Biochemistry (Moscow), Vol. 67, No. 4, 2002, pp. 446-450. Translated from Biokhimiya, Vol. 67, No. 4, 2002, pp. 535-540.

Polymerization Sites in the D-Domain of Human Fibrin(ogen)

E. V. Lugovskoy, E. N. Zolotareva, G. K. Gogolinskaya, P. G. Gritsenko, E. D. Moroz, and S. V. Komisarenko*

Palladin Institute of Biochemistry, Ukrainian Academy of Sciences, ul. Leontovicha 9, Kiev, 01601 Ukraine; fax: (380-44) 229-6365; E-mail: svk@biochem.kiev.ua

Received November 30, 2000
Revision received June 26, 2001

Abstract—The present work deals with localization of previously unknown polymerization sites of the fibrin DD-fragment. D-dimer we obtained has a pronounced inhibitory effect on fibrin polymerization (IC_{50} = 0.06 μM). The inhibitory effect of the D-fragment disappeared after reduction and carboxymethylation. However, polypeptide chains β_{DD} (Bβ134-461) and $\gamma\gamma_{DD}$ $(\gamma 63\text{-}411)_2$ of the DD-fragment, isolated by preparative electrophoresis, displayed their inhibitory activity. For instance, the rates of fibrin protofibril lateral association were decreased twice in the presence of β_{DD} and $\gamma\gamma_{DD}$ chains at their molar ratios to fibrin of 0.40 and 0.15, respectively. The IC_{50} values for β_{DD} and $\gamma\gamma_{DD}$ were 0.24 and 0.10 μM, respectively. Highly specific inhibition of protofibril lateral association suggests that the protofibril lateral association sites are located in Bβ134-461 and γ63-411 regions of the fibrin D-domain. Our data confirm those reported by Doolittle et al. regarding the γ-chain and a hypothesis about β-chain of fibrin D-domain (Yang, Z., Mochalkin, I., and Doolittle, R. F. (2000) *Biochemistry*, **97**, 14156-14161).

Key words: fibrin(ogen), D-domain, polymerization sites

When fibrinogen is cleaved by thrombin, two fibrinopeptides A are released and the fibrinogen turns into desAA fibrin, which is capable of spontaneous polymerization. At the intermediate polymerization phase of protofibril formation, two fibrinopeptides B are released from of desAA fibrin and, under sufficient amounts of thrombin, it turns into desAABB fibrin, with enhanced polymerization activity. The main mechanism of protofibril formation is the interaction of central E-domain of the fibrin molecule with two peripheral D-domains of two other fibrin molecules. A double-stranded protofibril is formed, where one strand is half molecule-staggered in relation to the other strand, while D-domains of the fibrin molecules in each strand are in intermolecular contact. Spontaneous fibrin self-assembly is performed via the interaction between two pairs of specific polymerization sites "A"–"a" and "B"–"b". "A" and "B" sites are located in the E-domain, whereas the complementary "a" and "b" sites are located in the fibrin D-domain [1].

It has been known that the main structural part of the "A"-site is located in the Aα17-19 amino acid sequence [2], and that of the "a"-site is in the C-terminal region of the D-domain γ-chain [3, 4]. The greatest challenge was to reveal the locations of the sites "B" and "b". Several active sites Bβ15-18 [4, 5], Bβ20-25 [6], Bβ43-47 [7], and Bβ40-49 [8] were found in the N-terminal sequence of Bβ-chain. Furthermore, there is proline enriched hydrophobic region Bβ27-33 containing five proline residues, which usually plays an important role in the protein–protein interactions [9]. It can be assumed that the active sites of the E domain named above have the corresponding complementary sites in the D domain. Doolittle et al. have only localized two polymerization sites in the D-domain using X-ray analysis: "a"-site (α-"hole") and a site in the β-chain C-terminal sequence of the D-domain (β-"hole") [4], which is complementary to the Bβ15-18 site in the E-domain.

There are also two Ca^{2+} binding sites in C-terminal regions of the β- and γ-chains of the D-domain [4], and amino acid residues of the γ-chain C-terminal region, involved in non-covalent intermolecular D–D interaction [10].

According to the X-ray data for the fibrin DD-fragment [4], modified bovine fibrinogen [11], and chicken fibrinogen [12], the recent work of Doolittle et al. shows a computer modeling of protofibril structure and their lateral association [13]. The conclusion is made that there are protofibril lateral association sites in the fibrin D-domains. There is also information available [14] that suggests the emergence of new polymerization sites during the various phases of the process. For instance, the

* To whom correspondence should be addressed.

