Equilibria in the Fibrinogen–Fibrin Conversion. VIII. Polymerization of Acceptor-Modified Fibrin Monomer*

G. F. Endres† and H. A. Scheraga

ABSTRACT: This work was undertaken in order to test a previous suggestion that covalent bonds might be formed, in the *reversible* stage of fibrin polymerization, through the functional groups which are subsequently involved in the enzymatic clot stabilization reaction. By development of a published procedure, a satisfactorily pure preparation of modified bovine fibrin monomer was made, in which an average of 3.0 acceptor groups/molecule were blocked by reaction with hydroxylamine. The ability of this acceptor-modified fibrin monomer to form a stabilized clot was greatly reduced, but in all other respects it resembled the unmodified monomer. A study of the ionization changes accompanying polymerization in 1.0 M NaBr over a wide range of initial pH values at 25.0° revealed no significant difference in the results for this modified fibrin and for an unmodified control preparation. This finding disproves the particular mechanism previously suggested, and leaves unsupported the hypothesis of covalent bonding in *reversible* fibrin polymerization. The present status of knowledge of the mechanism of reversible fibrin polymerization is discussed.

Previous papers in this series described experimental data for the heat evolution (Sturtevant *et al.*, 1955) and ionization changes (Endres *et al.*, 1966) accompanying the reversible polymerization of bovine fibrin monomer in 1.0 M NaBr at 25.0°. The heat evolution and earlier data for the ionization changes in 0.3 M KCl obtained by an indirect method (Mihalyi, 1954) were accounted for quantitatively by Sturtevant *et al.* (1955) on the basis of intermolecular hydrogen bonding between ionizable groups only, assuming the enthalpy of formation of hydrogen bonds in water to be −6 kcal/mole. The subsequent limitation of the accepted value for the latter quantity to −1.5 kcal/mole made this postulate untenable, and led us to propose instead a mechanism based on intermolecular coordinate covalent bonding between ionizable groups. It was suggested that these groups might be those believed at the time to be involved in the subsequent enzymatic clot stabilization reaction, *i.e.*, the α-amino groups of the four N-terminal glycine residues ("donor" groups) and the carbohydrate-bound side-chain amide groups of asparagine or glutamine residues ("acceptor" groups). The chemistry of clot stabilization has been reviewed recently by Loewy (1968).

Certain simple compounds having an amino group are known to inhibit the stabilization of the fibrin clot by the enzyme plasma transglutaminase (also known as activated fibrin-stabilizing factor, activated factor XIII, or Laki–Lorand factor). These substances become incorporated into fibrin at the monomeric level, indicating that the inhibition is due to competition with the donor groups of fibrin, resulting in blocking of the acceptor groups by reaction with the added amine (Loewy *et al.*, 1964; Lorand and Jacobsen, 1964; Lorand and Ong, 1966). These findings afforded a means for testing the validity of the covalent bonding mechanism which we had suggested, through study of the polymerization of fibrin monomer in which the acceptor groups have been blocked by reaction with an amine. If the mechanism were correct, the ionization changes and heat evolution in the polymerization of such an acceptor-modified fibrin monomer should be greatly reduced.

Lorand and Ong (1966) have described the preparation of fibrin monomer modified by glycine ethyl ester, but as a mixture with a large amount of by-product with a slightly higher sedimentation constant. The present approach was to develop their method to yield reasonably homogeneous acceptor-modified fibrin monomer with hydroxylamine as modifier, and investigate the ionization changes accompanying its polymerization in 1.0 M NaBr.

Experimental Section

Materials. All proteins employed in this work (fibrinogen, fibrin monomer, thrombin, and factor XIII) were of bovine origin. The "Tris buffer" referred to is 0.10 M NaCl–0.05 M Tris, pH adjusted to 7.5 with HCl.

Fibrinogen was prepared from plasma fraction I (Armour, lot no. U4704) by the procedure of Laki (1951), as modified by Sturtevant *et al.* (1955), retaining only the fraction between 0.21 and 0.25 $(NH_4)_2SO_4$ saturation. Solutions of fibrin monomer in 1.0 M NaBr at pH 5.3 were prepared from this purified fibrinogen essentially by the procedure of Donnelly *et al.* (1955), except that the fibrin was reclotted and redissolved only once. Such preparations of fibrin monomer yielded completely soluble clots in the absence of added factor

* From the Department of Chemistry, Cornell University, Ithaca, New York. *Received July 18, 1968.* This investigation was supported by a grant (HE-01662) from the National Heart Institute, U. S. Public Health Service.

† Public Health Service research career development awardee (K3-HE-15,829) of the National Heart Institute.

XIII in the standard stabilizability test described below, indicating at most a low level of contamination by factor XIII. These solutions of fibrin monomer were the starting materials for the preparations of acceptor-modified fibrin monomer.

