Half-of-the-Sites and All-of-the-Sites Reactivity in Human Plasma Blood Coagulation Factor XIIIα*

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The reactivities of human plasma factor XIIIa toward iodoacetone acid and toward α-bromo-4-hydroxy-3-nitroacetophenone have been studied under conditions where this dimeric enzyme reacts with the reagents in half-of-the-sites fashion and under conditions where it reacts with the reagents in all-of-the-sites fashion. Direct measurements of alkylation of active site —SH groups in the apparently identical subunits of the enzyme as functions of remaining catalytic activity are in agreement with the observed reactivities. In addition to extending earlier evidence for half-of-the-sites reactions in factor XIIIa (Chung, S. I., Lewis, M. S., and Folk, J. E. (1974) J. Biol. Chem. 249, 940–950), the present findings suggest that the all-of-the-sites reactivity results from a positively cooperative interaction between enzyme subunits.

Human plasma blood coagulation factor XIIIa, formed from its tetrameric zymogen, factor XIII (a2b2), through limited proteolysis by thrombin, is composed of two apparently identical catalytic α' subunits and two noncatalytic β subunits (for review see Ref. 1). Ca2+, at levels essential for catalytic activity of the enzyme, causes reversible dissociation of the tetrameric enzyme (a'2b2) to active α' chain dimers and β chain dimers. Factor XIII from blood platelets or placental tissue exists as a dimer composed of α chains only. Proteolysis by thrombin leads to formation of dimeric enzyme (α'2), the subunit structure of which is unchanged by Ca2+, but for which this metal ion is essential to the expression of enzymic activity. Although the biological function of these enzymes is not fully understood, their role in catalyzing cross-link formation between protein molecules is believed to be vital to normal blood clotting or to normal wound healing, or to both.

Several years ago, it was reported from our laboratory that total loss in catalytic activity of samples of enzyme generated from a number of preparations of plasma factor XIII and from several preparations of the platelet zymogen is associated with the incorporation of 1 molecule of sulphydryl reagent per molecule of enzyme, i.e. 1 molecule of reagent per dimer or per two catalytic α' chains (2). On the basis of this evidence of inactivation by derivatization of a cysteine residue in only one of the two apparently identical α' subunits, it was concluded that these enzymes exhibit half-of-the-sites reactivity (for reviews see Refs. 3 and 4) with certain reagents under defined experimental conditions (2). The findings of experiments carried out under somewhat different conditions in two other laboratories were reported to be inconsistent with our conclusion (5, 6). For this reason, we have carefully further investigated the inactivation of the plasma enzyme under several conditions. Because this enzyme contains a number of —SH groups in addition to those essential for catalytic activity, evidence is provided that the inactivations described here occur as a consequence of derivatization of active site —SH groups. The present findings support our original conclusion. In addition, it is reported that the enzyme exhibits half-of-the-sites reactivity with one reagent and all-of-the-sites reactivity with another and that both reagents can react with enzyme either in a half-of-the-sites manner or in an all-of-the-sites manner under the appropriate conditions.

EXPERIMENTAL PROCEDURES

Materials—Plasma blood coagulation factor XIII was purified from 96% clottable human fibrinogen that was prepared from outdated acid-citrate dextrose (USP Formula A) plasma. The procedure was as outlined earlier (2, 7). Several preparations of purified factor XIII were used for the present studies. Each of these preparations displayed the characteristic two-band staining pattern after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and each showed the expected change in mobility of catalytic subunits in gel electrophoresis following activation by thrombin (8). The stoichiometry of inactivation under the experimental conditions described was the same for enzyme prepared from each of the factor XIII preparations used.

Purified human thrombin was a gift from Dr. D. L. Aronson (Bureau of Biologics, Food and Drug Administration). [1-14C]Iodoacetamide (57 Ci/mol), [2-3H]Iodoacetic acid (57 Ci/mol), and [2-3H]Iodoacetic acid (82 Ci/mol) were purchased from Amersham Corp. Nonradioactive iodoacetic acid, obtained from Sigma, was recrystallized from benzene-pentane before use. α-Bromo-4-hydroxy-3-nitroacetophenone was prepared by a published procedure (9). Stock solutions of this reagent were made immediately before use in 0.01 M Tris-chloride, pH 7.0, and concentrations were determined from absorbances at 323 nm, using a molar extinction coefficient of 17,000 (10).

