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Bovine Fibrinogen. I. Effects of Amidination on Fibrin Monomer Aggregation†

H. M. Phillips and J. Lyndal York*

ABSTRACT: Eighty-four to ninety per cent of the lysine residues in bovine fibrinogen were accessible to ethyl acetimidate modification at pH 8 to 8.5. The pseudo-first-order plot for the amidination reaction was biphasic with 42% of the lysines reacting at the faster rate and 30% at the slower rate. All lysines were trinitrophenylated at pH 9–9.5 in 50% urea. The lysine residues accessible to ethyl acetimidate and distinguishable by their reactivity were also distinguishable on the basis of the effect of their amidination on the pH profile of clot opacity and syneresis. Opacity–per cent amidination studies indicated that some or all of the first 16% of the residues amidinated and that some or all of the residues modified between 57 and 84% play a role in the aggregation of fibrin monomers. The amidination of the middle 40% of the lysine residues had no observable effect on the properties of the clot. We have suggested that the pH shifts observed in the clot opacity–pH transition, clot syneresis–pH transition, and breaking weight maximum are a result of a change in hydrogen-bonding ability due to the change in basicity of lysine and/or amino terminal upon amidination.

Fibrinogen is a large protein involved in blood clotting; however, relatively little is known about its structure, particularly the topography of its functional groups. The biological role of fibrinogen involves the formation of a gel induced by proteolysis by thrombin to form fibrin monomers and subsequent aggregation of the fibrin monomers to form the gel. Mihalyi (1970a) has suggested that the surface features of the molecule should reflect this mechanical function (clot formation) with specific structural details in certain regions on the surface of the fibrin monomer determined by the nature of the forces, or the specific bonds, which are necessary for the aggregation phenomena.

In an attempt to elucidate the mechanism of fibrin polymerization, Mihalyi (1954) observed pH changes accompanying polymerization and suggested that hydrogen bonding may be involved. Sturtevant *et al.* (1955) and Scheraga (1958) have postulated that the links holding the individual fibrin monomers together were of the hydrogen-bond type, based on the observation that the reversible fibrin aggregation is exothermic. Endres *et al.* (1965) found that hydrogen bonding could not account for the magnitude of the heat of polymerization and suggested that reversible fibrin polymerization could be the result of covalent-bond formation between ionizable groups. More recently, Endres and Scheraga (1968) have reported that the concept of reversible covalent-bond formation is no longer tenable based on the ionization changes and heat evolution in the polymerization of an acceptor-modified fibrin monomer.

Many attempts have been made to modify fibrinogen in order to determine the effect on clot formation. Zieve and Solomon (1966) found that photooxidation of fibrinogen renders it unclottable. Also extensive iodination of fibrinogen (Laki and Mihalyi, 1949) and 35% acetylation of fibrinogen (Caspary, 1956) have been found to render fibrinogen nonclottable even though fibrinopeptides A and B were removed by thrombin. These data have been used to suggest a role for lysine and/or tyrosine in the polymerization of fibrin monomers. However, the reagents used in the experiments just quoted are either nonspecific or introduce a large change in charge on the protein which could alter its properties drastically. More recently Fuller and Doolittle (1966) have found that amidination of the amino groups in fibrinogen does not affect clottability or clotting time but does prevent cross-linking of fibrin. Amidination does not change the charge on amino groups but does shift the pK from about 10.5–11.0 to about 12–12.5 (Hunter and Ludwig, 1962).

Thus since amidination is specific for amino groups and

† From the Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas 72207. *Received April 13, 1973*. Supported by the National Science Foundation Grant Gb-30742 X. This work forms part of a dissertation presented by H. M. P. in partial fulfillment of the requirements for the Doctor of Philosophy.