Two preparations of thrombin were used in this work. Crude thrombin powder (bovine) supplied by the Upjohn Co., Kalamazoo, Mich., was employed for the preparation of fibrin monomer from fibrinogen. Thrombin from beef plasma (grade II, Sigma Chemical Co.) was clarified by centrifugation, and purified by chromatography on Sephadex G-25, followed by ion-exchange chromatography by the procedure of Rasmussen (1955). The product (specific activity 380 *p*-tosyl-L-arginine methyl ester hydrochloride units/mg of protein) was stored frozen at −20°, and freshly thawed and diluted portions were employed to activate factor XIII.

Factor XIII (plasma transglutaminase precursor) was "fibrinase fraction 5" (Loewy *et al.*, 1961), but prepared from bovine plasma. The lyophilized powder was stored at −20°. Solutions in Tris buffer may be kept at 4°, but appear to gradually lose their activity over a period of several weeks.

L-Cysteine (free base) and *p*-tosyl-L-arginine methyl ester hydrochloride were Assayed or C.P. grades of Mann Research Laboratories, and 1-naphthylamine was Certified grade of Fisher Scientific Co. All other materials were reagent grade, unless otherwise specified.

Methods. Preparative centrifugations were carried out at 25° in a Beckman Model L-2 ultracentrifuge, using the No. 30 rotor.

The acceptor-modified fibrin monomer used for the ionization change experiments was prepared by a procedure based on that described for glycine ethyl ester modifier by Lorand and Ong (1966). In the present work hydroxylamine (or, in a few exploratory experiments, hydrazine) was used as modifier, the fibrin clot (after modification) was synerized to a small volume before redissolving in 1.0 M NaBr and reclotting, and the final product was purified by high-speed preparative centrifugation. All operations through the beginning of dialysis (see below) were completed in a single day, and all solutions were as fresh as possible. The L-cysteine and *p*-tosyl-L-arginine methyl ester hydrochloride solutions were made up immediately before use, by solution in Tris buffer (see above) and readjustment of the pH to 7.5 with 2 N NaOH. The stock thrombin solution was thawed and an aliquot diluted with Tris buffer just before use. The factor XIII was dissolved in Tris buffer 1 day before, and the isolation of fibrinogen for preparation of the starting fibrin monomer was begun 9 days before the modification. The control fibrin for ionization change studies was prepared in the same manner, except that an equal volume of Tris buffer was substituted for the factor XIII solution. The procedure for preparation of modified fibrin was as follows. The following were mixed in order at room temperature in an 800-ml beaker: 75 ml of 0.001 M aqueous $CaCl_2$, 75 ml of 0.1 M L-cysteine solution, 60 ml of factor XIII solution containing 11.2 mg of protein (0.030 mg/ml in the final 375-ml reaction mixture), and 15 ml of thrombin solution containing 266 *p*-tosyl-L-arginine methyl ester hydrochloride units. After allowing 10 min for the conversion of factor XIII into plasma transglutaminase, 30 ml of 0.25 M *p*-tosyl-L-arginine methyl ester hydrochloride solution was added, followed immediately by 37.5 ml of Tris buffer, 37.5 ml of 0.18 M hydroxylamine (0.018 M in final reaction mixture, prepared by adjusting the pH of an aqueous solution of the hydrochloride to 7.5 with concentrated NaOH), and 45 ml of 1.5% fibrin monomer in 1.0 M NaBr (pH 5.3) (weight ratio fibrin/factor XIII was 60). The fibrin monomer solution was stirred in quickly but smoothly with a glass rod, and the solution was not agitated further. A very turbid clot appeared almost immediately, and after a reaction time of 1 hr the clot was cut up in the beaker with a spatula. As much liquid as possible was decanted, and the clot was poured out onto a 13-in. square of fine cloth over a layer of absorbent paper. Since syneresis was slow, the corners of the cloth were twisted into a bag, which was squeezed with the fingers until the clot volume was reduced to 55 ml (checked in a calibrated beaker). About 20 min was required for this operation, during which the plasma transglutaminase was presumably still active. The synerized clot was then dissolved with gentle magnetic stirring in 35 ml of 2 M NaBr, keeping the pH at 5.4–5.5 by dropwise addition of 1.0 M sodium acetate buffer (pH 5.0) in 1.0 M NaBr. The NaBr concentration was maintained at 1.0 M by portionwise addition of 2.0 g of the solid salt, taking care to dissolve the salt quickly and minimize contact with undissolved lumps of clot. The final solution volume of about 95 ml was centrifuged for 20 min at 20,000 rpm and reclotted by addition to 19 volumes of clotting buffer (0.0947 M KCl–0.0158 M Na_2HPO_4–0.0316 M KH_2PO_4, pH 6.4). The clot was removed promptly as formed by winding onto a glass rod, collecting nine portions at increasing time intervals over a period of 2 hr. The clot was redissolved as collected in 5 ml of 2 M NaBr with addition of 2.6 g of solid NaBr and acetate buffer as above. The final solution volume was about 35 ml, and the final pH was lowered to 5.3. This solution was dialyzed for 2 days at 4° with gentle rocking, against four 1-l. portions of 1.0 M NaBr, the pH of which had been adjusted to 5.3 with hydrobromic acid. Centrifugation was then carried out for 5.1 hr at 25° and 30,000 rpm, using two 25-ml capacity tubes (these conditions are critical). The final product consisted of 35 ml of solution containing 0.87% protein. The yield of modified fibrin monomer from the starting fibrin was 45%.