0006-2979/02/6704-0446$27.00 ©2002 MAIK "Nauka/Interperiodica"

release of fibrinopeptide B is followed by the structural stabilization of the D-domain and increase of fibrin polymerization activity. There are also data about the increase of D-domain mutual affinity after the interaction of B–b sites [15].

Thus, this suggests the existence of multiple active but not localized sites in the D-domain. The present investigation reports an attempt to discover those sites. The polypeptide constituents of DD-fragment of human fibrin were isolated and their influence on fibrin polymerization was studied for this purpose.

D-dimer was used instead of monomer D-fragment for two reasons. First, β- and γ-chains of D-fragment monomer are difficult to separate due to very similar molecular weight and a high degree of homology. Second, the formation of an additional polymerization site after γ-γ-cross-linking with factor XIII[a] cannot be excluded.

MATERIALS AND METHODS

Fibrinogen was isolated from human plasma by salting out with sodium sulfate [16] and stored in 0.15 M NaCl solution at −18°C. The product was electrophoretically and chromatographically pure and had the standard set and molar ratio of N-terminal amino acid residues as determined by the method described by us earlier [17]. Plasminogen was obtained according to a previously described method [18]. Plasmin was obtained by activation of plasminogen with streptokinase (Varidase N., Lederle, Germany). Proteolytic activity of plasmin was expressed in casein units (c. u.) [19].

Fibrin desAABB was obtained by thrombin treatment of fibrinogen followed by dissolution of the clot in 0.02 M acetic acid [20]. The absence of fibrinopeptides B was proved by the value of the molar ratio of N-terminal fibrin amino acid residues (4G/2Y) [17].

DD-fragment was obtained by plasmin cleavage of fibrin stabilized with XIII[a]-factor [21]. Factor XIII[a] was obtained as described earlier [22].

The mixture of α_{DD}-, β_{DD}-, and $\gamma\gamma_{DD}$-chains constituting the D-dimer was obtained by its reduction and carboxymethylation [23]. The material was purified by gel filtration using a Sephadex G-25 column, then concentrated and stored in 6 M urea. An aliquot of the material was used for obtaining isolated chains by preparative electrophoresis.

Fibrin polymerization was performed in standard cuvettes (1 × 1 cm) at 37°C. The buffer solution consisted of 0.05 M ammonium acetate buffer, pH 7.4, containing 0.1 M NaCl and 10^{-4} M $CaCl_2$. An amount of 0.05 ml of the examined protein inhibitors, dissolved in 6 M urea (the control sample contained the corresponding volume of 6 M urea) was placed in the cuvette, and then 0.90 ml of buffer solution was added. This mixture was thermostatted for 5 min. The reaction was initiated by addition of 0.05 ml of 12 μM desAABB fibrin solution in 0.15% acetic acid under vigorous stirring, and the absorbance at 350 nm was immediately measured. The curve illustrating the increase of turbidity during the polymerization was used to determine the length of the lag-period of the reaction (t), tangent of slope angles of tangent lines to the curve (tanα), and the clot optical density in 5 min after the reaction started (Δh) [24].

Electrophoresis was carried out in 10.5% polyacrylamide gel with 0.1% SDS [25]. The same method in our modification was used for the separation of β_{DD}- and $\gamma\gamma_{DD}$-chains. Plate thickness was 2 mm, electric current 50 mA, voltage 180 V. For detection of protein bands the gel edges were cut out and treated with Coomassie R-250, and later the corresponding lanes were cut out of the untreated gel. After they were crushed, the protein was extracted with 0.05 M sodium phosphate buffer, pH 7.4, containing 4 M urea and 0.15 M NaCl. To remove the SDS, the proteins were precipitated twice with a mixture of 76.5% acetone and 12.9 mM HCl. Isolated β_{DD}- and $\gamma\gamma_{DD}$-chains were stored in 6 M urea solution.

RESULTS AND DISCUSSION

The D-fragment of human fibrin consists of three polypeptide chains, α_D, β_D, and γ_D, with molecular weights of 12, 42, and 39 kD, respectively [23]. They correspond to the chain fragments Aα105-197, Bβ134-461, and γ63-411 [26]. Each monomer unit of D-dimer fully corresponds to the monomer D-fragment. C-termini of two D-monomer γ-chains are held together by two isopeptide bonds by factor XIII[a].