Methods—Factor XIII was converted to the active enzyme, factor XIIIa, by limited proteolysis with thrombin as outlined earlier (7). An extinction coefficient of ε275 = 13.8 (8) and a molecular weight of 300,000 (2) were used to determine factor XIII concentration.

Measurement of enzyme activity was carried out by [14C]putrescine incorporation into casein as outlined previously (2, 11) or by a coupled procedure in which ammonia released by the hydrolytic action of the enzyme toward protein-bound glutamine residues was measured. In this latter assay, ammonia formation was monitored by following the concomitant change in absorbance at 340 nm resulting from oxidation of NADH in the α-ketoglutarate to glutamate conversion catalyzed by glutamate dehydrogenase. Assays were conducted at pH 7.0 and 25°C in 0.2 M Tris-chloride buffer containing 0.1 M NaCl, 40 mM CaCl2, 0.1 mM EDTA, 5 mM α-ketoglutarate, 0.4 mM NADH, and 2.5 μg of succinylated β-casein (12) and 120 μg units of glutamate dehydrogenase (Boehringer Mannheim, dialyzed free of phosphate salts against 0.01 M Tris-chloride buffer, pH 8) per ml. Identical results were obtained in inactivation experiments with the use of the two assay procedures.

Peptide maps were prepared by the method of Katz et al. (13).
Radioactivity on maps was detected by autoradiography on Kodak X-2 film. In some cases ninhydrin-positive materials were located by the use of a cadmium-ninhydrin reagent (14). Isolation of radioactively labeled peptides from maps was carried out by extraction with dilute acetic acid. Amino acid compositions were determined by the use of an amino acid analyzer after hydrolysis of samples in 3 N p-toluene sulfonic acid containing 0.2%, 3-(2-aminoethyl)indole (24 h at 110°C) (15). For measurements of radioactivity, extractions were made directly in scintillation counting fluid (Hydromix, Yorktown). Radioactive areas located on maps by autoradiography were excised. The papers containing labeled peptides were shredded into about 2-mm sections, and these were placed in 10 ml of p-toluene sulfonic acid containing 0.2% 3-(2-aminoethyl)indole for enzymic activity, and the protein in the remainder of the sample was freed of reagents by dialysis against water and was taken to dryness by lyophilization. Digestion with α-chymotrypsin (Worthington) was carried out for 12 h at 25°C in water using a 1 to 100 weight ratio of enzyme to labeled protein. A second addition of an equal amount of chymotrypsin was made after 6 h. The pH was maintained at 7.0 ± 0.2 during digestion.

RESULTS

The results shown in Fig. 1 are those for the inactivation of plasma factor XIIIa by [14C]iodoacetic acid at pH 7.0 in the presence and absence of glycerol. Examination of the data indicates that, whereas complete inactivation in the absence of glycerol is associated with incorporation of 1 mol of carboxymethyl group per mol of the two-catalytic subunit enzyme, complete loss in activity in the presence of glycerol requires incorporation of 2 mol of this group per mol of the enzyme.

In Fig. 2 is presented a diagrammatic representation of a composite of the autoradiograms from two peptide maps. One of these was prepared from a chymotrypsin digest of active site [14C]carboxymethyl-labeled factor XIIIa. The other was from a chymotrypsin digest of enzyme that had been labeled at all of its —SH groups by reaction with [14C]-iodoacetic acid in the presence of guanidine. It is evident from the results shown in Fig. 2 that there is a single peptide formed by digestion of active site-labeled enzyme and that this active site peptide is well resolved from all other S-[14C]carboxymethyl peptides. A discrete positive area observed in the position of the active site peptide on a ninhydrin-stained map indicated that this peptide is also separated from unlabeled peptides. Analysis of the labeled active site peptide, following its elution from a map, showed an amino acid composition of carboxymethylcysteine, 1.0, glutamic acid, 0.9, glycine, 1.0, tryptophan, 0.8, in agreement with that reported by Holbrook et al. (5).