Protein concentrations were determined from the optical density of solutions at 280 mμ, using the following values of the extinction coefficient $E^{1\%}_{1cm}$: fibrin in 1.0 M NaBr, 16.0 (Ehrenpreis and Scheraga, 1957); factor XIII, 13.4 (Loewy *et al.*, 1961). The concentrations of the fibrin solutions used for ionization change experiments were also checked gravimetrically before dilution by the following procedure, from which the % clottability was also determined. Duplicate 2.00-ml samples of fibrin solution in 1.0 M NaBr were added to 38.0 ml of clotting buffer (see above) in 100-ml beakers, and allowed to stand overnight at room temperature. Samples were withdrawn from the supernatants and the concen-

tration of nonclottable protein was determined from the optical density at 280 mμ, read against clotting buffer. The clots were synerized onto cloth squares, washed in clotting buffer and water, rolled off the cloth, and dried to constant weight at 110°. The concentrations of clottable protein determined in this way and from optical density agreed to within the experimental error. The ionization change results were calculated on the basis of clottable protein concentration.

The procedure used to test fibrin stabilizability and estimate plasma transglutaminase activity was based on the system of Lorand *et al.* (1962). The conditions were similar to those described above for the modification of fibrin, but scaled down to a total reaction mixture of 2.5 ml. The following were mixed in order at room temperature in a test tube: 0.5 ml of 0.001 M aqueous $CaCl_2$, 0.5 ml of 0.1 M L-cysteine solution, 0.4 ml of factor XIII solution, and 0.1 ml of solution containing 1.8 *p*-tosyl-L-arginine methyl ester hydrochloride units of purified Sigma thrombin. After 10 min, 0.2 ml of 0.25 M *p*-tosyl-L-arginine methyl ester hydrochloride solution is added, followed immediately by 0.5 ml of Tris buffer and 0.3 ml of 1.5% fibrin monomer in 1.0 M NaBr (pH 5.3). After 30 min, 2.5 ml of 2% monochloracetic acid is added, the clot is carefully detached from the walls of the tube with a fine spatula, and the suspension is swirled gently. Nonstabilized clots dissolve readily under these conditions, while stabilized clots may shrink somewhat, but retain their shape and remain undissolved. The test was used as a visual "threshold assay," and the insoluble clot was not isolated for protein determination. Using the stock purified fibrin monomer under the above conditions, the threshold for factor XIII was between 0.009 and 0.018 mg/ml in the total reaction mixture, corresponding to weight ratios fibrin/factor XIII of 200 and 100. In the standard conditions adopted for testing the stabilizability of fibrin, 0.022 mg/ml of factor XIII was added.

Analytical ultracentrifugations were carried out in a Spinco Model E instrument, at 25° and 50,740 rpm. A Kel-F centerpiece with 12-mm light path was used, and exposures were taken at 16-min intervals at schlieren angle 70°.

The hydrazine or hydroxylamine contents of modified fibrin were determined by the colorimetric procedures of Seifter *et al.* (1960) as applied to fibrin by Lorand and Ong (1966), except that they were applied directly to fibrin solutions in 1.0 M NaBr. To 0.4 ml of 0.75% fibrin monomer in 1.0 M NaBr was added 0.50 ml of 0.18 M ammonium carbonate containing 0.3 mg of trypsin (Worthington crystallized–lyophilized), and the homogeneous solution was permitted to stand at room temperature for several days. The standards for the reference curve were prepared in the same way from stock purified fibrin monomer solution. After digestion, 0.10 ml of water was added, containing known amounts of hydroxylamine or hydrazine in the case of the standards. Determinations were usually run in duplicate. The concentration of unbound modifier remaining after dialysis in the solution of modified fibrin was determined from 0.90-ml samples of the last portion of dialysate, using pure 1.0 M NaBr for the standards.

Results

The principal difficulties encountered with the system used to modify fibrin monomer involved heterogeneity of the product or reduction in yield due to precipitation during work-up. It was found that precipitation could be avoided by not lowering the pH below 5.4 during the first redissolving of the clot in 1.0 M NaBr, and by continuing the work-up of this solution without delay. Evidently precipitation at this stage is due to the residue of the original reaction mixture which remains in the clot after syneresis.

