the maximum degree of proteolytic fragmentation of factor XII in the presence of DS500 under the experimental conditions used. It is evident that modification using 2.5-fold molar excess DEP (Lane 4) which modified 6 histidines inhibited factor XII autoactivation as indicated by the presence of remaining intact 80 kDa factor XII. In presence of 25-fold molar excess DEP (Lane 7) which modified 15 to 16 histidines, autoactivation is almost completely inhibited.

The possibility that modification of the catalytic histidine residue in factor XII could account for the inactivation observed was investigated by studying the effect of DEP on β-factor XIIa. It is a 28 kDa fragment of factor XII which contains the catalytic site and possesses

[4] We observed in our studies that the 28 kDa fragment of factor XII has stronger stain intensity than intact factor XII or the 52 kDa fragment at the same protein concentration when silver stained.

catalytic activity but lacks the surface binding domain. As shown in Figure 1 (closed triangles), modification of the histidines in β-factor XIIa at 2.5-fold molar excess of DEP could account only for the loss of 20% of the activity. Under similar conditions, intact factor XII lost 80% of the activity. DS500 did not protect any histidines in β-factor XIIa from DEP modification (data not shown). These suggest that the loss of the activity of intact factor XII is not due to the modification of the catalytic histidine residues. Rather, it is due largely to the loss of surface binding affinity because of the modification of histidine residues at the surface binding site. Since surface binding is the first step in the zymogen activation, the proteolytic fragmentation of factor XII is subsequently diminished. The protection of two histidines by DS500 and the diminished surface binding demonstrated by native PAGE strongly support this interpretation. Although these functional properties of factor XII are sensitive to histidine modification, they were not completely eliminated until about ten histidine residues were modified. This suggests that even if only two histidines were protected by DS500, perhaps more histidine residues are involved in the expression of these activities.

It is interesting to note that three peptide segments in factor XII have been implicated at the surface binding site. These are peptide sequences 1-28 (25), 38-57 and 134-153 (26,27). The peptide sequence 1-28 has two neighboring histidine residues at positions 10 and 17, and the peptide sequence 38-57 has two histidines at positions 40 and 44. Although the peptide sequence 134-153 has only one histidine, the complete "Type I" domain (homologous to fibronectin) of which this is a part, also contains two histidines. It is reasonable to speculate that two of these histidines could be the ones protected by DS500 in the present studies. Studies are continuing in this laboratory to identify the specific histidine residues involved in surface binding.

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