TABLE I: Comparison of the Per Cent of Lysine Trinitrophenylated in Various Fibrinogens.[a]

Bovine Fibrinogen Source	Purifcn	No. of Determinations	% Trinitrophenylation	σ
1. Sigma Fraction I	Laki	4	97.4	1.46
2. Calbiochem	Laki–Blombäck	2	102.5	0.7
3. Sigma Fraction I, amidinated at pH 8–8.3		5	15.9	0.9
4. Sigma Fraction I, amidinated at pH 8.5		4	7.87	1.56
5. Calbiochem amidinated at pH 8.5		3	10.6	2.6

[a] The solvent consisted of 8 M urea–0.2 M sodium borate (pH 9.0).

does not change the charge on the protein (Hunter and Ludwig, 1962; Wofsy and Singer, 1963) we felt that this reaction would allow the determination of the number of accessible lysine residues under milder conditions than used in the past Mihalyi, 1970b) and also would allow us to determine if any of the accessible lysine residues in fibrinogen were involved in the aggregation of fibrin monomers to form the soluble fibrin clot.

Materials and Methods

Bovine fibrinogen (Sigma fraction I, Calbiochem) was purified according to the method of Laki (1951). Recent lots of bovine fibrinogen required a further purification through fractionation steps I-3, I-4 of Blombäck and Blombäck (1956).

Ethyl acetimidate was prepared by the method of McElvain and Nelson (1942) or obtained from Eastman.

Fibrinogen was amidinated similar to the procedure described by Wofsy and Singer (1963). The reaction was conducted with a protein concentration of 20 mg/ml and 0.2–0.45 M ethyl acetimidate in 0.02 M sodium borate buffer–0.15 M NaCl (pH 8–8.5) for 2 hr at 0°. Ethyl acetimidate hydrochloride was adjusted to the desired pH (8.0–8.5) with 5 M NaOH just prior to addition to the reaction mixture. The amidinated fibrinogen was dialyzed three times at 4° against 0.3 M NaCl and frozen. The time course for amidination of fibrinogen was performed in 0.2 M sodium borate buffer (pH 8.2)–0.41 M ethyl acetimidate as described above. At the desired times 1-ml aliquots were removed and added to 1 ml of 2 M lysine (pH 7.0) and reacted 30 min at 0° to stop the reaction. These aliquots were exhaustively dialyzed against 0.3 M NaCl at 4° with six buffer changes over 64 hr. After dialysis the samples were centrifuged at 4° at 20,000 rpm for 30 min.

The number of unmodified lysine residues was determined by trinitrophenylation by a method similar to Ozols and Strittmatter (1966). The difference between the number of groups trinitrophenylated in modified *vs.* unmodified fibrinogen gives the number of lysine residues modified by ethyl acetimidate. The reaction with trinitrobenzenesulfonic acid (TNBS)[1] was performed at 4° for 24 hr or at room temperature for 2 hr in 0.1 M sodium borate buffer (pH 9 or 9.5) in the dark in the presence or absence of urea. The solutions were transferred quantitatively to dialysis bags by rinsing with 0.01% sodium dodecyl sulfate three times and then dialyzed at 25° against three changes of 0.01% sodium dodecyl sulfate and one time against 0.01 M sodium phosphate buffer (pH 7.0). The solutions were then transferred quantitatively to 10- or 25-ml volumetric flasks. The optical densities were recorded at 348 nm. Scatter was corrected by subtracting the optical density at 600 nm. Generally 0.3–3 mg of fibrinogen or 3–8 mg of amidinated fibrinogen was used in the reaction.

The $E_{1\%}^{1cm}$ for fibrinogen was taken to be 15 and 280 nm (Blombäck, 1958). The $E_{1\%}^{1cm}$ for 85% amidinated fibrinogen was calculated from the increase in molecular weight and found to be 14.7.

The sedimentation coefficient of amidinated fibrinogen (4 mg/ml) was determined in 0.3 M NaCl at 25°. The partial specific volume of fibrinogen was taken to be 0.71 (Johnson and Mihalyi, 1965).

Bovine thrombin (Parke-Davis) was prepared by dissolving 5000 NIH units in 1 ml of 0.05 M sodium phosphate buffer (pH 7.0) and by subsequent chromatography on a G-25 column. The thrombin fractions were pooled and diluted to 10 ml so that there were 500 units/ml. The solution was frozen and stored in 1-ml aliquots.