In their preparation of glycine ethyl ester modified fibrin, Lorand and Ong (1966) found that the product contained about 23% of material sedimenting at 8.5 S compared with 6.3 S for the monomer. We have attempted to reproduce their experiment, using 0.04 M glycine ethyl ester. The procedure was as described for hydroxylamine in the Experimental Section, except that quantities were scaled down to one-fifth size, the level of factor XIII was 22 mg/ml, and the final centrifugation of the product was carried out for 20 min at 20,000 rpm. Evidently extensive modification of fibrin had in fact occurred, since the isolated product did not form a stabilized clot under the standard test conditions. The sedimentation pattern showed the 8.5 S material, but in smaller quantity relative to the monomer than reported by Lorand and Ong. In addition, we observed material sedimenting at about 22 S, which increased the visible turbidity of the solution. This fast-sedimenting material was later found to be responsible for abnormal clotting behavior in clotting buffer, reducing the clotting time and giving a lumpy rather than gelatinous clot.

A series of exploratory preparations of modified fibrin monomer was carried out in an attempt to find the cause of the heterogeneity described above and to develop conditions for avoiding it. It was found that the 8.5S and 22S materials were formed to about the same extent with 0.018 M hydroxylamine or hydrazine modifier as with 0.04 M glycine ethyl ester. Even when added factor XIII or both factor XIII and thrombin were omitted from the reaction mixture, the amount of these materials in the product was unchanged. Evidently the heterogeneity is not associated with the action of plasma transglutaminase, and is not due to the action of impurities present in the enzyme preparations used. A further series of blank experiments was carried out, with 0.018 M hydroxylamine, using the absence of turbidity of the product solution as a measure of purity. The ratio of optical densities at 350 and 280 mμ was used as an index of the turbidity. It was found that most of the constituents of the modification reaction mixture contribute toward the product turbidity, but that *p*-tosyl-L-arginine methyl ester hydrochloride was a major cause.

The original reason for including *p*-tosyl-L-arginine methyl ester hydrochloride in the Lorand system was to inhibit thrombin after the activation of factor XIII to plasma transglutaminase, in order to clarify the mecha-

nism of action of the latter (Lorand and Konishi, 1964). It would be expected that the presence of *p*-tosyl-L-arginine methyl ester hydrochloride is not necessary for preparative purposes. We found, in fact, that the presence of *p*-tosyl-L-arginine methyl ester hydrochloride is not necessary in clot stabilization experiments, but it has a pronounced accelerating effect. *p*-Tosyl-L-arginine methyl ester hydrochloride was also found to reduce the clotting time and give a more readily synerizable clot in modification experiments. The clot-promoting effect of *p*-tosyl-L-arginine methyl ester hydrochloride in fibrin polymerization has been described previously (Abildgaard and Godal, 1964). Owing to these beneficial effects, it was decided to continue the use of *p*-tosyl-L-arginine methyl ester hydrochloride in preparations of modified fibrin. It was found that the 22S impurity can be removed from such preparations without prohibitive reduction in yield by carefully controlled centrifugation.

Several small-scale preparations were also run in order to explore the effects of some of the obvious reaction variables on the extent of modification. The results are summarized in Table I, and indicate that: (1) hydroxylamine is a more effective modifier than hydrazine, as found by Lorand and Ong (1966) by analysis of modified clots; (2) doubling the concentration of hydrazine to 0.036 M had a negative effect; (3) the extent of reaction is increased by doubling the reaction time; and (4) doubling the concentration of factor XIII greatly increases the extent of modification.

TABLE I: Some Effects of Reaction Variables on Extent of Modification.[a]

Modifier (M)	Factor XIII (mg/ml)	Time[b] (hr)	Extent of Modification[c] (Groups/Molecule)
H_2NOH (0.018)	0.022	1.25	2.5
N_2H_4 (0.018)	0.022	1.25	1.80 ± 0.05
N_2H_4 (0.036)	0.022	1.25	1.55 ± 0.01
N_2H_4 (0.018)	0.044[d]	1.25	2.52 ± 0.01
N_2H_4 (0.018)	0.022	2.5	1.95 ± 0.03

[a] Unless noted otherwise, conditions were as described for modification by H_2NOH in the Experimental Section. [b] Time elapsed from the addition of fibrin to the reaction mixture until the completion of syneresis. [c] The molecular weight of fibrin monomer is taken as 3.3×10^5. [d] The concentration of thrombin was also doubled in this experiment.

