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Research Articles

Photo-oxidation of histidine as a probe for aminoterminal conformational changes during fibrinogen-fibrin conversion

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Abstract. Fibrinogen is known to become unclottable when irradiated with light in the presence of methylene blue, the loss of clottability being due to photo-oxidation of the histidine at position 16 of the Bβ chain. In the present investigation it could be demonstrated that not only this histidine but also the one at position 24 of the Aα chain was modified and that the rates of modification could be modulated by fibrinopeptide release, polymerization inhibition and denaturation. Accordingly, the histidine modifications can be used as probes for surface accessibility of and conformational differences among the various forms of the protein. Both histidines are shielded by the fibrin polymer structure. Fibrinopeptide A release alone leads to maximal protection of the one in the Aα chain, but only partial protection of the one in the Bβ chain. Subsequent fibrinopeptide B release leads to maximal protection of the one in the Bβ chain. The differential effects indicate that two conformational changes have occurred. Polymerization inhibition reverses the protective effect. Denaturation leads to maximal and equal modification in all samples as a consequence of the loss of native conformation.
Key words. Fibrinogen; histidine modification; photo-oxidation; methylene blue; conformation; surface accessibility.

Fibrinogen is a central protein in the blood coagulation system as it is the precursor of fibrin which forms the scaffold of the blood clot [1, 2]. The fibrinogen molecule is composed of three pairs of nonidentical peptide chains, and the overall structure is therefore $(A\alpha, B\beta, \gamma)_2$. During blood clotting the enzyme thrombin cleaves two pairs of peptide chains, i.e. Aα and Bβ, within their aminoterminal regions, releasing the fibrinopeptides A and B and forming a fibrin monomer with the structure $(\alpha, \beta, \gamma)_2$. The monomer will then polymerize into the insoluble fibrin clot. However, release of fibrinopeptide A alone is sufficient for the induction of clot formation. A selective release of this peptide is obtained with the snake venom enzyme batroxobin. Fibrin polymerization is a highly ordered process which depends on completely specific interactions between at least two sets of complementary affinity sites. Within each set one site is located in the carboxyterminal region of both fibrinogen and fibrin. The complementary sites are located at the aminoterminal ends of the α and β chains, respectively, and only available in fibrin, i.e. after the removal of the corresponding fibrinopeptides [1].

It has been well established that fibrinogen is rendered unclottable when irradiated with visible light in the presence of the dyes rose bengal [3, 4] or methylene blue [4–6]. This type of dye-mediated photo-oxidation has often been shown to affect primarily histidine residues in proteins, though tryptophan, tyrosine and methionine residues may also be modified [3, 4, 7]. In the case of fibrinogen a clear correlation was observed between the oxidative destruction of the histidine residue in position 16 of the Bβ chain and the loss of the ability to polymerize on thrombin treatment [4–6]. Employing fibrin-specific affinity-chromatographic techniques, it could be demonstrated that this special histidine residue had a distinctive role in the end-to-end association during fibrin polymerization [6]. Furthermore, it has been shown that even in blood plasma the coagulability of fibrinogen is lowered by irradiation in the presence of methylene blue [8, 9]. The present study was prompted by the recent observation that when a fibrin clot is formed in the presence of methylene blue in the dark, it will not dissolve when exposed to light, even for a much longer time than is required to cause a corresponding fibrinogen solution to become unclottable [10]. Experiments were then designed to test the hypothesis that differences between fibrinogen and fibrin in conformation and surface accessibility could explain the unequal susceptibility to dye-mediated photo-oxidation. The results indicated that surface exposure as well as additional unidentified factors must influence fibrin clot stability.

Materials and methods

Materials. Human fibrinogen, 97% clottable, was purchased from KABI (Stockholm, Sweden), human

thrombin, 3300 NIH units/mg, from Sigma (St. Louis, MI), batroxobin (as Reptilase-reagent) from Boehringer (Mannheim, Germany) and methylene blue from Merck (Darmstadt, Germany). The peptide Gly-Pro-Arg-Pro was kindly provided by Dr G. R. Matsueda (Princeton University, NJ).