Salting out curves were obtained by adding 0.375 ml of ammonium sulfate solution to 0.75 ml of fibrinogen or amidinated fibrinogen (0.45 mg/ml) in 0.02 M sodium barbital buffer (pH 7.5). The samples were centrifuged and the optical density at 280 nm was recorded on the supernatant.

Per cent clottability of fibrinogen and modified fibrinogen were determined as described by Laki (1951). Clotting times were determined by noting the time for rigidity of the clot to develop and also by measuring the time elapsed before the increase in opacity was observed spectrophotometrically at 600 nm.

Clots for breaking weight measurements were prepared by adding 10 units of thrombin to 1 ml of fibrinogen or 84% amidinated fibrinogen (5 mg/ml) in 0.02 M sodium phosphate–0.02 M sodium borate–0.15 M NaCl buffer of the desired pH. After 1 hr (2 hr at pH 5.6) the clot was removed and synerized between two pieces of moistened filter paper under a weight of 43 g. The clot was then clamped at both ends with small clips, containing a ring of latex tubing. The breaking weight was then determined by fastening one clamp to a support and attaching the second clamp to a force transducer. The clot was then stretched at a rate of 1 cm/70 sec until the clot snapped. The force required to stretch the clot to its breaking point was recorded on a Grass recorder previously calibrated with known weights.

The development of clot opacity was followed spectrophotometrically at 600 nm. Ten units of thrombin was added to 1 ml of fibrinogen and amidinated fibrinogen (2.5–5 mg/ml) in 0.15 M NaCl–0.02 M sodium phosphate–0.02 M sodium borate at the desired pH in a 1-cm path-length cuvette.

A measure of the differences in ability to synerize was determined by the compression of the clots under a centrifugal force of 100,000 rpm for 15 min in a Beckman microfuge centrifuge at room temperature. Five units of thrombin was

[1] Abbreviation used is: TNBS, trinitrobenzenesulfonic acid.

TABLE II: Per Cent of Lysine Residues Amidinated at pH 8.5.

Fibrinogen Prepn	No. of Amidination Expt	Mean % Lys Amidinated	σ
Sigma Fraction I, Laki purification	2	91.5	0.7
Calbiochem Laki–Blombäck purifications	3	89.0	1.2
Sigma Laki–Blombäck purifications	1	92.8	
Average of the three preparations		91.1	1.93

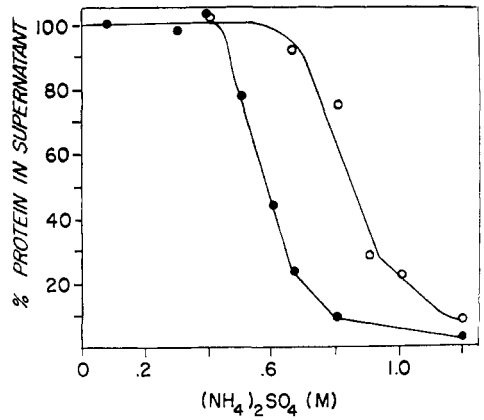

FIGURE 1: Comparison of salting out curves of fibrinogen (○) and 84% amidinated fibrinogen (●). One milliliter of protein in 0.02 M sodium barbital at pH 7.5 was mixed with 0.375 ml of ammonium sulfate solution. Soluble protein was measured at 280 nm. The final protein concentration was 0.3 mg/ml.

added to 0.4 ml of fibrinogen or amidinated fibrinogen (2.5 mg/ml) in 0.15 M NaCl–0.02 M sodium phosphate–0.02 M sodium borate at the desired pH in a polyethylene tube. The samples were clotted for 1 hr at all pH's except pH 5.6 which was clotted 3 hr before centrifugation. The volume of buffer extruded by the centrifugal force was removed with a pasteur pipette and measured.