A large-scale preparation of acceptor-modified fibrin monomer for ionization change studies was carried out using the procedure detailed in the Experimental Section. Hydroxylamine (0.018 M) was the modifier, with factor XIII added at 0.030 mg/ml, and a total reaction time of 1.33 hr. These conditions were not expected to produce the maximum possible extent of modification, but were designed to utilize the purified factor XIII efficiently, and minimize exposure of fibrin to the other components of the reaction mixture. A control preparation was also carried out using the identical procedure, except that no factor XIII was added. Some of the properties of these preparations are summarized in Table II, which also includes data for the purified fibrin monomer used as starting material. The extent of modification with added factor XIII is seen to be quite high, an average of 3.0 groups modified/mole of fibrin. That the hydroxylamine detected is actually bound to fibrin is evidenced by the fact that the last portion of the dialysate contained no detectable hydroxylamine. The limit of detection for the assay conditions used is equivalent to about 0.2 mole/mole of fibrin. No hydroxylamine was found in the control preparation beyond the limit of detection. This shows that the hydroxylamine was bound to the modified fibrin through the action of the added factor XIII, and not through some side reaction. It also shows that the fibrin starting material was not significantly contaminated by factor XIII originating in plasma fraction I.

The sedimentation patterns for modified fibrin and the control are shown in Figure 1, and the sedimentation coefficients of the principal boundaries are given in Table II. The coefficients in both cases are the same as for untreated fibrin monomer, within the experimental uncertainty. On this basis there is no indication that modification of the acceptor groups of fibrin causes any extensive change in conformation. In both cases only a single boundary is seen in the first photograph, showing that the preparative centrifugation had removed the 22 S material. This is also shown by the low turbidities indicated by the optical density ratios in Table II. The 8.5 S material is still evident in Figure 1, since its sedimentation rate is only a little larger than for the monomer. The amount relative to the monomer, however, is quite small and about the same in both cases.

The ability of the modified and control fibrin monomer to form stabilized clots was determined by the standard test described in the Experimental Section, with factor XIII added at 0.022 mg/ml, but using 0.75% rather than 1.5% fibrin samples. The control gave an insoluble clot while the clot from modified fibrin was completely soluble, confirming that extensive blocking of acceptor groups had in fact occurred. Insoluble clots could be obtained from the modified fibrin, however, by increasing the amount of added factor XIII. The threshold ratio fibrin/factor XIII is 30–40 for the modified material, compared to 100–200 for untreated fibrin.

The results of the ionization change studies are given in Figure 2, expressed as the release of protons during polymerization as a function of the initial pH. These studies were carried out as described previously (Endres *et al.*, 1966), except that the protein concentration was 0.59% rather than 0.74%. Briefly, the method consists of rapidly increasing the pH of a solution of fibrin monomer in 1.0 M NaBr at pH 5.3 by the addition of alkali, and following the pH change accompanying the resultant polymerization. From a series of such experiments

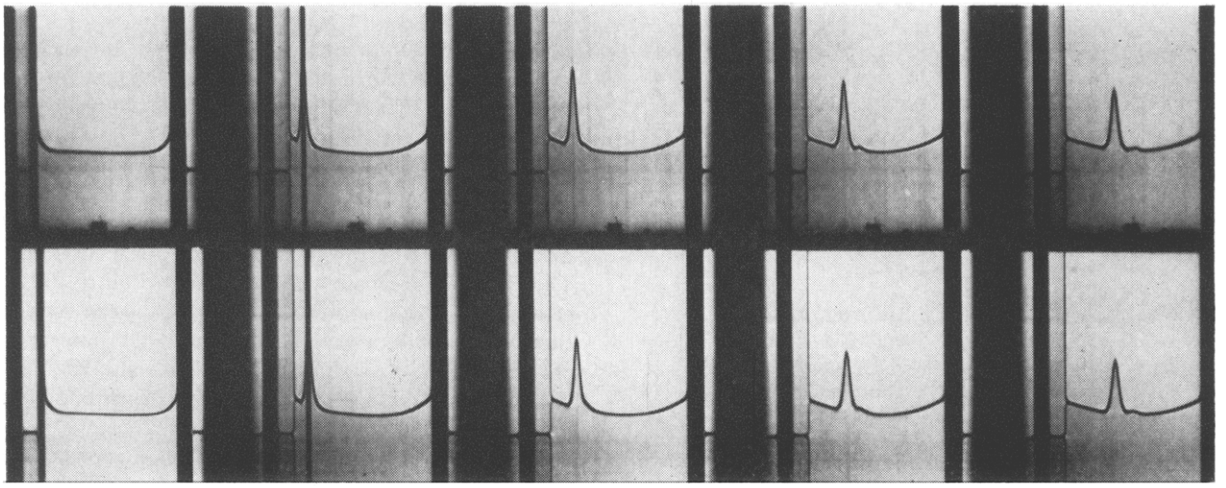


FIGURE 1: Sedimentation of fibrin preparations, 0.61% in 1.0 M NaBr (pH 5.3) at 50,740 rpm at 25°. Upper: control preparation. Lower: acceptor-modified fibrin, 3.0 moles of hydroxylamine bound/mole of fibrin monomer. The photographs were taken at 16-min intervals.

TABLE II: Fibrin Preparations for Ionization Change Studies.