After the reduction and carboxymethylation of D-dimer using the preparative electrophoresis, the single β_{DD}- and $\gamma\gamma_{DD}$-peptide constituents of the D-dimer were obtained.

Figure 1 shows electrophoregrams of D-dimer and β_{DD}- and $\gamma\gamma_{DD}$-isolated chains alone. Inhibitory activity of these chains on fibrin desAABB polymerization was further investigated. One of these experiments for β_{DD}- and $\gamma\gamma_{DD}$-chains at their molar ratio to fibrin 0.2 is presented in Fig. 2. As can be seen in this figure, the material exhibited a significant inhibitory activity, with $\gamma\gamma_{DD}$-chain influencing desAABB fibrin polymerization much more efficiently. These curves were obtained at varied molar ratios of polypeptide chains to fibrin. The lag-periods and tangents of slope angles of tangent lines to turbidity were determined from the turbidity curves. As previously shown from the comparative turbodimetric and electron microscopy investigations of fibrin polymerization, the lag phase corresponds to the phase of fibrin monomer units self-assembling into protofibrils, and the slope of the curve of turbidity increase corresponds to the rate of protofibril lateral association [27]. As seen in Fig.

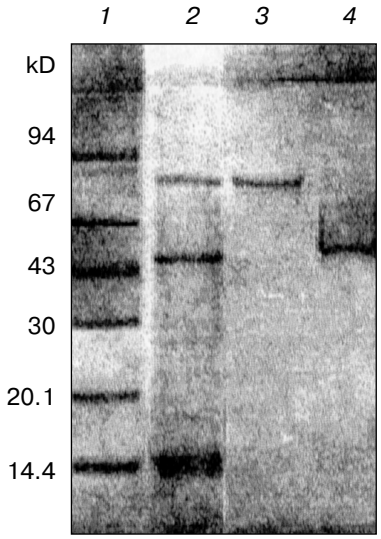

Fig. 1. Electrophoresis in 10.5% polyacrylamide gel with 0.1% SDS: *1*) molecular weight standards; *2*) DD-fragment after the reduction and carboxymethylation; *3*) single γγ-chain of DD-fragment; *4*) single β-chain of DD-fragment.

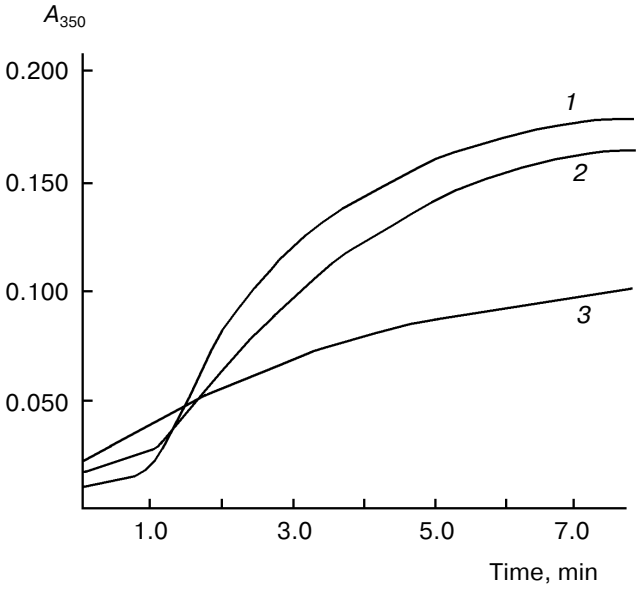

Fig. 2. Variations in optical density during the fibrin desAABB polymerization in the absence (*1*) and in the presence of β_{DD}- (*2*) and $\gamma\gamma_{DD}$-chains (*3*) at their molar ratio to fibrin equal to 0.2.

3, the effects of the chains displayed in the increase of self-assembling rate of monomer molecules into protofibrils (Fig. 3a), also in the decrease of protofibril lateral association rate (Fig. 3b) and maximum optical density of the clot (Fig. 3c). With β_{DD}- and $\gamma\gamma_{DD}$-fragments to fibrin molar ratios of 0.40 and 0.15, respectively, the rate of protofibril lateral association decreased twice.