Results

Recent lots of bovine fibrinogen from Sigma and Calbiochem were found to be unstable when purified by the Laki method. However, subsequent purification by fractionation step I-3, I-4 of Blombäck and Blombäck (1956) gave stable preparations with 95–97% clottabilities. Table I lists the average per cent of lysines trinitrophenylated in 8M urea for the two types of fibrinogen preparations and for fibrinogen previously modified with ethyl acetimidate. It is seen that the per cent of lysines trinitrophenylated is the same for fibrinogen obtained from Sigma purified by the Laki (1951) procedure as for fibrinogen obtained from Calbiochem purified by the Laki (1951) procedure followed by Blombäck and Blombäck (1956) fractionation I-3, I-4. These two preparations of fibrinogen were also amidinated to the same extent as determined by the per cent of lysines left to react with TNBS as seen in Table I. It was observed that in the absence of urea an average of 200 ± 10 residues in fibrinogen are modified by TNBS, but in 8 M urea 210 ± 4 lysine residues are modified by TNBS. This latter value agrees well with the lysine content found by amino acid analysis and has a smaller standard error than found in the absence of urea. Thus 8 M urea is necessary to consistently modify all lysines in fibrinogen with TNBS. The same was found to be true for the trinitrophenylation of all the lysine remaining after amidination. The data in Table I also demonstrate the degree of reliability of this method of lysine determination by trinitrophenylation.

The data in Table II show the reproducibility with which lysine can be amidinated in 0.02 M sodium borate–0.15 M NaCl (pH 8.5) for several fibrinogen preparations. The data in Table III suggest that 84% of the lysines can be amidinated at pH 8 to 8.3 and 88.5% of the lysines is amidinated at pH 8.5. The 84% amidinated fibrinogen was found to sediment as a single sharp peak with an $s_{20,w}$ of 7.88 S which is in the range of unmodified fibrinogen. Figure 1 shows that 84% amidinated fibrinogen is less soluble in ammonium sulfate than fibrinogen. Amidinated fibrinogen is half-precipitated at 0.6 M salt and fibrinogen at 0.8 M. However they both have sharp salting out curves indicating homogeneity of the preparations.

The data show that 85–90% of the lysines of fibrinogen are readily available for reaction with ethyl acetimidate (0.2–0.45 M) in the pH ranges of 8–8.5 while 88.5–97.5% of the lysines of fibrinogen are trinitrophenylated at pH 9. This larger variability in the lysines in fibrinogen trinitrophenylated in buffer may be due to variability of denaturation in the molecule due to change in charge on lysine. Trinitrophenylated fibrinogen in buffer precipitates completely in about 5–10 hr at 4° and the yellow precipitate is soluble in water. The delay in precipitation indicates a change in conformation from a change in charge due to trinitrophenylation. Thus 85–90% and probably no more of the lysine residues in native fibrinogen are completely exposed to the solvent in the pH range of 8.0–8.5.

The time course for amidination of fibrinogen is shown in Figure 2. A pseudo-first-order plot of the data is also shown. The plot is biphasic with a break at 5 min when 42% of the lysine residues have been modified. This indicates that the exposed lysines do not all have the same environment.

A plot of opacity *vs.* pH for fibrinogen and 22 and 84% amidinated fibrinogen is shown in Figure 3. The resulting curve for the 22% amidinated fibrin clot parallels that for the unmodified fibrin clot except that it is shifted to higher pH by about 0.5 pH unit. The opacity curve for 84% amidinated fibrin clot is biphasic: at pH 6.5–7 the curve parallels the un-

TABLE III: Effect of pH on the Per Cent of Lysine Residues Amidinated, 0.2 M Borate.

pH	No. of Amidination Expt	Mean % Lys Amidinated	σ
8	2	84.3	0.7
8.3	2	83.7	0.5
8.5	2	88.5	2.1
8–8.3	1	84	

