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Purification of Human Factor VIII:C and Its Characterization by Western Blotting Using Monoclonal Antibodies†

Frances Rotblat,*,‡ Donogh P. O'Brien,‡ Fergal J. O'Brien,‡ Alison H. Goodall,§ and Edward G. D. Tuddenham‡
Haemophilia Centre, Academic Department of Haematology, and Department of Immunology, Royal Free Hospital and School of Medicine, London NW3 2QG, U.K.

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ABSTRACT: Human factor VIII:C has been purified over 300 000-fold from cryoprecipitate by polyelectrolyte purification followed by affinity chromatography on Sepharose linked to antibody to factor VIIIR:Ag (monoclonal or polyclonal) and Sepharose linked to monoclonal antibody to factor VIII:C. The purified material has been analyzed by polyacrylamide gel electrophoresis (PAGE) and Western blotting using monoclonal antibodies. PAGE shows predominant bands at 360K (unreduced), 210K, and 90K and an 80K/79K doublet; Western blotting showed all the monoclonal antibodies used bound the 360K form. In a small-scale purification, plasma from blood taken directly into thrombin inhibitor Kabi S-2581 was applied directly to the monoclonal anti-factor VIII:C column. Western blot analysis of this material showed the 360K band on reduction. The purified factor VIII:C could be activated 13-fold by human thrombin. Gel analysis of the activated material showed intensification followed by fading of the band at 90K and generation of bands at 70K/69K, 55K, and 40K. Western blotting shows that the 70K/69K doublet derives from the 80K/79K moiety and the 40K peptide derives from the 90K and is presumed to contain the active site. From these studies an epitope map of the factor VIII:C molecule has been constructed.

The commonest severe congenital bleeding disorder in all races is haemophilia A. This condition is inherited as a sex-linked recessive trait, affecting approximately 1 in 5000 males (Rizza, 1972). The characteristic defect is lack of coagulation factor VIII:C. Factor VIII:C is a glycoprotein (Tuddenham et al., 1979) that functions as a cofactor for the activation of factor X by activated factor IX in the intrinsic coagulation cascade (Mertens & Bertina, 1980) in the presence of phospholipid and calcium. Factor VIII:C is highly susceptible to proteolysis by thrombin, plasmin, and other serine proteases (Atichartakarn et al., 1978). In plasma it is noncovalently linked to von Willebrand factor (Hoyer, 1981). The latter is a high molecular weight protein designated factor VIII related antigen (VIIIR:Ag) consisting of a series of oligomers ranging in size from 10^6 to 20×10^6 through covalent linkage of a 200K protomer (Ruggieri & Zimmerman, 1981; Hoyer & Shainoff, 1982). VIIIR:Ag probably stabilizes factor VIII in vivo by protecting it from proteolysis (Weiss et al., 1977) but is also necessary for platelet adhesion (Weiss et al., 1978). The plasma concentration of factor VIII:C is extremely low, and this combined with its marked tendency to proteolytic degradation has frustrated many attempts to purify it to homogeneity. These problems may be overcome by using very large quantities of starting material as was demonstrated first by Vehar & Davie (1980) for bovine factor VIII. From 125 L of bovine plasma these authors obtained 400 μg of protein that appeared as a triplet of bands of M_r 93K, 88K, and 85K on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

A new approach to purification of factor VIII using specific polyclonal and monoclonal antibodies was reported by Fass et al. (1982) using porcine plasma as starting material. The end product appeared on SDS–PAGE to consist of subunits with M_r 160K, 130K, and 76K. Fulcher & Zimmerman (1982) modified the method of Tuddenham et al. (1978) by substituting a monoclonal antibody to factor VIIIR:Ag for polyclonal antibody and further purified the material on (Aminohexyl)agarose as earlier described by Austin (1979). The starting material for this purification was commercial factor VIII concentrate, and the end product consistently contained high molecular weight contaminants. Also there were strong indications of partial proteolysis. The majority of their material was shown to be factor VIII by binding of specific antibody.

In this paper we describe a method for purifying human factor VIII:C from cryoprecipitate, in which potent proteolytic inhibitors were used throughout. The polyelectrolyte procedure of Johnson et al. (1978) was used as a preliminary step before immunoaffinity chromatography with antibody to factor VIIIR:Ag followed by adsorption to a monoclonal antibody specific to factor VIII:C (Rotblat et al., 1983).

Materials and Methods

Protein was estimated by a modification of the method of Lowry (Hartree, 1972). Factor VIII:C was assayed by a one-stage technique using plasma from a patient with severe haemophilia A (factor VIII:C 0%) as substrate (Breckenridge & Ratnoff, 1962). Factor VIIIR:Ag was measured by a one-stage immunoradiometric assay using rabbit antibody to

† This work was sponsored by a research grant from Speywood Laboratories, Wrexham, U.K.
‡ Haemophilia Centre, Academic Department of Haematology, Royal Free Hospital and School of Medicine.
§ Department of Immunology, Royal Free Hospital and School of Medicine.