Along with the inhibition of protofibril lateral association, the presence of β_{DD} and $\gamma\gamma_{DD}$ chains in fibrin polymerization medium leads to a certain decrease of lag period, i.e., to the acceleration of protofibril formation from the monomer fibrin molecules (Fig. 3a). Considering the decreased turbidity of the clot (Fig. 3c), the fact of the lag-period decrease is evidence for the acceleration of the first phase of the reaction, with formation of elongated protofibrils. The interaction of fibrin monomers with β_{DD} and $\gamma\gamma_{DD}$ polypeptide chains probably increases the mutual affinity of fibrin monomers, as results from the acceleration of polymerization first phase.

The most interesting fact was a strong inhibitory influence of β_{DD} and $\gamma\gamma_{DD}$ chains constituting the D-dimer on the fibrin protofibril lateral association phase. The inhibitory effect of $\gamma\gamma_{DD}$-chain determined via tgα was approximately 2.5-fold greater than that of β-chain: IC_{50} values for $\gamma\gamma_{DD}$- and for β_{DD}-chains were equal to 0.10 and 0.24 μM, respectively (the IC_{50} value for the intact DD-fragment was 0.06 μM).

It is well known that the denaturation of D- and DD-fragments leads to the loss of their anti-polymerizing activity [23]. Because of this, the inhibitory activity of D-fragment is usually attributed to the functioning of conformation-dependent polymerization sites "a" and "b" [28]. Therefore, the presence of inhibitory properties in the denatured samples of isolated β_{DD}- and $\gamma\gamma_{DD}$-chains,

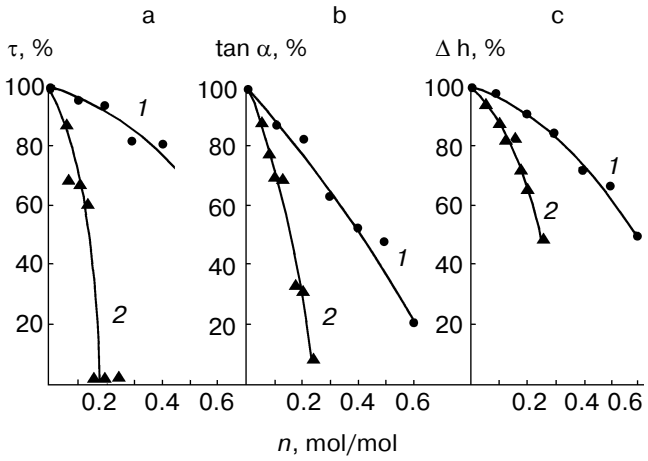

Fig. 3. Dependence of the main parameters of fibrin desAABB polymerization on molar ratio of β_{DD}- (*1*) and $\gamma\gamma_{DD}$-constituents (*2*) of D-dimer to fibrin: a) lag period of fibrin polymerization (τ); b) rate of protofibril lateral association (tanα); c) clot turbidity (*h*). Control parameters (fibrin polymerization rates in the absence of the inhibitor) are taken as 100%.

in which "a" and "b" sites cannot display any functional activity, was not expected.

Based on the data, it can be assumed that polypeptide chains β_{DD} and $\gamma\gamma_{DD}$, constituents of the fibrin DD-fragment, contain the sites of fibrin protofibril lateral association. Supposed sites exist in the native fibrin molecule and in the D- and DD-fragments. Obviously they are not dependent on native conformation and are preserved in D-dimer polypeptide chains after reduction and carboxymethylation of the DD-fragment. The functional activity of these sites is apparently displayed in the fibrin polymerization and inhibitory activity of isolated β_{DD}- and $\gamma\gamma_{DD}$-chains.

Reduced and carboxymethylated D-dimer, which is a mixture of α_{DD}, β_{DD}, and $\gamma\gamma_{DD}$ polypeptide chains, has no anti-polymerizing activity (Fig. 4). Considering that isolated β_{DD}- and $\gamma\gamma_{DD}$-chains of DD-fragment displayed an inhibitory activity, the assumption was made that the active sites of these denatured chains are inter-complementary and, therefore, are mutually blocked in the samples containing their equal amounts (denatured D-fragment or a mixture of chains). (DD)E-complex released from the plasmin stabilized fibrin can be taken as an example. D-dimer involved in the complex formation is a strong inhibitor and E-fragment is a mild inhibitor of fibrin polymerization. (DD)E-complex, however, having its polymerization sites mutually blocked, is completely inert [29].