Photo-oxidation of native proteins. Fibrinogen and methylene blue were separately dissolved in 0.05 M Tris-0.1 M sodium chloride, pH 7.4 with HCl. The solutions were mixed in the dark to a final protein concentration of 1.0 mg/ml and dye concentration of 50 μM. Samples of 0.5 ml were transferred to beakers having a diameter of 2 cm, providing liquid layers of 0.16 cm. Thrombin or batroxobin were added to some samples in sufficient concentrations to cause clotting to occur in about 20 s, i.e. corresponding to 2 NIH units of thrombin. The samples were left in the dark for 1 h to insure a complete fibrinopeptide release by the enzyme treatment. All samples were then placed 25 cm below a 60-W incandescent light source and left there for specified periods of time at room temperature. No external supply of oxygen was used. Subsequently, the fibrinogen samples were mixed with 2 units of thrombin, and the batroxobin-clotted samples overlayered with 2 units of thrombin in 0.1 ml of buffer and left in the dark for 1 h.

Photo-oxidation of polymerization-inhibited proteins. The peptide Gly-Pro-Arg-Pro was included in the fibrinogen solution to a final concentration in the samples of 0.01 M, which is sufficient to prevent fibrin polymerization [11]. The samples were otherwise treated as described above.

Photo-oxidation of denatured proteins. The solutions of fibrinogen and methylene blue were mixed to the final concentrations of 2.0 mg/ml and 100 μM, respectively. A part of the mixture was clotted by the addition of 4 NIH units of thrombin per ml of sample and left for 1 h. Subsequently, solid urea and some Tris-sodium chloride buffer were added to the unclotted and clotted parts of the mixture to give the final concentrations of 1.0 mg/ml of protein, 50 μM of methylene blue and 6 M of urea. The urea concentration was sufficient to dissolve the fibrin gel. All procedures were carried out in the dark. Samples of 0.5 ml were then transferred to beakers and irradiated as described above. After the irradiation the samples were precipitated with 10 vol of cold ethanol, and the fibrinogen samples mixed with 0.2 ml of the Tris-sodium chloride buffer and digested with 2 NIH units of thrombin for 2 h, all again in the dark.

Amino acid sequence analysis. The amounts of histidine and other amino acid residues were determined by quantitative sequence analysis, the yields being calculated in relation to unmodified amino acids directly consecutive in and characteristic of the corresponding sequence. In order to reduce the background during sequence analysis and to solubilize certain samples, all samples were digested by incubation with 100 mg of cyanogen bromide in 1.0 ml of 99% formic acid for 2 h in the dark. The aminoterminal region of the protein, which is contained in the largest cyanogen bromide fragment [1], was purified by gel filtration chromatography and directly used for sequence analysis. The analyses were carried out on fragments derived from 0.3 nmol of the original fibrinogen material employing a Hewlett-Packard G1005A protein-sequencing system. No characteristic oxidation-specific products were detected. All samples were sequenced for nine cycles after thrombin-induced removal of the fibrinopeptides.

Results

Effect on histidines in the native proteins. The methylene blue-mediated photo-oxidation of histidine residues in the aminoterminal region of fibrinogen and fibrin was studied by means of quantitative amino acid sequence analysis. There are only two histidine residues present within those sections of the Aα and Bβ chain believed to function as or contribute to the thrombin-activated polymerization sites, i.e. the first 50 to 60 residues of these peptide chains [1]. The positions of the two relevant histidines are Aα24, corresponding to α8, and Bβ16, corresponding to β2, after fibrinopeptide release (fig. 1). There are no histidines present within the first 40 or more residues of the γ chain. The irradiation time-dependent loss of histidine is displayed in figure 2. The analysis of the photo-oxidized fibrinogen confirmed that the histidine Bβ16, earlier demonstrated to be essential for fibrin polymerization [5, 6], was indeed rapidly destroyed (fig. 2, left). However, in contrast to the earlier report [5], the histidine Aα24 was found to disappear even faster (fig. 2, right). At the irradiation time when fibrinogen clottability was lost, which occurred after about 7 min under the conditions used, 20% of the histidine in the Bβ chain and 24% of the one in the Aα chain had been destroyed.

An explanation was then sought for the irradiation resistance of the methylene blue-containing fibrin clots preformed in the dark. For this purpose methylene blue-containing fibrinogen was clotted in the dark with batroxobin to release only fibrinopeptide A or with thrombin to release both fibrinopeptides A and B. The two types of fibrin are designated desAA-fibrin and desAABB-fibrin, respectively. The increase in light ab-

Aα chain	A D S G E G D F L A E G G G V R ↓ G P R V V E R **H** Q . .
Bβ chain	Z G V N D N E E G F F S A R ↓ G **H** R P L D K K R . .
γ chain	Y V A T R D N C C . .