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VIIIR:Ag (Hoyer, 1972). Factor VIII:C inhibition was assayed in the Bethesda system (Kasper et al., 1975). The production and characteristics of the monoclonal antibodies to factor VIII:C have been previously described (Rotblat et al., 1983). Antibodies were radiolabeled with ^{125}I by the chloramine T method (Hunter & Greenwood, 1962). Immunoglobulin was prepared from ascites by Rivanol–ammonium sulfate precipitation (Heide & Schwick, 1978). All chemicals were of reagent grade and obtained from BDH, Poole, Dorset, U.K. or Sigma London Ltd., Poole, Dorset, U.K. Polyelectrolyte E5 was a gift of Speywood Laboratories, Wrexham, Clwyd, Wales.

Purification of VIII:C. The starting material was from 1–3 kg of cryoprecipitate obtained from several commercial sources. This was defrosted into 0.02 M tris(hydroxymethyl)aminomethane (Tris) and 0.15 M NaCl, pH 6.5, with 10 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM benzamidine to a final volume of 10 L. The cryoprecipitate was then adsorbed with 1/10th volume of 2% aluminium hydroxide (Alhydrogel). After centrifugation at 3900*g* for 20 min, the supernatant was made 0.02 M citrate and applied to a column containing 750 g of polyelectrolyte E5 (which had been protonated at pH 3.5 for 15 min and washed extensively with 0.02 M citrate–0.15 M NaCl, pH 6.5) (Rotblat et al., 1983) at a flow rate of 120 mL/min.

The polyelectrolyte was washed with 15 L of 0.02 M citrate–0.2 M NaCl, and then the factor VIII (PE VIII) was eluted in a volume of 18 L of 0.02 M citrate–1 M NaCl, pH 6.5. All buffers contained 10 mM PMSF and 10 mM benzamidine. PE VIII was concentrated at 20% PEG 4000 with human serum albumin as a coprecipitant. After centrifugation at 3000*g* for 20 min, the resultant precipitate was redissolved in 300 mL of barbital saline, pH 7.5, containing 2 mM diisopropyl fluorophosphate (DFP) and 10 mM benzamidine.

The concentrated PE VIII was then applied to a 2.2 × 30 cm column containing 80 mL of Sepharose 2B linked to antibody to factor VIIIR:Ag, at a flow rate 1.5 mL/min. The preparation of this column has been previously described (Rotblat et al., 1983). The outflow was monitored at 280 nm, and after the column was washed down to base line with barbital saline, pH 7.5, the factor VIII:C was eluted in 0.02 M imidazole–0.24 M calcium chloride, pH 7.5, containing 2 mM DFP and 10 mM benzamidine. Fractions of 6 mL were collected and assayed for factor VIII:C. The peak fractions were pooled and applied to a bank of three columns connected in series as follows: 5-mL Sepharose 2B–rabbit polyclonal anti-VIIIR:Ag (DAKO), 12-mL Sepharose–2B monoclonal antibody to fibronectin (the gift of Dr. L. Tredjosowitz, Department of Immunology, Royal Free Hospital), and 18-mL Sepharose 2B–monoclonal antibody to factor VIII:C No. 8 (Rotblat et al., 1983). The antibodies were coupled to Sepharose by the method previously described (Rotblat et al., 1983) at a ratio of 2 mg of immunoglobulin to 1 mL of settled Sepharose. The flow rate of the columns was 1 mL/min. The columns were then washed with the 0.02 M imidazole–0.24 M calcium chloride, pH 7.5, until the optical density of the eluate began to fall. Then, the first two columns were removed, and the factor VIII:C column was washed with barbital saline containing 2 M NaCl, 2 mM DFP, and 10 mM benzamidine, pH 7.4. The factor VIII:C was eluted by a multicomponent buffer consisting of 0.05 M imidazole, 1 M potassium iodide, 20% ethylene glycol, 2 mM DFP, and 10 mM benzamidine, pH 6.5, after reversal of the direction of flow of the column. Fractions of 2 mL were collected, and factor VIII:C was assayed immediately. Peak fractions were pooled and dialyzed against barbital saline, pH 7.5. The purified factor VIII:C was concentrated by lyophilization after dialysis against ammonium bicarbonate or by dialysis against solid PEG 8000.

Small-Scale Factor VIII:C Purification. A 450-mL aliquot of blood was drawn from a normal volunteer into 50 mL of 3.8% trisodium citrate containing 20 mM S-2581 (Kabivitrum Ag, Sweden) and 1 mM benzamidine. After centrifugation at 3000*g* for 20 min the plasma was separated and made 2 mM in DFP and 240 mM in calcium chloride. This plasma was applied to a column containing 10 mL of Sepharose 2B–monoclonal antibody to VIII:C (see above) at a flow rate of 1 mL/min. The column was washed with 0.05 M imidazole–2 M NaCl, pH 7.5, and after reversal of the flow direction, factor VIII:C was eluted in 0.05 M imidazole, 1 M potassium iodide, 20% ethylene glycol, 2 mM DFP, and 10 mM benzamidine, pH 6.5. Fractions of 2.2 mL were collected and assayed immediately for activity. Peak fractions were pooled and dialyzed overnight at 4 °C against barbital saline, pH 7.5, with 2 mM DFP. The material was then concentrated to 300 μL by dialysis against solid PEG 8000.