The lack of the inhibitory activity in the combined mixture of polypeptide chains α_{DD}, β_{DD}, and $\gamma\gamma_{DD}$, which are the components of DD-fragment, is taken in this case to be a negative control for a pronounced inhibitory effect of the isolated β_{DD}- and $\gamma\gamma_{DD}$-chains.

Earlier an assumption was made by Blomback that the lateral association of fibrin protofibrils is performed through inter-protofibrillar bonds between "B"–"b" sites [30]. Based on fibrinogen complexes with desAA, desBB, and desAABB fibrins formation, Shainoff and Dadrik, however, proposed that "B"–"b" interactions are realized within a protofibril and are not directly involved in the lateral association. These interactions lead to the increased stability of protofibril structure and, hence, to reinforcement of the lateral association [31]. Fowler et al. also assumed that all possible D–E contacts are realized in a protofibril, and the protofibril lateral association is carried out via the D-domain pairs of different protofibrils brought close together [32].

Based on the previously reported X-ray data, Doolittle et al. in their recent work performed a computer modeling of protofibril structure and lateral association. The conclusion is that the protofibril primary lateral association is accomplished via the intermolecular interaction between γC-subdomains of fibrin D-domain, and the site of the primary lateral association of fibrin protofibrils is located in the fragments γ350-360 and γ370-380 [13]. The results permitted the authors to propose the existence of a secondary auxiliary site of fibrin protofibrils lateral association, which is located in the Bβ330-375 region of βC-subdomain of fibrin D-domain.

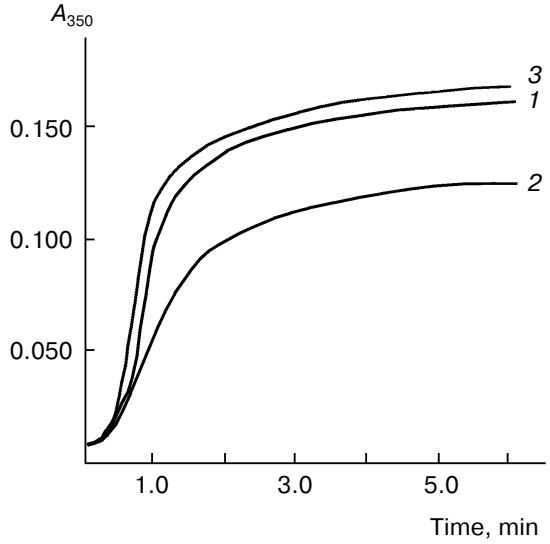

Fig. 4. Variations in optical density during the polymerization process: *1*) fibrin desAABB; *2*) fibrin desAABB in the presence of DD-fragment; *3*) fibrin desAABB in the presence of a reduced DD-fragment (molar ratio of original DD-fragment and reduced DD-fragment to fibrin desAABB is 0.1).

In the present work we have obtained an experimental proof that the sites of fibrin protofibril lateral association are located in the carboxy-terminal regions of β- and γ-chains which are the constituents of D-domain. In our opinion, the most valuable is the detection of functional activity of the protofibril lateral association site in β-constituent of D-domain, the existence of which was proposed by Doolittle et al.

The real situation may be more complex than it appears from the X-ray data. For instance, there are data indicating the possibility for protofibril lateral association sites to exist in a D-domain fragment different from those suggested by Doolittle et al. It was recently reported that the substitution BβR166→C in fibrinogen Longmont results in a significant decrease of fibrin protofibril lateral association [33]. On the other hand, the substitution of BβN160→S in fibrinogen Nigata leads to the additional glycosylation of BβN158 amino acid residue and therefore to the inhibition of protofibril lateral association [34]. These facts obviously prove that one of the protofibril lateral association sites is damaged, which results in the inhibition of this polymerization phase.

Thus, our conclusion that protofibril lateral association sites are located in the β- and γ-chains of the fibrin D-domain are in accordance with X-ray data and experiments with defective fibrinogens.