Figure 1. Aminoterminal sequences of the three peptide chains of human fibrinogen, the arrows pointing at the thrombin cleavage sites in the Aα and Bβ chain during fibrinogen-fibrin conversion. Only the site in the Aα chain is cleaved by batroxobin. The two histidine residues relevant to the present work are underlined.

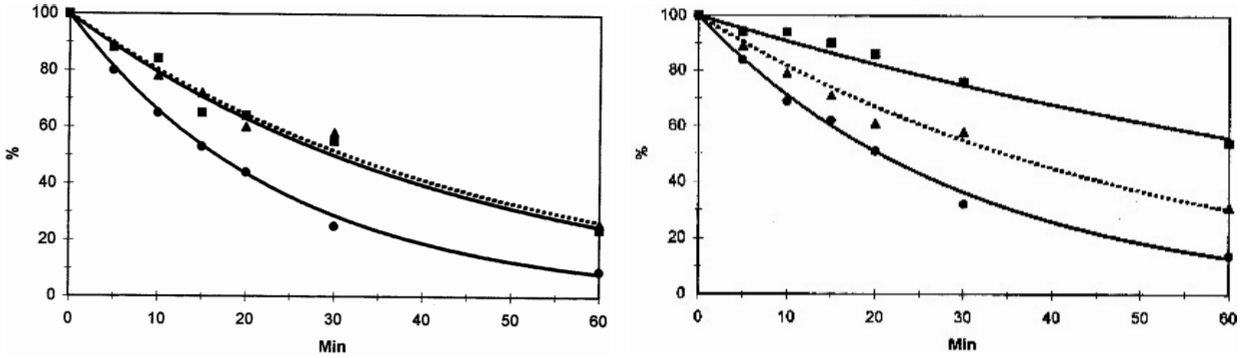

Figure 2. Time dependence of methylene blue-mediated photo-oxidation of histidines Aα24/α8 (left) and Bβ16/β2 (right) in native human fibrinogen (circles, solid lines), desAA-fibrin (triangles, broken lines) and desAABB-fibrin (squares, solid lines), the yield of histidine being determined by sequence analysis and expressed as percentage of initial amount. Fibrinogen became unclottable after about 7 min of irradiation under the present conditions.

sorbance due to fibrin polymerization in the wavelength range corresponding to the maximal absorbance of the dye, i.e. 610 to 680 nm, was only 3% of that of the dye itself under the conditions used; this slight increase in absorbance seemed of no relevance to the experiments. The preformed clots were irradiated in parallel with unclotted material, and the photo-oxidized proteins were examined by amino acid sequence analysis. The results are presented in figure 2, the upper curves in each part of the figure being derived from the clotted samples. It was obvious that both histidines were destroyed much more slowly in the two types of fibrin than in fibrinogen. For the histidine in the Aα chain the increase in resistance to photo-oxidation was virtually identical in the two types of fibrin, i.e. the release of fibrinopeptide A alone accounted for the change in susceptibility, and the release of fibrinopeptide B was without further consequence. In contrast, the resistance of the histidine in the Bβ chain increased to a very different extent in the two types of fibrin. Thus, the release of both fibrinopeptides A and B had a much greater effect on the susceptibility of this histidine than the release of only fibrinopeptide A.

The data were interpreted as an indication of the protective effect of the fibrin gel structure against photo-oxidation of the histidines Aα24/α8 and Bβ16/β2. The data also appeared to imply that there are two separate conformational changes taking place during the fibrinogen-fibrin conversion, one change related to the release of fibrinopeptide A and a second one related to the release of fibrinopeptide B. Furthermore, it was obvious from the results that even if these histidines, one or both, are essential for clot structure formation, they must be irrelevant to clot structure maintenance. All clots once formed were stable even when their histidines had been oxidized to an extent much exceeding the one rendering fibrinogen unclottable. The level of 20% destruction of the histidine in position Bβ16/β2, which makes fibrinogen unclottable after 7 min of irradiation, was reached after 25 min in the fibrin lacking both types of fibrinopeptides. However, there was no change in the appearance of the clots even after 24 h of irradiation.

Effect on histidines in the polymerization-inhibited proteins. In order to obtain additional evidence for the protective effect of the fibrin gel structure, photo-oxidation was also carried out in the presence of the fibrin polymerization inhibitor peptide Gly-Pro-Arg-Pro [11]. The results are shown in figure 3 (upper curves). As the time-dependent losses of the histidines were virtually identical in fibrinogen and fibrin, only a single curve has been drawn for each of the histidine residues. Thus the data confirm that the differences between the destruction rates in fibrinogen and fibrin are indeed due to conformational dissimilarities related to fibrin polymerization. The presence of the polymerization inhibitor seemed to make the histidine in the Aα chain somewhat less vulnerable to oxidation, but the histidine in the Bb chain was not affected by its presence (see table 1).