Activation of Purified Factor VIII:C by Thrombin. Purified factor VIII:C was diluted in barbital saline, pH 7.5, with 0.1% bovine serum albumin to a final concentration of 240 units/mL. A total of 80 μL of this solution was incubated at 4 °C with 2 units of purified human thrombin, sp act. 3000 units/mg (Sigma London Ltd., Poole, Dorset). The final thrombin concentration was 8.25 μg/mL. Aliquots were sampled at the following times; pre thrombin addition and 2, 5, 10, 20, 30, 60, and 120 min after thrombin addition. Dilutions were made and immediately added to tubes of phospholipid, kaolin, and factor VIII:C deficient plasma that had been preincubated at 37 °C for 7.5 min as in the one-stage assay system (Breckrenridge & Ratnoff, 1962), and the one-stage assay was performed immediately.

At each time interval, a 10-μL aliquot was subsampled into 15 μL of reducing sample buffer containing 25 mg/mL DTT. The samples were boiled at the end of the experiment and then analyzed on polyacrylamide gradient gels.

Polyacrylamide Gel Electrophoresis by the Method of Laemmli. Polyacrylamide gels (4–10%) were prepared by the method of Laemmli (1970). Gradients were formed in the following manner. A 4% acrylamide separating gel solution was poured into one chamber of an LKB gradient mixer. A 10% acrylamide/10% glycerol separating gel solution was poured into the second chamber. The polymerization catalysts were added to each chamber, and the solution from the second was delivered via a cannula to the gel plates. A mechanical mixer stirred the solutions from both chambers. Electrophoresis was carried out in a Bio-Rad Protean cell, and all chemicals were of electrophoresis grade (Bio-Rad Laboratories, Watford, Herts, U.K.).

Western Blotting. Western blotting was performed by a modification of the method of Towbin et al. (1979). Following electrophoresis, 4–10% polyacrylamide gradient gels were washed in 25 mM sodium phosphate buffer, pH 6.5, for 30 min with two changes of buffer. Gels were then overlaid with presoaked nitrocellulose membrane and electroeluted in a Bio-Rad Trans Blot cell at 0.6 A for 18 h. The nitrocellulose membrane was then removed and soaked in quench buffer for 1 h (25 mM sodium phosphate, pH 6.5, 0.02% Triton-X-100, 0.25% gelatin). The membrane was then incubated with antibody (1 μL of ascitic fluid/mL of quench buffer) for 2 h. Following a 1-h wash in quench buffer, the membrane was incubated with goat anti-mouse Ig radiolabeled with ^{125}I, approximately 5 million cpm per membrane for 2 h. A final

Table I: Purification of Factor VIII:C

	VIII:C		VIIIR:Ag	protein		sp act. of VIII:C (units/mg of protein)	recovery of VIII:C from cryo (%)	purification from plasma (x-fold)
	total units	units/mL	(units/mL)	mg/mL	total mg			
plasma (nominal value)		1	1	70		0.0143		1
$Al(OH)_3$ adsorbed cryoprecipitate	21 600	2.7	7.9	13.25	106 000	0.204	100	14
PE VIII	8 400	84	18.1	14	1 400	6	39	420
Sepharose–anti-VIIIR.Ag eluate	4 900	175	*a*	0.75	21	233	23	16 317
Sepharose–anti-VIII:C eluate	4 219	474	*a*	0.100	0.89	4 740	19.5	331 468

[a] Undetectable.

wash procedure consisted of three changes of quench buffer. After drying, the membrane was exposed to X OMAT-RP film (Kodak, Hemel Hempstead, Herts) for 72 h at −80 °C. All volumes were 100 mL of buffer/membrane.

Results

Table I shows the result of a typical purification procedure that has now been repeated 36 times. The largest losses occur at the polyelectrolyte purification stage. However, this step removes large amounts of fibrinogen and fibronectin, which often contaminate purified factor VIII preparations. Polyelectrolyte purified factor VIII also has a reversed ratio of factor VIII:C to VIIIR:Ag compared to conventionally purified concentrates, and this allows more factor VIII:C to be captured by the anti-VIIIR:Ag column for each unit of VIIIR:Ag that attaches to it. This obviates the need for the massive immunoaffinity columns used by some other workers (Fulcher & Zimmerman, 1982). Recovery over this stage of the procedure has varied from 50% to 70% of the applied factor VIII:C activity. A small amount of factor VIIIR:Ag is assayable in the eluate from the anti-RAg column, and contaminating fibronectin can be seen on polyacrylamide gels at this stage of the purification.