Fig. 3 presents the results of experiments in which loss in factor XIIIa enzymatic activity in the presence and absence of glycerol is related directly to the degree of derivatization of the enzyme active site —SH groups. In these experiments samples of enzyme were first inactivated to varying degrees with [14C]-iodoacetic acid. Their remaining —SH groups were then carboxymethylated by reaction with [3H]iodoacetic acid under denaturing conditions. Following chymotrypsin digestion, the active site peptide from each sample was isolated by peptide mapping and its content of 14C- and 3H-labeled carboxymethyl groups measured. The findings given in Fig. 3 clearly show that total inactivation of factor XIIIa by iodoacetic acid at pH 7.0 in the absence of glycerol results from derivatization of the active site —SH group in one of the two catalytic subunits. Further, they show that the total loss in enzymic activity that results from treatment with this reagent in the presence of glycerol is associated with incorporation of
in a manner similar to that outlined in Fig. 1.

The values in parentheses are those for the percentage of initial moles/mol of protein.

Additional evidence that carboxymethylation of the active site —SH group in a single catalytic subunit of factor XIIIa is responsible for loss in enzymic activity in the absence of glycerol was obtained through separation and measurement of $[^3H]$carboxymethyl and $[^14C]$carboxamidomethyl active site peptides. Peptide maps (not shown) were prepared from enzyme protein that was fully alkylated in 7 M guanidine-HCl with $[^3H]$- and with $[^14C]$iodoacetic acid, respectively.

Amounts of $[^14C]$- and $[^3H]$-labeled carboxymethyl group were determined based on the radioactivity measured in active site peptide from a map prepared from enzyme protein that was fully alkylated in 7 M guanidine-HCl with $[^14C]$- and with $[^3H]$iodoacetic acid, respectively.

**TABLE I**

<table>
<thead>
<tr>
<th>Reagent added</th>
<th>Enzymic activity</th>
<th>Remaining active site —SH groups</th>
<th>Theory for $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol/mol of protein</td>
<td>% of initial</td>
<td>mol/mol of protein</td>
<td>All-of-the sites</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>23</td>
<td>0.40 (20)</td>
<td>0.46 (23)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>20</td>
<td>0.15 (7.5)</td>
<td>0.40 (20)</td>
</tr>
</tbody>
</table>

$^a$ Remaining active site —SH groups are defined as those converted to $[^14C]$carboxymethyl derivatives by treatment of the enzyme protein with $[^3H]$-labeled iodoacetic acid as described in the paragraph above.

$^b$ The values in parentheses are those for the percentage of initial active site —SH groups.

Per two catalytic subunits.

**Fig. 3.** Stoichiometry of $[^14C]$carboxymethyl group incorporation and $[^3H]$carboxymethyl group incorporation at the active site —SH groups of factor XIIIa in experiments where $[^3H]$-labeled iodoacetic acid was employed for inactivation and where the remaining protein —SH groups were alkylated with $[^3H]$iodoacetic acid. Inactivations were carried out as outlined in Fig. 1 in the presence of, and in the absence of, glycerol. Reagent-free, partially inactivated enzyme samples were further alkylated in 7 M guanidine-HCl as outlined in Fig. 2, except by the use of $[^3H]$-labeled reagent instead of $[^14C]$-labeled reagent. The active site peptide was prepared and isolated in each case by chymotrypsin digestion and peptide mapping, respectively, as described in Fig. 2. Amounts of $[^3H]$- and $[^14C]$-labeled carboxymethyl group were determined on the basis of radioactivity measured in active site peptide from maps prepared from enzyme protein that was fully alkylated in 7 M guanidine-HCl with $[^3H]$- and with $[^14C]$iodoacetic acid, respectively.