REFERENCES

1. Olexa, S. A., and Budzynski, A. Z. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1374-1378.
2. Laudano, A. P., Cottrell, B. A., and Doolittle, R. F. (1983) *Ann. N. Y. Acad. Sci.*, **408**, 315-329.
3. Varadi, A., and Sheraga, H. A. (1986) *Biochemistry*, **25**, 519-528.
4. Everse, S. J., Spraggon, G., Veerapandian, L., Riley, M., and Doolittle, R. F. (1998) *Biochemistry*, **37**, 8637-8642.
5. Pandya, B. V., Gabriel, J. L., O'Brien, J., and Budzunski, A. Z. (1991) *Biochemistry*, **30**, 162-168.
6. Hsieh Kun-hwa (1997) *Biochemistry*, **36**, 9381-9387.
7. Cierniewski, C. S., and Poniatowski, J. (1990) *Acta Biochim. Polonica*, **37**, 31-37.
8. Moskowitz, K. A., and Budzynski, A. Z. (1994) *Biochemistry*, **33**, 12937-12944.
9. Williamson, M. P. (1994) *Biochem. J.*, **297**, 249-260.
10. Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997) *Nature*, **389**, 455-462.
11. Brown, J., Volkmann, N., Jun, G., Henschen-Edman, A., and Cohen, C. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 85-90.
12. Yang, Z., Mochalkin, I., Veerapandian, L., Riley, M., and Doolittle, R. F. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 3907-3912.
13. Yang, Z., Mochalkin, I., and Doolittle, R. F. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 14156-14161.
14. Donovan, J. W., and Mihalyi, E. (1985) *Biochemistry*, **24**, 3434-3443.
15. Lugovskoy, E. V., Makogonenko, E. M., Chudnovets, V. S., Derzska, S. G., Gogolinska, G. K., Mihailovskaya, L. I.., and Komissarenko, S. V. (1995) *Ukr. Biokhim. Zh.*, **4**, 57-64.
16. Varetska, T. V. (1960) *Ukr. Biokhim. Zh.*, **32**, 13-24.
17. Lugovskoy, E. V., Derzska, S. G., Gogolinska, G. K., and Litvinova, L. M. (1975) *Ukr. Biokhim. Zh.*, **47**, 760-768.
18. Deutsch, D., and Mertz, E. (1970) *Science*, **170**, 1095-1096.
19. Robbins, K. C., and Summaria, L. (1970) *Meth. Enzymol.*, **9**, 184-186.
20. Varetska, T. V. (1965) *Ukr. Biokhim. Zh.*, **37**, 194-206.
21. Musyalkovskaya, A. A., Khodorova, E. L., and Pozdnyakova, T. M. (1976) *Ukr. Biokhim. Zh.*, **48**, 143-146.
22. Curtis, C. G., and Lorand, L. (1976) *Meth. Enzymol.*, **45**, Pt. B, 171-191.
23. Cierniewski, C. S., Kloczewiak, M., and Budzynski, A. Z. (1986) *J. Biol. Chem.*, **261**, 9116-9121.
24. Bouvier, S. A., Beretta-Piccoli, M., and Giacometti, N. (1975) *Progress in Chemical Fibrinolysis and Thrombolysis* (Davidson, J. F., ed.) Vol. 1, Ravon Press, New York, pp. 281-288.
25. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.*, **244**, 4406-4412.
26. Takagi, T., and Doolittle, R. (1975) *Biochemistry*, **14**, 940-946.
27. Hantgan, R., Fowler, W., Ericson, H., and Hermans, J. (1980) *Thromb. Haemost.*, **44**, 119-124.
28. Pozdnyakova, T. M., Rybatchuk, V. N., and Ugarova, T. P. (1987) *Biokhimiya*, **52**, 592-598.
29. Budzynski, A. Z., Olexa, S. A., and Brizuela, B. S. (1979) *Biochim. Biophys. Acta*, **584**, 284-287.
30. Blomback, B. (1979) *Plasma Proteins* (Blomback, B., and Hanson, L. A., eds.) John Wiley and Sons, pp. 223-253.
31. Shainoff, J. R., and Dardik, B. M. (1983) *Ann. N. Y. Acad. Sci.*, **408**, 254-268.
32. Fowler, W. E., Hantgan, R. R., Hermans, J., and Erickson, H. P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4872-4876.
33. Lounes, K. C., Lefkowitz, J. B., Coates, A. I., Hantgan, R. R., and Lord, S. T. (2000) *Fibrinogen 2000*, *XVI Int. Fibrinogen Workshop*, *Poster Abstracts*, Leiden, The Netherlands, Ab. 5.
34. Sugo, T., Nakamikawa, C., Takano, H., Mimuro, J., Yamaguchi, S., Mosesson, M. W., Meh, D. A., DiOrio, J. P., Takahashi, N., Takahashi, H., Nagai, K., and Matsuda, M. (1999) *Blood*, **94**, 3806-3813.