Effect on histidines in the denatured proteins. In order to evaluate the influence of the native spatial structure on the reactivity of the histidines during photo-oxidation, the irradiation treatment was also performed in the presence of 6 M urea. The results are shown in figure 3 (lower curves). As there were no obvious differences in destruction rates between fibrinogen and fibrin, only single curves have been drawn. The histidines were lost much faster, and there was no clear difference in reaction rate between the one in the Aα chain and the one in the Bβ chain once the protective effect of the native conformation had been abolished. In table 1 the disappearance rates of the histidines Aα24/α8 and Bβ16/β2 in fibrinogen and fibrin under native, polymerization-inhibited and denatured conditions are compared as the number of minutes of irradiation required for 50% loss.

Effect on fibrinopeptide release. Photo-oxidation of fibrinogen in the presence of rose bengal has previously been demonstrated to lead to an impaired fibrinopeptide release, as concluded from the decrease in aminoterminal glycine after thrombin digestion [3]. In the present study fibrinopeptide release was also

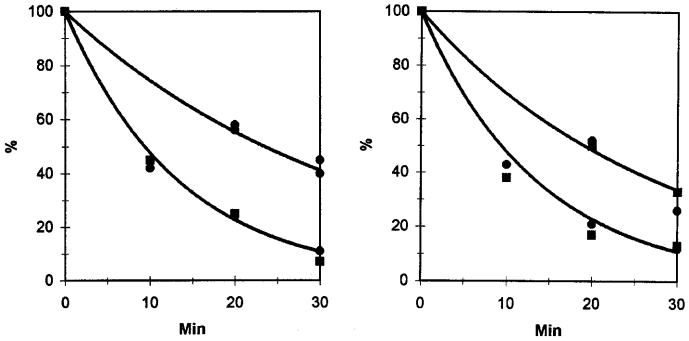

Figure 3. Time dependence of methylene blue-mediated photo-oxidation of histidines Aα24/α8 (left) and Bβ16/β2 (right) in polymerization-inhibited (upper curves) and denatured (lower curves) human fibrinogen (circles) and desAABB-fibrin (squares), the yield of histidine being determined by sequence analysis and expressed as percentage of initial amount.

evaluated as the appearance of aminoterminal glycine and, in addition, as the appearance of amino acid residues characteristic of the fibrin α chain and β chain, respectively, during later cycles of the sequence analyses (see fig. 1). The yields obtained before and after photo-oxidation were compared. There was no indication of an impairment to fibrinopeptide A release during the first 30 min of irradiation. However, fibrinopeptide B release decreased by about 50% during the same period of time, leading to an overall diminution in aminoterminal glycine.

Effect on other amino acids. It has been observed that photo-oxidation of various proteins in the presence of a dye will have a detrimental effect not only on histidine residues but on tryptophan, tyrosine and methionine residues as well [7]. In fibrinogen photo-oxidized in the presence of rose bengal, the overall contents of histidine, tryptophan, tyrosine and methionine have also been reported to be considerably decreased [3]. In the present study the yields of the amino acid residues appearing during sequence analysis in photo-oxidized and untreated samples were compared. The data clearly indicated that histidine was the only modified amino acid in the region of the molecule examined, i.e. the first nine amino acid residues of the α, β and γ chain (see fig. 1). Thus, there was no decrease in yield of, e.g. tyrosine, threonine or lysine, amino acids reported to be affected in other studies [3, 7]. However, the gel filtration chromatography profiles obtained after cyanogen bromide treatment of the photo-oxidized samples proved that the methionine residues had become resistant to cleavage, presumably by oxidation to the methionine sulfoxide form. After only 15 min of irradiation of the native proteins the chromatography components corresponding to the smaller fragments were decreasing in relative size, and larger fragments, i.e. fragments containing uncleaved methionine residues, were primarily observed. The oxidation of the denatured proteins occurred even faster.

Table 1. Methylene blue-mediated photo-oxidation of histidines Aα24/α8 and Bβ16/β2 in human fibrinogen, desAA-fibrin and desAABB-fibrin in the native state, in 0.01 M Gly-Pro-Arg-Pro, i.e. polymerization-inhibited, and in 6 M urea solution, i.e. denatured, expressed as minutes of irradiation required for 50% loss. The values are derived from the curves in figures 2 and 3.