**Fig. 4.** Inactivation of factor XIIIa by $\alpha$-bromo-4-hydroxy-3-nitroacetophenone at pH 6.2 ± 0.05 and pH 7.3 ± 0.1. Inactivations were carried out at 25°C in Tris-acetate buffers containing enzyme from 1 mg of factor XIII per ml, 0.1 mM EDTA, 40 mM CaCl$_2$, and various levels of reagent. Enzymic activity was measured after 120 min. It was observed in trial experiments that the reactions were complete within 20 min. Control samples incubated in the absence of reagent showed no losses in enzymic activity in 120 min.
was measured at two pH levels. At pH 6.2 total loss in enzymic activity results from treatment of the enzyme with 1 mol of reagent per dimer or per two catalytic subunits, in agreement with an earlier report (2). At pH 7.3, however, inactivation appears to require 2 mol of reagent per dimer, i.e. 1 mol per catalytic subunit. In order to determine if the inactivation by α-bromo-4-hydroxy-3-nitroacetophenone at the higher pH is indeed a consequence of reaction of the reagent with active site —SH groups in both of the catalytic subunits of factor XIII, we measured the amount of active site —SH group remaining after reaction. The results of two separate experiments are presented in Table I. In each of these experiments, conditions were adjusted so that the enzyme was largely, but not completely, inactivated by reagent. These conditions were chosen because the differences in expected values for remaining active site —SH groups in half-of-the-sites and in all-of-the-sites reactions become progressively greater with increasing degrees of inactivation. The results given in Table I supply evidence that inactivation of factor XIIIa by α-bromo-4-hydroxy-3-nitroacetophenone at pH 7.3 requires reaction of this reagent with the active site —SH groups in both of the enzyme catalytic subunits, i.e. that inactivation occurs in an all-of-the-sites manner.

**DISCUSSION**

The findings reported here are consistent with, and are strong support for, our earlier contention regarding half-of-the-sites reactivity in factor XIIIa (2). The present experiments examine directly the stoichiometry of active site —SH group alkylation of this two-catalytic subunit enzyme under conditions where the enzyme responds to alkylation reagents in a cooperative (half-of-the-sites) fashion and under conditions where it responds to alkylation in all-of-the-sites, but not necessarily noncooperative, fashion. The results obtained under each condition and with the two alkylation reagents used are in accordance with the observed reactivities.

The present findings supply no information as to whether the observed half-of-the-sites reactivity in plasma factor XIIIa results from nonidentical conformations at the active sites in the otherwise identical catalytic subunits of native enzyme (Fig. 5, IA1) or from ligand-induced conformational changes in identical catalytic subunits that contain identical active sites in the absence of ligand (Fig. 5, IA2). Work in progress, however, suggests that, whatever the basis for this phenomenon, it is not dependent upon the dissociation of catalytic from noncatalytic subunits or upon conformational alterations in dimeric enzyme that occur in the presence of calcium ion, nor is it dependent upon thrombin-catalyzed limited proteolysis that results in formation of enzyme from zymogen. We find that the plasma enzyme in its 4-subunit form in the absence of Ca2+ can be inactivated in a half-of-the-sites manner by iodoacetamide, albeit much more slowly than the enzyme in the presence of this metal ion. We also find that total loss in potential enzymic activity of the plasma zymogen occurs upon treatment with iodoacetamide as a result of derivatization of the active site —SH group in one of its two catalytic subunits. This reaction also proceeds slowly and only in the presence of Ca2+. However, it does not require, or cause, dissociation of catalytic from noncatalytic subunits.

Detailed alkylation and acylation experiments on rabbit muscle apoglycereraldehyde 3-phosphate dehydrogenase have revealed that the reagents used can be divided into two classes, those that react in a half-of-the-sites manner and those that react in an all-of-the-sites manner (16, 17). The half-of-the-sites behavior is believed by the author (16, 17) to be caused by the modifying reagent interacting at the binding site with a region through which conformational changes can be transmitted to neighboring subunits, thus rendering them less active to modifying reagent and enzymically inactive (Fig. 5, IA2). The reagents that behave in an all-of-the-sites fashion are thought to do so because they are unable to transmit to neighboring subunits conformational changes that affect the reactivity or enzymic activity (16, 17). Thus, the latter reagents may react in a noncooperative manner (Fig. 5, IA2).