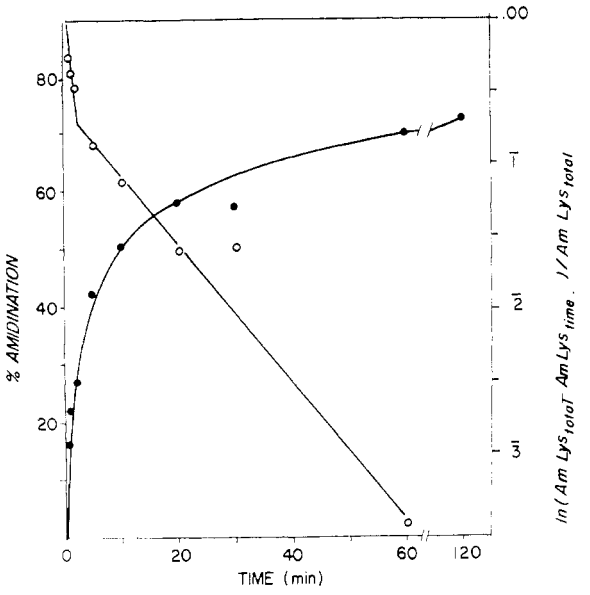

FIGURE 2: Rate of reaction of lysine in fibrinogen (20 mg/ml) with 0.41 M ethyl acetimidate in 0.2 M sodium borate buffer (pH 8.2) at 0° (●). (○) Pseudo-first-order representation (ordinate to the right).

modified clot but is shifted to higher pH by 1.0 unit; however, in the range of pH 7 to 9.5 the opacity is relatively independent of pH and above pH 10 the opacity begins to decrease again. A plot of opacity *vs.* per cent amidination is shown in Figure 4a. All degrees of amidination studied give clots with higher

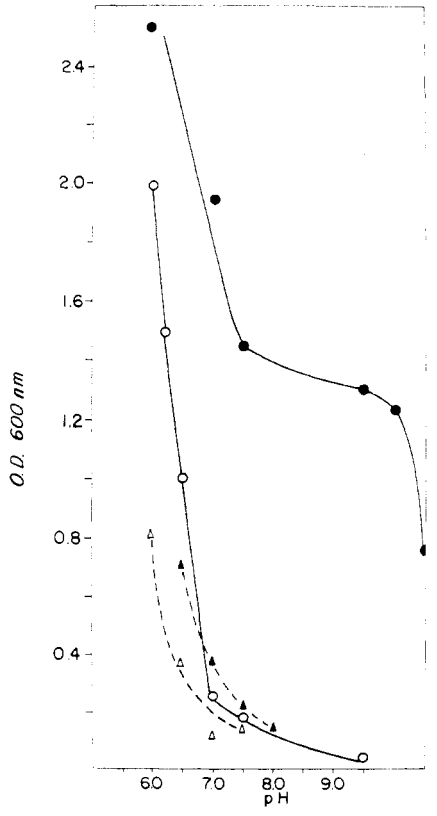

FIGURE 3: Clot opacity–pH curves of fibrinogen and amidinated fibrinogen in 0.2 M sodium phosphate–0.02 M sodium borate–0.15 M NaCl: (○) fibrinogen, 5 mg/ml; (●) 84% amidinated fibrinogen, 5 mg/ml; (△) fibrinogen, 2.5 mg/ml; (▲) 22% amidinated fibrinogen, 2.5 mg/ml.

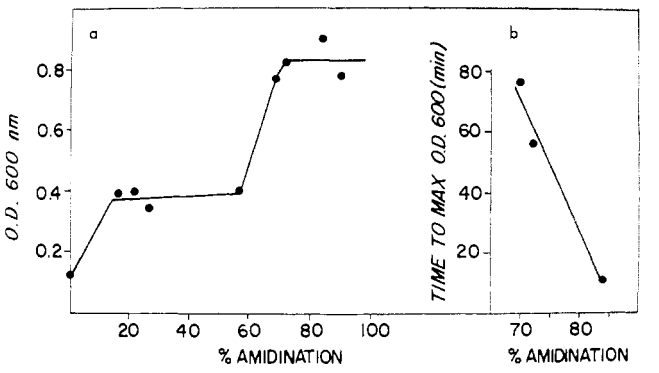

FIGURE 4: (a) Clot opacity *vs.* per cent amidination at pH 7 in the buffer described in Figure 3. Protein concentration was 2.5 mg/ml. (b) Plot of the time required for development of maximum opacity *vs.* percent amidination. Opacity was measured under conditions of part a.

opacity than unmodified fibrinogen at pH 7. Interestingly, there are two opacity plateaus, one from 16 to 57% amidination and a second from 70 to 90% amidination. The time to attain maximum optical density in the second plateau depends on the per cent amidination as shown in Figure 4b. These data suggest that two different classes of functional groups (lysines and/or amino terminal) may be responsible for the observed changes in opacity due to amidination.