Monomer	H_2NOH Assay (moles/mole of fibrin)	Clottability (%)	Sedimentation Coefficient (S)[a]	OD_{350}/OD_{280} ($\times 10^3$)
Untreated[b]		96–97	6.11	5.5–6.0
Control	0 ± 0.2	96	6.09	4.3
Modified	3.04 ± 0.07	97	6.16	4.4

[a] Protein concentration 0.61% in 1.0 M NaBr (pH 5.3). The value for untreated fibrin monomer is interpolated from data at 0.5 and 0.75%. [b] Purified fibrin monomer used as starting material for modification or control preparations.

at various initial pH, the titration curves of fibrin monomer and the polymer at equilibrium are constructed. The number of protons released or absorbed during polymerization is then computed from the pH change and the slope of the titration curve at that point. The initial and final pH values obtained in the present work were in good agreement with the previously published titration curves for untreated fibrin.

Figure 2 clearly shows that there is no significant difference between the ionization changes accompanying the polymerization of acceptor-modified and control fibrin, within the experimental error. The results also agree satisfactorily with the previous finding for untreated fibrin of a maximum proton release of 1.10 ± 0.03 equiv/10^5 g at initial pH 6.39 ● 0.05, and no net release at pH 7.65 ± 0.015.

Discussion

In our previous analysis (Endres and Scheraga, 1966) of the ionization and enthalpy changes in reversible fibrin polymerization in 1.0 M NaBr, we showed that the data could be accounted for quantitatively on the assumption of intermolecular interactions between ionizable groups. It was found, however, that the available data did not yield a unique set of equilibrium constants and standard enthalpy changes for the process. The data could be equally well accounted for by a large number of moderately exothermic interactions, or by a relatively small number of strongly exothermic interactions. The latter requirement could be met, for example, by coordinate covalent bonds. We favored such a mechanism, since it yielded thermodynamic parameters from the experimental data which appeared to correspond to the functional groups believed at the time to be involved in the subsequent clot stabilization; the "donor" groups were believed to be the α-amino groups of the four N-terminal glycine residues (Lorand *et al.*, 1962), and the "acceptor" groups to be the side-chain amide groups of asparagine or glutamine residues linked to oligosaccharide chains (Chandrasekhar *et al.*, 1964). The mechanism suggested for fibrin polymerization and clot stabilization was as follows, where G_2 is an N-terminal amino acid residue, G_1 is an amide side chain bound to an oligosaccharide through an imido ester linkage, and FSF* is plasma transglutaminase (reactions 1–4). In this mechanism, covalent-bond formation in reversible polymerization is shown in reaction 3 as addition of a free α-amino group (donor group) to a protonated imido ester (acceptor group). The final step,

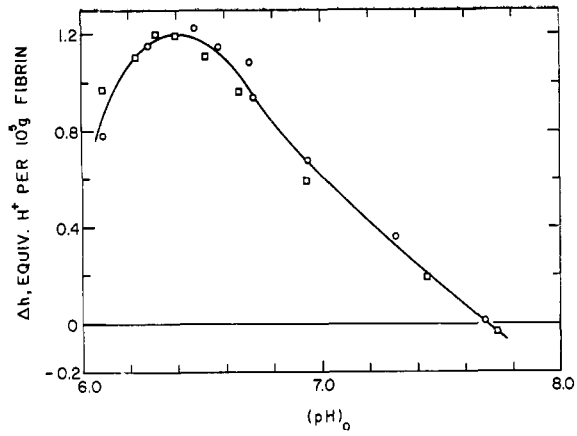
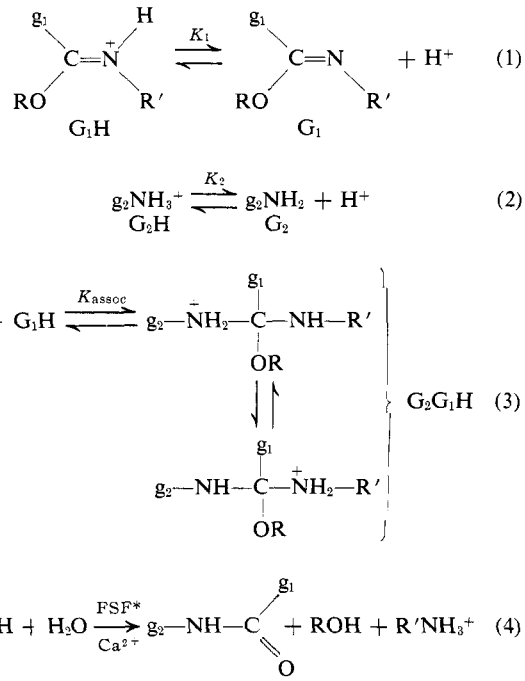


FIGURE 2: Proton release in the polymerization of fibrin (0.59% clottable protein) in 1.0 M NaBr, 25.0°, as a function of initial pH. (○) Control preparation. (□) Acceptor-modified fibrin, 3.0 moles of hydroxylamine bound/mole fibrin monomer.



which is the clot stabilization catalyzed by plasma transglutaminase, is the irreversible formation of an intermolecular amide linkage. If G_1 has the imido ester structure, R′ = H, R is a glycosyl moiety, and the reported liberation of ammonia (Loewy *et al.*, 1964) and oligosaccharide (Chandrasekhar and Laki, 1964) is accounted for.