The anti VIIIR:Ag eluate (from a starting batch of 3 kg of cryoprecipitate) can be applied to a 10-mL column of monoclonal anti-factor VIII:C. No activity flows through the column, and up to 100% of the activity can be recovered in the multifactor elution buffer.

After purification, the peak fractions must be immediately dialyzed, as there is rapid loss of activity if the material is left in the elution buffer. Some activity is lost on dialysis, probably due to loss of protein on the dialysis membrane in a sample with a low concentration of protein. The remaining activity (about 70%) is stable in liquid nitrogen for at least 5 weeks.

Figure 1 shows this purified preparation electrophoresed on 4–10% SDS–polyacrylamide gradient gel and stained with a sensitive silver stain. On reduction with DTT (Lane C), the highest molecular weight band seen is at 210K, with a series of bands between 210K and 110K, a predominant band at 90K, and a strongly staining doublet at 80K and 79K. Further faintly staining breakdown products in the range 66K–45K are also seen. In the absence of DTT, a similar pattern is seen with an additional band at 360K.

This pattern was reproduced in all 36 preparations. After Western blotting a series of monoclonal antibodies to factor VIII:C (Rotblat et al., 1983) were used to confirm that all the bands seen in the silver-stained gel are part of the factor VIII native protein. Column chromatography using ^{125}I-labeled monoclonal antibodies shows the specificity of these monoclonals is guaranteed by the fact that they bind to antigens in normal plasma but to none in CRM^{-ive} haemophilic plasma, thus meeting the operational definition of factor VIII (the protein absent from CRM^{-ive} haemophilic plasma) (Rotblat et al., 1983). When monoclonal antibodies to factor VIIIR:Ag

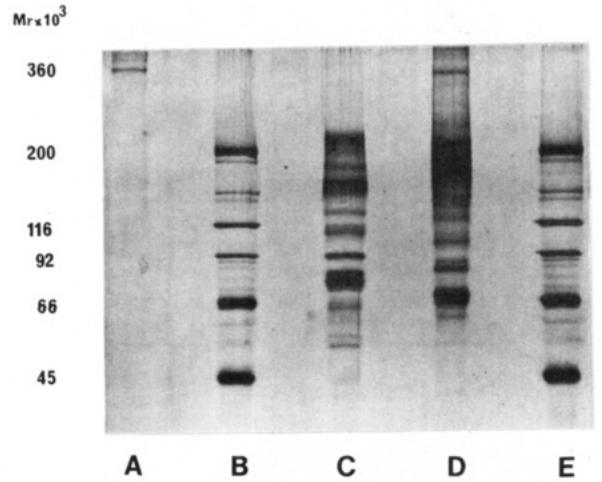

FIGURE 1: A 4–10% SDS–polyacrylamide gradient gel by the method of Laemmli (1970). Silver stain by the method of Morrisey (1981). A total of 1 μg of protein was applied to each lane. Lane A is α_2-macroglobulin standard; unreduced M_r 360K. Lanes B and E are molecular weight markers reduced with DTT (Bio Rad, Watford, Herts, U.K.): myosin, 200K; β-galactosidase, 116K; phosphorylase B, 92K; bovine serum albumin, 66K; ovalbumin, 45K; Lane C is purified factor VIII:C preparation reduced with DTT. Lane D is purified factor VIII:C preparation unreduced.

or fibronectin were used, no bands were seen on the autoradiograph.

In Figure 2A an unreduced preparation of factor VIII:C was used, and all the monoclonal antibodies bound to the 360K band. On reduction (Figure 2B), a variety of binding patterns are seen. All monoclonal antibodies except antibody 5 bind to the 210 000 band, but their patterns of binding to the bands between this and 110K vary. Monoclonal antibodies 2, 3, 6, and 8 bind all these bands but 2 is only faintly seen. The lowest molecular weight band in this series bound by monoclonal antibody 12 is the 170K band and for monoclonal antibody 10 is the 150K band. The 92K band is identified by monoclonal antibodies 2, 6, and 8 only.

Monoclonal antibody 5 is the only antibody reacting with the doublet at 80K. It does not bind any of the other bands in the reduced preparation.

Figure 3 shows the results of the thrombin activation of the purified factor VIII:C in a biological assay system. Within 2 min of addition of the thrombin, the activity of factor VIII:C rose 13-fold from 240 to 3100 units/mL and thereafter decayed rapidly in the first 20 min and then gradually to a level of 100 units/mL at 2 h.

Figure 4 shows a sequential time-course analysis on a 4–10% polyacrylamide gradient gel, using samples aliquoted during the activation described above. Prior to the addition of thrombin, the typical pattern of bands is seen. Upon activation there is a rapid loss of high molecular weight forms (210K–110K). The band at 90K rapidly intensifies to a maximum at 30 s and then gradually diminishes.