A plot of breaking weight *vs.* pH for fibrinogen and 84% amidinated fibrinogen is shown in Figure 5. Modification of fibrinogen with ethyl acetimidate did not cause a decrease in breaking weight, but increased the pH of the maximum breaking weight by 1.3 units.

The extent of syneresis as a function of pH as determined by the volume extruded by centrifugal force is shown in Figure 6b. Amidination shifts the pH for the transition between synerizable to nonsynerizable clot to higher pH. The degree of syneresis as a function of per cent amidination is shown in Figure 6a at pH 6 and 6.5. At pH 6 all amidinated clots are sedimented compared to the nonamidinated clot. At pH 6.5 the clots formed from 16 to 72% amidination are only partially sedimented and the 84 and 90% amidinated clots are completely sedimented. Thus the syneresis of amidinated fibrin is decreased as the pH increases as for unmodified fibrin, however the pH at which the transition occurs is higher and depends on the degree of amidination.

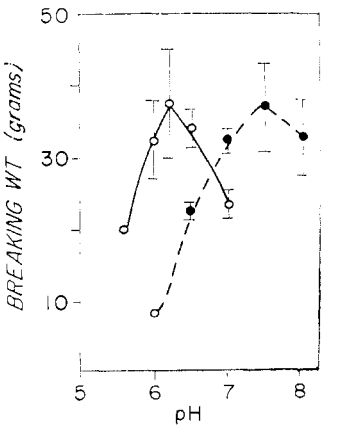

FIGURE 5: Breaking weight *vs.* pH of clot (5 mg/ml) in the buffer described in Figure 3: (○) fibrinogen; (●) 84% amidinated fibrinogen.

Thus the data presented here show that the opacity transition, extent of syneresis and maximum breaking weight are similar in modified and unmodified clots but are shifted to higher pH by about 0.5–1.0 pH unit. This shift of 0.5–1.0 pH unit is similar to the shift in pK for the ϵ-amino of lysine upon amidination (10.5–11.00 to 12–12.5).

Discussion

Accessibility of Lysine Residues. Reaction of native fibrinogen with ethyl acetimidate results in modification of 84–90% of the lysine residues (Tables II and III). A pseudo-first-order plot of the time course of the reaction in Figure 2 shows that the reaction is biphasic. Forty-two per cent of the lysines is amidinated at the faster rate and thirty per cent at the slower rate. This difference in rate of modification shows that some lysines have a different environment. Since all lysines and α-amino groups are accessible to TNBS at pH 9–9.5 in 50% urea, these data are consistent with classification of the fibrinogen lysine residues into three categories based on reactivity and accessibility to ethyl acetimidate: (1) those exposed in native fibrinogen which react quickly, (2) those that react slower, and (3) those exposed by unfolding in 50% urea.

Characteristics of Amidinated Fibrinogen. The physiological role of fibrinogen is as a substrate for thrombin, which converts fibrinogen to fibrin. Modification of fibrinogen with ethyl acetimidate results in a fibrinogen that behaved identically with native fibrinogen as a substrate for thrombin as measured by the clotting time at any given pH and by per cent clottability. The clotting time *vs.* pH curve we found for fibrinogen agrees with that reported by Shulman and Ferry (1950). Fuller and Doolittle (1966) reported that they also observed no change in clotting time and clottability for amidinated fibrinogen. Furthermore the sedimentation coefficient determined for amidinated fibrinogen (7.88) agrees with that found for unmodified fibrinogen.