The above mechanism was attractive because it rationalized most of the experimental facts available at that time. However, since the thermochemical treatment had not yielded a unique set of parameters, more direct experimental evidence was necessary before the mechanism could be accepted. The most direct experimental test of the mechanism appeared to be the study of the polymerization of an acceptor-modified fibrin monomer of the type described by Lorand and Ong (1966). Evidently, certain low molecular weight amines can compete with the natural donor groups (G_2). The above mechanism predicts that reaction of the acceptor groups of fibrin with a simple amine $R''NH_2$ in the presence of plasma transglutaminase would convert them to substituted amide groups g_1CONHR''. Hydroxylamine, for example, would yield hydroxamic acid groups (R″ = OH). Such modified acceptor groups would no longer be expected to participate in covalent bond formation during the polymerization of fibrin monomer. The proposed mechanism predicts that if all of the acceptor groups are modified in this way, the polymerization of fibrin will be accompanied by neither ionization change nor heat evolution. With partially modified fibrin, these effects should be reduced, depending upon the extent of modification.

The work of Lorand and Ong (1966) suggests that a maximum of about 4 moles of hydroxylamine could be incorporated/mole of fibrin (mol wt 3.3×10^5) through the action of plasma transglutaminase, and that this is the number of acceptor groups in one molecule. In the present work we prepared and studied fibrin monomer containing an average of 3.0 bound hydroxylamine residues/molecule. This was evidently bound through the action of plasma transglutaminase, since no hydroxylamine was detected in the control preparation where this enzyme was absent. The stabilization of the clot from the purified modified fibrin by added plasma transglutaminase was found to be strongly retarded, showing that most of the acceptor groups had in fact been blocked. On the basis of the mechanism under consideration, the ionization changes accompanying the polymerization of this material should be only a minor fraction of those observed for unmodified fibrin.

The fact that no significant difference was found in the ionization changes for modified, control, and untreated fibrin is taken as a demonstration that the above mechanism is not operative in reversible fibrin polymerization. It could still be maintained that covalent bonding occurs in reversible polymerization by some mechanism not involving the stabilization acceptor groups. In the absence of supporting chemical evidence, however, this would be no more than an unsupported hypothesis.

The present results are also in accord with recent further investigations of the chemistry of the clot stabilization reaction. It is now believed that carbohydrates are not released at any stage of the conversion of fibrinogen to insoluble fibrin, and that the acceptor groups in clot stabilization are unsubstituted amide side chains of glutamine residues (reviewed by Loewy, 1968). Modification of fibrin monomer by hydroxylamine in the presence of plasma transglutaminase thus appears to simply involve nucleophilic displacement of ammonia, converting these amide groups to hydroxamic acid groups. It has also become clear that the donors are ε-amino groups of lysine residues (Fuller and Doolittle, 1966; Lorand *et al.*, 1966, 1968; Doolittle and Fuller, 1967; Matacic and Loewy, 1968; Pisano *et al.*, 1968). The previous belief that the donors were the α-amino groups of the four N-terminal glycine residues of fibrin led us to assume that the number of group pairs per molecule involved in our suggested covalent mechanism

was necessarily 4. This consideration is clearly no longer valid.

The lack of supporting evidence for the hypothesis of covalent bonding in reversible fibrin polymerization leaves open the question of the origin of the heat evolution and the ionization changes. Even if the suggested mechanism had received support, however, the larger question of the driving force of polymerization (the origin of the negative free-energy change) would have remained. As discussed in the previous paper of this series (Endres and Scheraga, 1966), a covalent mechanism cannot explain the tendency of urea and certain neutral salts to reverse the polymerization. This limitation was also inherent in the particular mechanism suggested, since inhibition of clot stabilization by blocking the acceptor groups does not inhibit reversible polymerization. This was evident in the original work of Lorand *et al.* (1962) and confirmed in the present investigation.

In general, ionization changes during polymerization must result from changes in the environment of ionizable groups of the protein. In the case of fibrin, protons can be either released or absorbed, depending upon the initial pH. This fact, as well as the apparent symmetry of the dependence upon pH (Mihalyi, 1954), suggests that the environmental changes are of a particular kind. The shape of the experimental curve can be accounted for by bonding interactions between two kinds of ionizable groups, one kind in its protonated form and the other kind deprotonated (Sturtevant *et al.*, 1955; Endres and Scheraga, 1966). The hydrogen-bonding mechanism originally proposed was a very attractive one, since it accounted for the effects of salts and urea, and thus was a possible source of the negative free energy of polymerization. (The case for the hydrogen-bonding mechanism was described in detail in 1957 by Scheraga and Laskowski). Its only real shortcoming is its inadequacy in accounting for the *magnitude* of the heat evolution during polymerization. It can be argued that it was too much to expect a single type of intermolecular interaction to account completely for the thermodynamics of polymerization. Even if hydrogen bonding is the dominant interaction, it would be expected that other types of interaction, such as hydrophobic bonding and ion pair bonding, would also occur. The additional heat evolution might come from hydrogen bonds involving nonionizable groups and from ion pair bonds (Kauzmann, 1959). It might also arise from conformational changes during polymerization, as suggested by Mihalyi (1968), although there is apparently no firm evidence for this as yet (Bang, 1967).