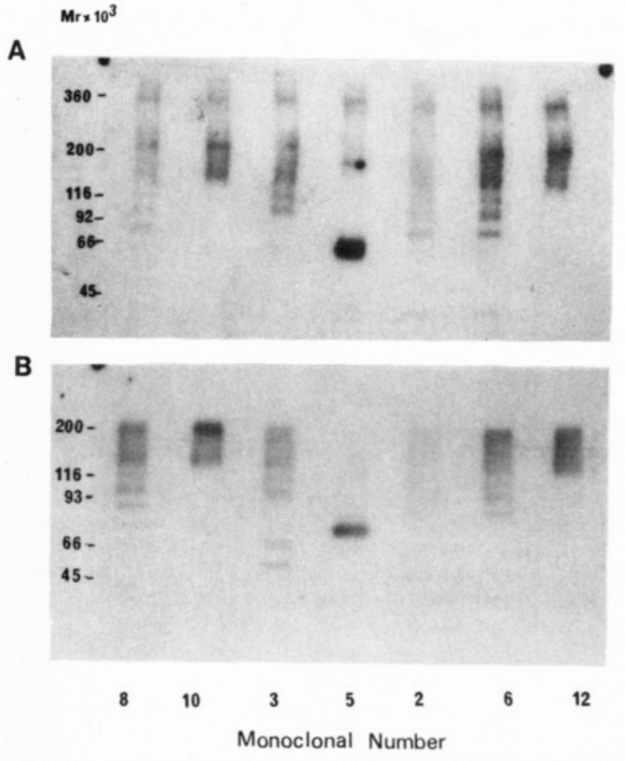

FIGURE 2: Autoradiograph of Western blot of 4–10% SDS–polyacrylamide gradient gel onto nitrocellulose membrane. A total of 5 μg of purified factor VIII:C was applied to each of seven lanes. After quenching unbound sites with gelatin, individual lanes were cut out and separately incubated with seven monoclonal antibodies to factor VIII:C (Rotblat et al., 1983). After washing, the strips were incubated with ^{125}I-labeled goat anti-mouse immunoglobulin. Strips were exposed to Xomat RP film (Kodak, Hemel Hempstead, Herts) at −80 °C for 72 h. The numbers refer to different monoclonal antibodies. Panel A shows unreduced factor VIII:C, and panel B shows factor VIII:C that has been reduced with dithiothreitol. Bio-Rad high molecular weight standards (see Figure 1) were blotted from the same gel, and the strip of nitrocellulose membrane was stained with Coomassie brilliant blue to place the molecular weight markers.

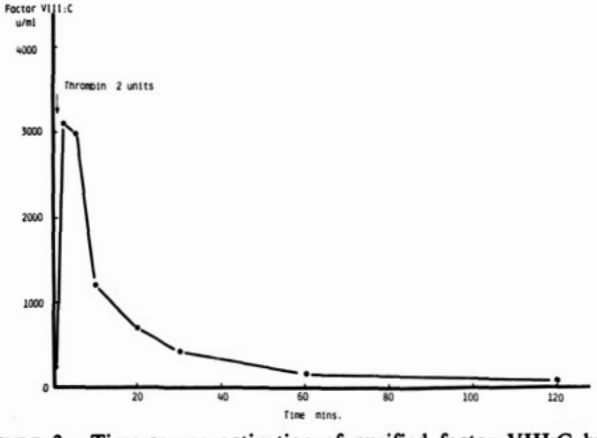

FIGURE 3: Time-course activation of purified factor VIII:C by thrombin. Factor VIII:C (20 units, 240 units/mL) was incubated at 4 °C with 2 units of thrombin. Aliquots were subsampled into the one-stage assay at time intervals indicated.

The 79K/80K doublet shows a steady decline in intensity, and a doublet at 96K/70K appears gradually. Bands at 55K and 44K also appear, and all these bands continue to intensify throughout the time course of the experiment. Addition of Kabi thrombin inhibitor S-2581 to the activation mixture arrested further breakdown of the 90K and 80K/79K peptides (data not shown).

Figure 5 shows autoradiographs of a Western blot of an identical experiment to that seen in Figure 4. Monoclonal

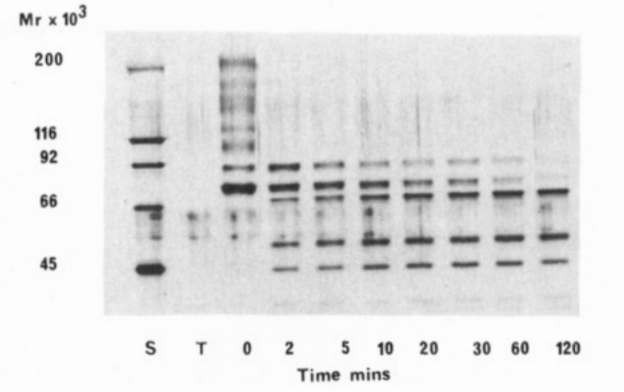

FIGURE 4: A 4–10% polyacrylamide gradient gel silver stained by method of Morissey (1981). A total of 1 μg of protein was applied to each lane; 10-μL aliquots were subsampled from a mixture of purified factor VIII:C (240 units/mL) and 2 units of human thrombin incubated at 4 °C, at the times stated beneath the lanes. Subsamples were placed into 15 μL of reducing buffer to stop the reaction and boiled before application to the gel. S, standards; T, thrombin alone; 0, factor VIII:C prior to addition of thrombin.

