THE FREE AND BURIED TYROSYL AND HISTIDYL GROUPS IN BOVINE FIBRINOGEN

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Bovine fibrinogen has been examined by chemical modification techniques using N-acetyl imidazole and 1-H-diazonium tetrazole to determine the free and buried tyrosyl and histidyl residues in the molecule. The data indicates that bovine fibrinogen contains 6 to 8 buried histidyl and 20-21 buried tyrosyl residues. Reaction of bovine fibrinogen with N-acetyl imidazole indicated that 35 tyrosyl groups are exposed in the molecule. At lower urea concentrations this value increases to 80 tyrosyl groups. The remaining tyrosyl groups are inaccessible to the reagent at urea concentrations up to and including 8 M.

In the initial stages of blood coagulation, intermediate polymers of fibrin are formed by the action of the enzyme thrombin on the fibrinogen molecule. This event is followed by gelation and visible phase separation. If the fibrin is warmed or dissolved in urea, and then cooled or dialyzed free of urea, the clot will reform. Several theories have evolved concerning the possible amino acid residues responsible for the association of fibrin monomers. Possible explanations are found in both hydrogen (Sturtevant et al., 1955) and intermolecular co-ordinate covalent bonding (Endus and Scheraga, 1966). Recently, the postulate of co-ordinate covalent bonding for fibrin formation has been questioned (Endus and Scheraga, 1968). The true mechanism by which fibrin polymerizes is still unresolved. It has been noted that photo-oxidation of the fibrinogen molecule renders this protein unclottable (Belister and Kotbova, 1960 and Zieve and Solomon, 1966). Fibrinogen does not form normal polymers at pH values below 5.0 or above 10.0. From these findings, implication of tyrosine and histidine

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in the formation of fibrin polymers has evolved. Little is known of the various states of the amino acid moieties in the fibrinogen molecule or their possible role in the formation of fibrin.

In this communication we wish to report preliminary results employing N-acetyl imidazole (Riordan et al., 1965) and 1-H-diazonium tetrazole (Horinishi et al., 1964 and Sokolovsky and Vallee, 1966) in the determination of the free and buried histidyl and tyrosyl residues in bovine fibrinogen.

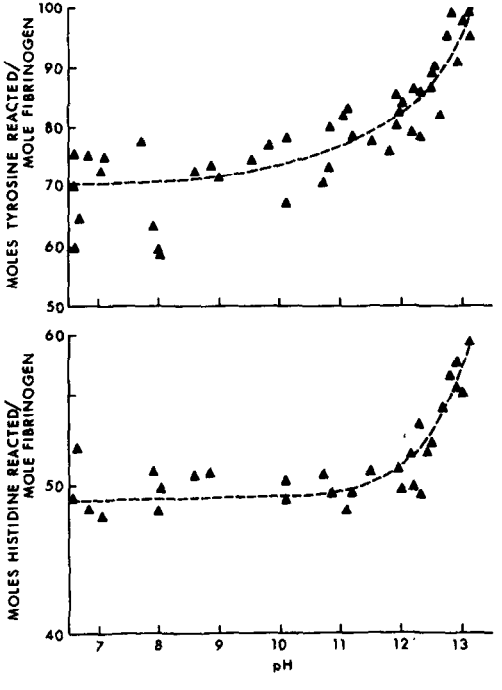
Materials and Methods

Purified bovine fibrinogen (Blombäck and Blombäck, 1956) of 97 percent clottability or greater was dialyzed against 0.1 M NaCl and frozen in small amounts at -20°C.

N-acetyl imidazole (K & K Laboratories) was purified from benzene-petroleum ether and stored over P_3O_5 · 5-Amino-1-H-tetrazole (Eastman) was used without further purification.

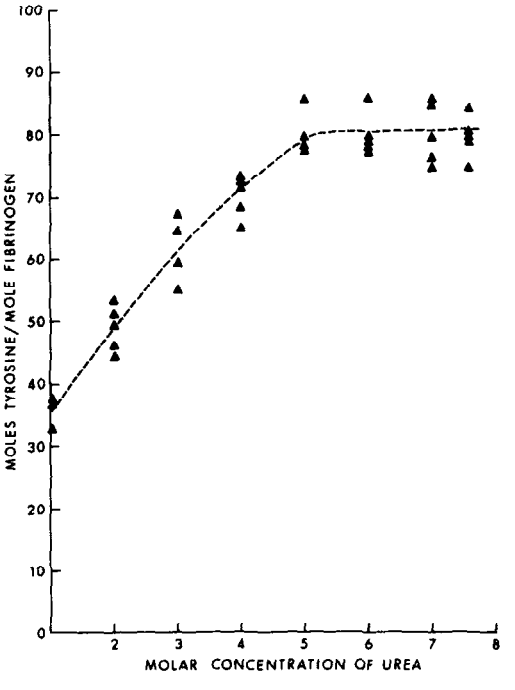
In typical experiments with 1-H-diazonium tetrazole, bovine fibrinogen solutions were diluted to a final concentration of 0.3 mg. protein/ml. with 0.065 M NaCl of the desired pH. The solutions were incubated at 40°C. for two hours and then cooled to 25°C. The solutions containing the protein were then rapidly mixed with 1 M $KHCO_3$, pH 8.8, to a dilution of 1:9 and 2 mls. of 1-H-diazonium tetrazole (Sokolovsky and Vallee, 1966) were added. The pH was adjusted to 8.8, if necessary, after the addition. A second 2 ml. portion of the diazonium reagent was added after 10 minutes and, again, the pH was adjusted to 8.8. After one hour the protein solutions were diluted to a readable optical density with 1 M K_2HPO_4 and the absorption spectra of the solutions were recorded at 550 and 480 mμ. Calculations of the molar fractions of histidine and tyrosine reacted were performed as previously described (Sokolovsky and Vallee, 1966). Validation of the results for histidine at 600 mμ, as suggested by Sokolovsky and Vallee (1966), were made, and agreement with the values as determined at 480 mμ were within ±3 percent.