These results are in contrast to those found by Caspary (1956) for acetylation of fibrinogen. In this case it was found that the fibrinogen became nonclottable by thrombin after 35% acetylation even though peptides A and B were removed by the thrombin. The same disparity between the aggregation properties of acetylated fibrin monomers as compared to amidinated fibrin monomers is also observed in the aggregation properties of amidinated or acetylated TMV protein monomers (Perham and Richards, 1968). These workers suggest that modification of lysine wtih ethyl acetimidate does not change the charge on lysine and allows aggregation whereas acetylation removes the positive charge and also prevents aggregation, thereby suggesting that the charge on lysine plays a role in aggregation. In general an alternative explanation is that the change in charge on lysine is sufficient preturbation to cause a conformational change. This point is discussed in the following paper (Phillips and York, 1973) with respect to acetylation of fibrinogen.

In view of the mildness of the amidination conditions and the aforementioned native properties of amidinated fibrinogen, it is interesting to observe that properties of the clot such as breaking weight, opacity, and syneresis are affected by amidination. The maximum breaking weight of the amidinated fibrin clot is shifted 1.3 pH units higher than for the unmodified fibrin clot and the opacity curves are shifted to one pH unit higher for 84% amidinated than for unmodified fibrinogen. Also the maximum in the breaking weight curves occurs when the opacity of the clots are the same. Thus we can conclude that amidination of fibrinogen does not affect the net attractive forces between fibrin monomers but shifts the pH of maximum attractive force to a higher pH since the breaking weight curve (a measure of net attractive force) is shifted to a higher pH.

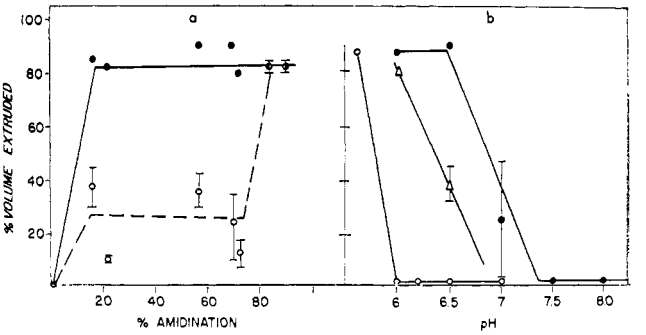

FIGURE 6: (A) Per cent volume extruded from clot (amount of syneresis) *vs.* per cent amidination: (●) pH 6; (○) pH 6.5. (B) Per cent volume extruded *vs.* pH. (○) fibrin clot; (△) 16% amidinated fibrin clot; (●) 84% amidinated fibrin clots. The protein concentration was 2.5 mg/ml in the buffer described in Figure 3.

The shift in pH of the properties of the amidinated clot is comparable to the shift in pK upon amidination of lysine (10.5–11 for lysine and 12–12.5 for amidination of the ϵ-amino group of lysine (Hunter and Ludwig, 1962).

Ferry and Morrison (1947) suggested that opacity of fibrin clots is determined by the type of aggregation that occurs upon clot formation. They observed that fibrin clots are more opaque at low pH than at high pH and suggested that the opaque clot (coarse clot) is formed by larger amounts of side-to-side aggregation whereas the clear clot (fine clot) is formed by more end to end aggregation. This postulate has been confirmed by electron microscope studies (Hawn and Porter, 1947) on clots as a function of pH. The opacity–pH curve presented here on fibrinogen agrees with those of Ferry and Morrison (1947). The opacity–pH curve for the 22% amidinated fibrin clot parallels that for the fibrin clot but is shifted to higher pH by 0.5 unit whereas the opacity–pH curve for the 84% amidinated fibrin clot is biphasic being shifted by about 1.0 pH unit up to pH 7 where it becomes relatively independent of pH up to 9.5 at which point a further decrease in opacity is observed. If one accepts the study of Hawn and Porter (1947) as sufficient confirmation of the hypothesis that opacity of fibrin clots is a measure of the proportion of side-to-side aggregation relative to end-to-end aggregation, then the shift to higher pH of the opacity curves (Figure 3) suggests that certain lysine residues may play a role in aggregation, particularly side-to-side aggregation. Furthermore the opacity–per cent amidination curve (Figure 4a) indicates that some or all of the first 16% of the residues modified and that some or all of the residues modified between 57 and 84% may play a role in the aggregation of fibrin monomers but that the modification of the middle 40% has no observable affect on the properties of the clot. Thus the residues accessible to ethyl acetimidate and distinguishable by their reactivity are also distinguishable on the basis of the effect of their amidination on clot opacity and on clot syneresis.