	Fibrinogen		Fibrin (desAA)		Fibrin (desAABB)	
	Aα24	Bβ16	α8	Bβ16	α8	β2
Native	17	20	30	35	30	70
Polymerization-inhibited	23	19			23	19
Denatured	9	9			9	9

Discussion

Fibrinogen, desAA-fibrin and desAABB-fibrin have been subjected to methylene blue-mediated photo-oxidation for various periods of time in their ordinary native form as well as in their polymerization-inhibited and denatured form. The effects on the amino acids in the aminoterminal region of the molecules (fig. 1) have been studied by quantitative amino acid sequence analysis. Histidine residues Aα24/α8 and Bβ16/β2, which are the only histidines within the first 40 to 60 amino acid residues of the three peptide chains, were found to be selectively modified by the treatment in an irradiation time-dependent manner. However, the extent of histidine loss varied greatly depending on the presence of the fibrinopeptides A and B, on the types of fibrin polymers formed and on the environment of the proteins. The time-dependence of the histidine loss in the various types of samples under the environmental conditions selected is displayed in figures 2 and 3. The data for the different sets of samples and conditions are compared in table 1 by the irradiation time required for 50% loss of each of the two histidines.

The data demonstrate that the influence of the protein conformation on the reactivity of the histidines is highly significant. The various conformational forms may be

expected to provide different degrees of protection or surface exposure. In the denatured state, i.e. in the environment of 6 M urea, the two histidines are maximally accessible and accordingly reactive. There is no noticeable difference between the denatured fibrinogen and fibrin conformations in this respect, and the reaction rate is independent of the surrounding amino acid sequences, as the histidines in the Aα and Bβ chain show the same reactivity. In fact, all four time-dependence curves can be superimposed (fig. 3, lower curves). In the native state all reaction rates are much lower, indicating a general, partial protection by the native conformations. Depending on the type of sample, two to eight times as long was needed for the corresponding degree of loss of histidine. When the fibrin monomer polymerization is inhibited, i.e. in the environment of 0.01 M Gly-Pro-Arg-Pro, there is no obvious difference in conformation between fibrinogen and fibrin as far as the reactivities of the two histidines are concerned. Here the fibrinogen and fibrin time-dependence curves for each of the two histidines can be superimposed, though the histidines in the two peptide chains differ slightly from each other (fig. 3, upper curves).

In contrast, when fibrin polymerization has not been inhibited, there are very considerable differences in histidine susceptibility to photo-oxidation among fibrinogen, fibrin lacking only fibrinopeptide A and fibrin lacking both fibrinopeptides A and B. Both the histidine in the Aα chain and the one in the Bβ chain are protected against photo-oxidation by the fibrin gel structure, but to different degrees. Under comparable conditions the histidine in the Aα or α chain is always lost at a higher rate than the one in the Bβ or β chain. Fibrinopeptide A release, independent of fibrinopeptide B release, leads to the maximal, almost twofold decrease in accessibility of the histidine in the Aα chain. Fibrinopeptide A release alone gives an almost twofold decrease in accessibility of the histidine in the β chain, but only the additional fibrinopeptide B release provides the maximal, three- to fourfold decrease in susceptibility.

The data indicate that during fibrinogen-fibrin conversion the removal of fibrinopeptide A induces a first conformational change, and the subsequent removal of fibrinopeptide B a second such change. The aminoterminal ends of the Aα chains receive their full protection once the fibrinopeptide A is absent, presumably by the interaction of the α chain aminotermini with their complementary polymerization sites. The aminoterminal ends of the Bβ chains become partly hidden at this stage, but receive their full protection only when fibrinopeptide B is absent, so that the β chain aminotermini can interact with their own complementary sites. The histidine residue(s) in the aminoterminal region seem to be important for fibrin polymerization [4–6], but not for retaining fibrin in polymerized form, as preformed fibrin polymers are stable even when these histidines have become completely modified by photo-oxidation.

Methylene blue has been widely used for medical diagnostic and therapeutic purposes [see 8, 12] and in addition for blood plasma virus inactivation [9]. It is obvious that attention should be paid to the modifying effect of methylene blue, especially in the presence of light, on the functional properties of the proteins with which it comes into contact. It seems highly likely that the impairment of fibrinogen clottability [3–6, 8, 9], the alteration of fibrin clot structure [12] and the decrease in activity of several blood clotting factors [9] may just be examples of a general detrimental influence methylene blue could have on protein function in vivo.

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