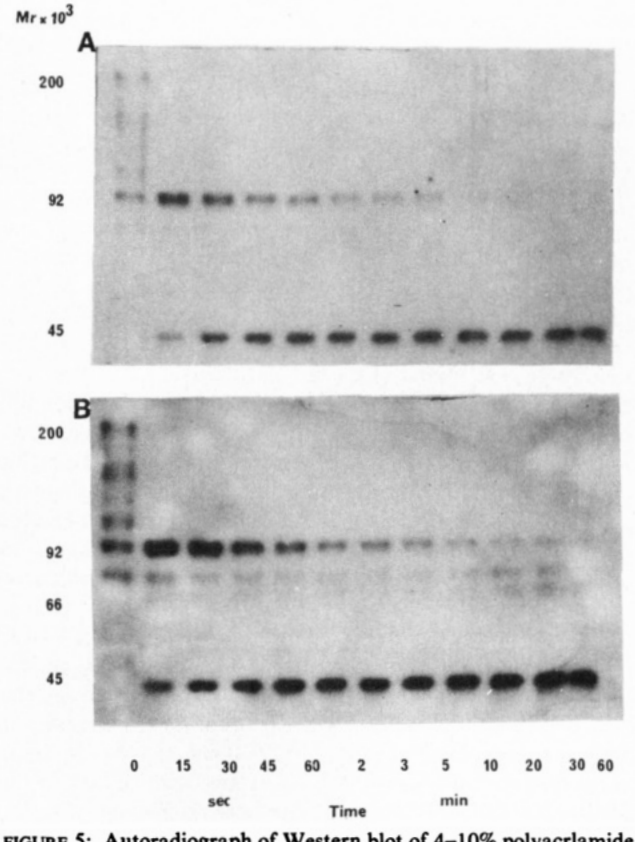

FIGURE 5: Autoradiograph of Western blot of 4–10% polyacrlamide gradient gel. Samples were from time-course activation as described above. 0 is factor VIII:C before addition of thrombin. In (A), monoclonal 8 is used as the probe and ^{125}I-radiolabeled goat anti-mouse immunoglobulin. In (B), monoclonals 5 and 8 are used as probes and ^{125}I-radiolabeled goat anti-mouse immunoglobulin.

antibodies 5 and 8 are used as probes. In Figure 5A with monoclonal antibody 8 alone, all bands normally seen, excepting the 80K doublet, are present in the pre-thrombin sample (the 80K doublet is not bound by this antibody). After the addition of thrombin, the same pattern is seen as in the silver gel except that the 80K/79K doublet and the 70K/69K doublet are absent. The addition of monoclonal antibody 5 (Figure 5B) to the same blot visualizes the 80K/79K and 70K/69K doublets. The 55K band is not seen and does not bind any of our monoclonal antibodies.

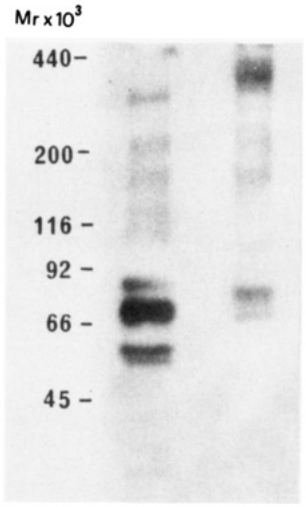

FIGURE 6: Autoradiograph of Western blot of 4–10% polyacrylamide gradient gel. Blood from one normal donor was taken into citrate containing the specific thrombin inhibitor S-2581 (Kabi Vitrum, Stockholm). The plasma was made 2 mM in DFP and 10 mM in benzamidine and applied directly to the monoclonal anti-factor VIII:C column (see Materials and Methods). A total of 14 μg of protein was applied to each lane on the gel. The probe used is a mixture of monoclonal antibodies to factor VIII:C (3, 5, 8, and 10) and ^{125}I-labeled goat anti-mouse immunoglobulin. (Lane A) Factor VIII:C boiled and reduced with dithiothreitol. (Lane B) Factor VIII:C unreduced (boiled). The 440K marker was unreduced fibronectin.

In the small-scale purification of factor VIII:C from 220 mL of plasma (see Materials and Methods) a total of 132 units of factor VIII:C was applied to the monoclonal antibody 8 column. No detectable factor VIII:C flowed through this column or was eluted in the 2 M salt wash.

Only 20 units of factor VIII:C was recovered in the multifactor elution buffer, and on PAGE this material was shown to contain many contaminants. However, by use of the highly specific Western blotting technique, an autoradiograph showing factor VIII:C components only was produced (Figure 6). The typical pattern of bands is seen in both lanes. In addition, however, a band at 360K is seen in the reduced preparation.