If the only data available here were those for half-of-the-sites inactivation of factor XIIIa by iodoacetic acid (Figs. 1 and 3, in the absence of glycerol) and for all-of-the-sites inactivation of this enzyme by α-bromo-4-hydroxy-3-nitroacetophenone (Fig. 4, at pH 7.3 and Table I), one might suppose that this enzyme also is inactivated in a noncooperative all-of-the-sites manner by the latter reagent. That is, one might expect that this reagent reacts randomly with active site —SH groups and that dimeric enzyme in which the active site —SH group in only one subunit is derivatized by this reagent is one-half as active enzymically as unmodified dimeric enzyme. Taken together, however, the results presented here are not consistent with this type of conclusion. This becomes evident when one compares the models illustrated in Fig. 5 and bears in mind the important fact that all enzymic activity measurements were conducted using the same assay conditions. Total inactivation of factor XIIIa with iodoacetic acid in the absence of glycerol (Figs. 1 and 3) or with a-bromo-4-hydroxy-3-nitroacetophenone at pH 6.2 (Fig. 4) results from reaction of reagent with the active site —SH group in one subunit of the dimeric enzyme (Fig. 5, IA1 or IA2). Therefore, dimer containing a derivatized active site —SH group in one subunit and a free active site —SH group in the other is catalytically inactive under these assay conditions. If the all-of-the-sites reaction of iodoacetic acid with enzyme in the presence of glycerol and that of α-bromo-4-hydroxy-3-nitroacetophenone with enzyme at pH 7.3 occurred in a noncooperative fashion, the relationships between percentage of initial enzymic activity and remaining free active site —SH groups would be

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1. G. F. Seelig and J. E. Folk, manuscript in preparation.
expected to deviate widely from linear ones. This follows from two facts. a) Equal reactivity of active site—SH groups in the two subunits of dimeric enzyme predicts that when an average of 1 molecule of reagent has reacted covalently with 1 molecule of dimer the molecular composition will be approximately 25% monoderivatized enzyme, 50% monoderivatized enzyme, and 25% dimerized enzyme, as shown in Fig. 5. II. b) It is implicit that monoderivatized enzyme is the only catalytically active form of enzyme under the conditions of assay. Therefore, about 25% of the initial enzymic activity would remain when one-half of the total active site—SH groups had reacted.

The results presented in Fig. 3 and Table 1 do not show this type of relationship and, thus, seem inconsistent with the noncooperative mechanism for all-of-the-sites reactivity. Depicted in model IB of Fig. 5 is what appears to be the simplest mechanism that is in accordance with the data presented here. In this model the modifying reagents under the proper reaction conditions, through interacting at the binding site in one subunit of the dimeric enzyme, transmit conformational changes to the other subunit. The result is to render the second subunit more reactive toward reagent. The data collected and presented here are not extensive enough, or of such precision, to allow the relationships between active site groups reacted and enzymic activity remaining to be defined as linear and, therefore, evidence for extreme positive cooperativity, i.e. $k << k_2$ in Fig. 5. IB. They are, however, indicative of a positively cooperative interaction between enzyme subunits.

It is conceivable, on the other hand, that monoderivatized dimeric enzyme formed under one reaction condition retains one-half of the enzymic activity of native enzyme, whereas that formed with the same reagent at another reaction condition is totally inactive, even though the conditions for measurement of catalytic activity in each case are identical. This alternative seems implausible as an explanation for agreement of the data for inactivation by two reagents of somewhat different chemical structure, under quite different solution conditions, with a noncooperative all-of-the-sites reactivity. However, it cannot be totally eliminated on the basis of the present findings.

Regardless of the origin for this all-of-the-sites reactivity, the fact is that factor XIIIa reacts in this fashion under certain experimental conditions with two reagents. That both of these reagents under different conditions yield catalytically inactive forms of enzyme in which only half of the potentially reactive sites are derivatized is a firm foundation for classification of this enzyme as one that possesses half-of-the-sites reactivity.

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REFERENCES