Additional experimental work will obviously be required to elucidate further the mechanism of reversible fibrin polymerization. We feel that the method of selectively modifying specific functional groups of the monomer is a powerful one, and we are continuing investigations along this line. Although recent investigations have established that the α-amino groups of fibrin are not involved in clot stabilization, they may well be involved in reversible polymerization by an electrostatic mechanism, as proposed by Ferry *et al.* (1954). We are currently testing this hypothesis by a study of the polymerization of fibrin monomer in which the α-amino groups have been neutralized by carbamylation.

The origin of the heat evolution in reversible fibrin polymerization has become an increasingly important problem. Although it appeared originally that this was an isolated phenomenon, a number of other cases of exothermic protein polymerization have been discovered. These include flagellin (Erlander *et al.*, 1960), β-lactoglobulin (Townend and Timasheff, 1960; Timasheff and Townend, 1961), and lactic dehydrogenase (Millar, 1962). In each of the cases cited, the standard entropy change for polymerization was found to be negative, showing that the negative enthalpy change is responsible for the polymerization.

Acknowledgments

We are indebted to Dr. Ariel G. Loewy for a generous gift of purified factor XIII, to Miss M. C. Oliveira for the purified thrombin, and to Drs. L. Lorand and H. H. Ong for disclosure of their preparative method prior to publication.

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Conformational Aspects of Polypeptide Structure. XXVI. Azoaromatic Side-Chain Effect from Poly-L-*p*-(*p'*-hydroxyphenylazo)phenylalanine*

Murray Goodman and Ettore Benedetti

ABSTRACT: We have undertaken conformational analyses of polymers of L-*p*-(*p'*-hydroxyphenylazo)phenylalanine and copolymers of this amino acid with *N*-(3-hydroxypropyl)-L-glutamine. We compared the ultraviolet, optical rotatory dispersion, and circular dichroism spectra of these materials under neutral, acidic, and basic conditions with results obtained from the model compound, *N*-acetyl-L-*p*-(*p'*-hydroxyphenylazo)phenylalanine methyl ester. In trimethyl phosphate the homopolymer and copolymers exist as right-handed α helices. We have evidence for exciton resonance coupling of spatially adjacent azoaromatic chromophores. In aqueous solutions above pH 10 but below pH 11.9, the homopolymer also exists as a right-handed α helix. Above pH 12 we observe a major change in the circular dichroism spectra. At high salt concentration the magnitude of the Cotton effect is reduced to the values assumed by the model compound under the same conditions. Lastly, in trifluoroacetic acid the azoaromatic residues are protonated. The circular dichroism spectrum provides evidence for an ordered polymer structure, perhaps of the polyelectrolyte type.

It is known that polypeptides containing large chromophores such as the indole, imidazole, and substituted aromatic groups exhibit side-chain in addition to main-chain (peptide) Cotton effects. These side-chain Cotton effects arise because the normally symmetric chromophores are in a dissymmetric environment. The sources of such environments include the asymmetric α-carbon of the peptide group, ordering of the main chain and/or with respect to each other, and intermolecular interactions between adjacent aggregated chains.

It is not completely clear what contribution the side chains make to the optical rotatory dispersion or circular dichroism spectra of polypeptides because side-chain Cotton effects often overlap those arising from the peptide groups. Several aromatic amino acids with auxochromic substituents on the aromatic ring have been investigated in our laboratories such as L-*p*-nitrophenylalanine (M. Goodman, unpublished results), L-*p*-aminophenylalanine (Goodman and Peggion, 1967), and L-*p*-(phenylazo)phenylalanine (Goodman and Kossoy, 1966). In our present report we extend our work on azoaromatic polypeptides and describe the synthesis and the conformational characterization of poly-L-*p*-(*p'*-hydroxyphenylazo)phenylalanine and copolymers of

* Contribution from the Polymer Research Institute and the Department of Chemistry, Polytechnic Institute of Brooklyn, Brooklyn, New York. *Received July 11, 1968*. Presented in part before the 155th National Meeting of the American Chemical Society, March 31–April 5, 1968, San Francisco, Calif., Abstract p-145. This research was supported by the National Science Foundation (Grant GB-7558) and the National Institutes of Health (Grant GM 08974).