Figure 7 shows a proposed epitope map of the factor VIII:C molecule as derived from the patterns of binding of the various individual monoclonal antibodies in the Western blot system. Also shown are the inhibitory activities of these antibodies in Bethesda units (Rotblat et al., 1983). It can be seen that those antibodies with the highest inhibitory activities all bind to the 90K peptide induced by thrombin activation.

Monoclonal antibody 3, which has moderate inhibitory activity, binds to all high molecular weight peptides except 90K and would therefore seem to have its epitope close to the thrombin cleavage site, which is essential for conversion to activated factor VIII:C. Monoclonal antibodies 12, which has low inhibitory activity, and 10, which does not inhibit factor VIII:C in a biological system, only bind to the highest molecular weight peptides, and their epitopes are therefore located further away from the active site. Monoclonal antibody 5, which binds the 80K/79K doublet and its thrombin cleavage product, has moderate inhibitory activity.

Discussion

On analysis of purified factor VIII:C, the largest molecular weight band found after reduction of disulfide bonds was 360K. It is concluded that this represents the native chain of factor

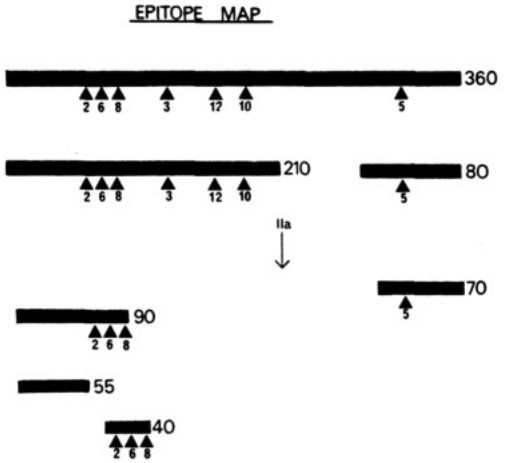

FIGURE 7: Epitope map of the factor VIII:C molecule. The arrow heads represent binding sites of different monoclonal antibodies. The bars represent peptides ($M_r \times 10^3$). The following are inhibitory activities (Bethesda units/mg of IgG) for the antibodies used: (2) 4600; (3) 35 μL/ascites IgM antibody; (5) 79; (6) 9700; (8) 10 200; (10) none; (12) 0.6. IIa represents addition of thrombin.

VIII:C from which other fragments are derived by limited proteolysis. Clearly exact determination of the molecular weight of such a large protein on polyacrylamide gels is limited by the availability of standards in this molecular weight range. However, the factor VIII consistently comigrates with undreduced α_2-macroglobulin which has a M_r of 360K. Recently, results from gene cloning experiments (Vehar et al., 1984) have shown a derived M_r of 264 763 for the unglycosylated protein, which would be consistent with a circulating M_r of 330K. This native form of factor VIII could only be detected by taking stringent precautions against proteolysis from the venesection onward. Other authors have used commercial factor VIII concentrate as starting material (Fulcher & Zimmerman, 1982; Fass et al., 1982; Hamer et al., 1983). This has already been subjected to at least three preparative steps without proteolytic inhibitors. It is not surprising that these authors have found no material with a molecular weight above 188K on reduction or in one 100K (Fay et al., 1982).

A unique feature of our procedure was the combination of polyelectrolyte E5 chromatography with use of an immunoaffinity column containing immobilized antibody to factor VIIIR:Ag. The first allowed most of the fibrinogen and fibronectin in the cryoprecipitate to be separated from factor VIII and also reversed the ratio of VIIIR:Ag to factor VIII:C. The anti-VIIIR:Ag immunoaffinity column could then operate with maximum efficiency since each adsorbed unit of VIIIR:Ag bound many units of factor VIII:C. We found that either immobilized polyclonal rabbit antibody to VIIIR:Ag or mouse monoclonal antibody could be used at this stage of the purification with no appreciable difference in efficacy. For maximum recovery this column should only be used once and then discarded.

The monoclonal antibody specific to factor VIII:C (8) used in the purification is of high affinity, which enabled the column to be washed in high ionic strength buffer. Subsequent elution of factor VIII:C required a multicomponent buffer, which progressively denatured the protein causing loss of activity over a few hours (data not shown). On analysis of material purified from cryoprecipitate, the 360K protein was only seen prior to reduction, despite the use of high concentrations of proteolytic inhibitors throughout the procedure. This suggests that a degree of proteolysis occurred during preparation of bulk cryoprecipitate without inhibitors in the commercial frac-

tionation plant. Evidently, the conversion from a single-chain 360K to the 210K and 80K forms occurs very readily ex vivo. Also, this conversion is continually proceeding in vivo since both single-chain and multiple molecular weight breakdown products were present in the material separated from blood drawn directly into potent inhibitors. Similar results were reported by Weinstein et al. (1981), who found factor VIII molecular weights ranging from 260K to 100K with a polyclonal human inhibitor. The assessment of molecular weight made in this latter system must be viewed with considerable caution since the preformed antibody–antigen conjugates necessitated the use of nondenaturing conditions for electrophoresis. Also, the molecular weight of Fab needed to be subtracted to derive the supposed M_r of factor VIII:C antigens. Nevertheless, Weinstein's data strongly support the idea that factor VIII:C is continually undergoing degradation in the circulation. This is in accord with the short half-life of factor VIII:C in vivo (8–10 h) and its known in vitro sensitivity to thrombin, plasmin, and other proteases (Atichartakarn et al., 1978).