In conclusion these data imply that certain lysine residues and perhaps the amino terminal on the γ chain play a role in fibrin polymerization. The greatest change on lysine or amino terminal upon amidination is in the pK of the amidino group of about 12–12.5 compared to that of 10.5–11.0 for lysine and about 8 for amino terminal. Since a change in basicity can change hydrogen bonding characteristics, perhaps it is this

parameter which is responsible for the pH shifts observed for the clot opacity, syneresis, and breaking weights.

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Bovine Fibrinogen. II. Effects of Tyrosine Modification on Fibrin Monomer Aggregation†

H. M. Phillips and J. Lyndal York*

ABSTRACT: Contrary to earlier reports of 35–40 free tyrosines we have found 15–20 tyrosine residues exposed in fibrinogen. No additional residues are exposed on conversion of fibrinogen to fibrin, indicating no large conformational change as a result of this conversion. In addition it is shown that modification of tyrosine does not change the clotting time and clottability of fibrinogen, but does decrease the clot strength. The evidence indicates that only two to three modified tyrosine residues are responsible for the change in clot strength. Since modification of these tyrosine residues does not inhibit clotting but changes the physical properties of the clot formed, tyrosine hydrogen bonding is not the driving force for aggregation but rather may serve in the alignment of the fibrin monomers preceeding aggregation.

Studies by several investigators leading to the suggestion that lysine and/or tyrosine may play a role in fibrin polymerization have been reviewed (Phillips and York, 1973). If tyrosine residues are involved in fibrin polymerization they must certainly be exposed in fibrin and perhaps in fibrinogen but not necessarily so. Thus the determination of the number of available tyrosine residues in fibrinogen and fibrin and the effect of tyrosine modification on clottability and the physical properties of the resulting clots is meaningful in understanding fibrin polymerization.

Aside from the possible role of tyrosine in fibrin polymerization, a knowledge of the number of accessible tyrosine and lysine residues as well as other functional groups is evidence which would be useful in distinguishing between various models that have been suggested for the structure of fibrin. For instance, the model of three globular units connected by thin rods proposed by Hall and Slayter (1959) suggests a more compact structure than the "birdcage" of Köppel (1970) which predicts 90% of the molecule is exposed to solvent.

Previous attempts to determine the number of accessible tyrosine residues in fibrinogen by acylation with *N*-acetylimidazole (Huseby and Murray, 1967, 1969) or by spectrophotometric titration (Mihalyi, 1968) yielded values in the range of 35–40.

Chemical modification of amino acids on proteins has long been used to distinguish between freely available and buried residues; however, this approach may yield anomalous results if the reagent has low specificity and reaction occurs with another residue such as lysine resulting in a conformational change in the protein and the exposure of a normally buried residue such as tyrosine. Likewise, spectrophotometric titration will give high values if the protein conformation is sensitive to pH as is the case with fibrinogen (Mihalyi, 1965).

To circumvent these problems we wish to report the number of free tyrosine residues in fibrinogen using a tyrosine-specific reagent, tetranitromethane (Sokolovsky *et al.*, 1966), and in amidinated fibrinogen and amidinated fibrin using *N*-acetylimidazole. Data are also presented on the breaking weight, a measure of clot strength, and clottability of tyrosine-modified fibrin clots.

† From the Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas 72207. *Received April 13, 1973.* Supported by the National Science Foundation Grant GB-30742X. This work forms part of a dissertation presented by H. M. P. in partial fulfillment of the requirements for the Doctor of Philosophy.