Figure 1



eaction of fibrinogen with 1-H-diazonium tetrazole fter incubation of the protein for 2 hours at the pH alues indicated in Figure 1. Fibrinogen solutions 10^{-6} M) were incubated as indicated above and then djusted to pH 8.8 with 1 M $KHCO_3$. The reaction of the pH adjusted solutions with 1-H-diazonium tetrazole was performed as described in the text.

Figure 2



Reaction of fibrinogen with N-acetyl imidazole at various urea concentrations. Fibrinogen (10^{-6} M) in 0.02 M veronal-urea solutions, pH 7.0, reacted with N-acetyl imidazole (130 M excess/mole tyrosine). After reaction with the reagent as described above the solutions were dialyzed and calculations of mole ratios reacted were made as described in the experimental section.

N-acetyl imidazole was reacted with fibrinogen solutions in a similar manner as described above. The fibrinogen solutions were diluted to a final concentration of 0.3 mg. protein/ml. with 0.02 M veronal buffer, pH 7.5, containing urea of varying molar concentrations. The protein solutions were incubated at 40°C. for two hours in the presence of N-acetyl imidazole (130 M excess per mole tyrosine), cooled, and dialyzed against 1800 volumes of 0.1 M sodium phosphate buffer, pH 7.0. The number of acetylated tyrosine groups/mg. of protein was determined spectrophotometrically at 278 mμ, using 1 M hydroxyl amine (Simpson et al., 1963).

Fibrinogen concentrations in all experiments were determined at 280 mμ using the previously reported $E^{280}_{1\%}$ of 15.0 (Pollara, 1963).

Results and Discussion

The reaction of 1-H-diazonium tetrazole with bovine fibrinogen is shown in Figure 1. The tyrosine curve shows a general increase in the number of tyrosyl groups available to react from a minimum value of 70 groups per molecule at pH 7.0 to a maximum of 98-100 groups after unfolding of the protein under alkali conditions. A sharp rise in the slope of the curve is observed above pH 12.0. The histidyl curve shows that 49 histidyl groups are accessable at neutral pH. At pH values above 12.0 this value increases to 58-59 groups. The results for total histidyl and tyrosyl groups determined by this method are in excellent agreement with values found by amino acid analysis (Huseby and Murray, 1967).

The reaction of N-acetyl imidazole with fibrinogen is shown in Figure 2. At neutral pH, 35 tyrosyl groups are seen exposed in the molecule. With increasing concentrations of urea, a constant value of 80 tyrosyl groups reacting is seen above a urea concentration of 5 M. No distinction has been made between different residues in different environments during the course of unfolding of the protein. Only the

total free and buried residues before and after unfolding of the protein, under the experimental conditions outlined, are considered.

As shown earlier (Huseby and Murray, 1967), the number of available tyrosine groups for titration in the native state for the bovine fibrinogen molecule ranges between 30 and 35. If unfolding of the protein occurs, this value increases from 75 to 77 groups. These values as determined by spectrophotometric titration are in close agreement to the values obtained with N-acetyl imidazole as seen in Figure 2.

The observed rate of the availability of tyrosyl groups at pH 12.5 during spectrophotometric titration has been shown to be very rapid (Huseby and Murray, 1967). It was thus concluded that under these conditions a major, rapid, conformational change occurs. From the above data, it may be seen that some 35 to 40 tyrosyl groups in bovine fibrinogen are exposed in the molecule. An additional 35 to 40 tyrosyl groups are easily accessable after unfolding of the molecule in lower urea concentration. The remaining 20 tyrosyl groups are buried. In a like manner, Figure 1 shows that only 6 to 8 histidyl groups and 20 to 21 tyrosyl groups are buried within the bovine fibrinogen molecule after the protein has been allowed to unfold under alkaline conditions. As has been shown by Mihalyi and Godfrey (1963), trypsin treatment of fibrinogen results in the production of a refractory "core" of molecular weight of 95,000. This "core" may contain the buried tyrosyl and histidyl residues in bovine fibrinogen. Optical rotatory dispersion studies by Mihalyi (1965) have shown that incomplete unfolding of bovine fibrinogen occurs in 8 M urea, with retention of 20 percent of the secondary structure of the molecule. These data indicate that this area of the molecule could contain the buried histidyl and tyrosyl residues in bovine fibrinogen. Studies are in progress to determine the location of the buried histidyl and tyrosyl residues in the molecule and their role in the structure and polymerization characteristics of bovine fibrinogen.

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