The fact that all our monoclonal antibodies bind to the 360K band as well as to one or another of the lower molecular weight derivatives is strong evidence for a precursor product relationship. From the epitope map (Figure 7) and the inhibitory effect of different monoclonal antibodies, it can be concluded that the 40K fragment of the 90K subunit is important in the functional activity of factor VIII:C. All three antibodies binding this region are potent inhibitors. Monoclonal antibody 5, which binds the 80K subunit, also inhibits factor VIII:C, which indicates that it is part of the active complex.

The effect of thrombin is to rapidly generate the 90K band with disappearance of all higher molecular weight material. This coincides with evolution of maximum activity at a time when 90K and 80K bands are most prominent. These bands would correspond to those seen at 92K and 79K/80K by Fulcher and her co-workers (1983). Activated factor VIII:C therefore appears to consist of a heavy chain and a light chain nearly identical in size with those found after activation of factor V by thrombin (Nesheim et al., 1984). The functional homology of factors VIII:C and V has long been apparent, and this structural homology is not unexpected.

Factor VIII:C quite rapidly loses activity after further incubation with thrombin, and this loss is coincident with the conversion of the 90K to the 40K and 55K bands and the appearance of the 70K doublet derived frm the 80K doublet. This likewise corresponds to the results seen by Fulcher (1983), where loss of activity was accompanied by generation of bands at 54K and 44K and a doublet at 71K/72K. Data obtained by Western blotting has confirmed the supposition made by Fulcher in 1983 that the 55K band derives from the 90K active peptide (Fulcher & Zimmerman, 1984). Since the breakdown of the peptides can be arrested by the addition of a specific thrombin inhibitor (Kabi S-2581), the biphasic response of factor VIII:C activity to thrombin is clearly accounted for by specific proteolytic cleavages. Factor VIII cannot be activated by thrombin in the presence of S-2581.

Fass et al. (1982) have presented evidence that factor VIII:C subunits are stabilized in a complex by calcium ions. The light and heavy chains of active factor V are held together via calcium-dependant bonds, and these observations account for the destruction of factor V and VIII:C activity by the calcium-chelating agent EDTA. Recent data (Vehar et al., 1984; Mann et al., 1984) have shown sequence homology between factor VIII, factor V, and the copper binding protein caeruloplasmin. Factor V has been shown to contain copper ions at a ratio of one ion/mol. It has been suggested that these copper ions may play a role in stabilizing the active complex, and this may also be the case with factor VIII. The moleclar mechanism by which factor VIII:C enhances proteolysis of factor X by factor IXa in the presence of phospholipid remains to be elucidated. however, present data suggest that specific binding sites for factor X, factor IXa, and phospholipid should be sought in the heavy (90K) and light (80K) subunits of thrombin-activated factor VIII:C. Monoclonal antibodies could prove to be useful probes in this endeavor.

Registry No. Blood coagulation factor VIII, 9001-27-8.

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Complete Assignment of the Imino Protons of *Escherichia coli* Valine Transfer RNA: Two-Dimensional NMR Studies in Water†

Dennis R. Hare, N. Susan Ribeiro, David E. Wemmer, and Brian R. Reid*
Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195
Received October 12, 1984

ABSTRACT: The imino proton spectrum of *Escherichia coli* valine tRNA has been studied by two-dimensional nuclear Overhauser effect spectroscopy (NOESY) in H_2O solution. The small nuclear Overhauser effects from the imino proton of an internal base pair to the imino protons of each nearest neighbor can be observed as off-diagonal cross-peaks. In this way most of the sequential NOE connectivity trains for all the helices in this molecule can be determined in a single experiment. AU resonances can be distinguished from GC resonances by the AU imino NOE to the aromatic adenine C2-H, thus leading to specific base-pair assignments. In general, the NOESY spectrum alone is not capable of assigning *every* imino proton resonance even in well-resolved tRNA spectra. Multiple proton peaks exhibit more than two cross-peaks, resulting in ambiguous connectivities, and coupling between protons with similar chemical shifts produces cross-peaks that are incompletely resolved from the diagonal. The sequence of the particular tRNA determines the occurrence of the latter problem, which can often be solved by careful one-dimensional experiments. The complete imino proton assignments of *E. coli* valine tRNA are presented.

The hydrogen-bonded imino protons of helical nucleic acids offer two major advantages in the analysis of nucleic acid structure and dynamics by high-resolution NMR.[1] First, the imino protons resonate in a uniquely low-field region of the spectrum, and there is only one such proton per Watson–Crick pair. Second, their exchange with solvent requires some form of transient opening of the corresponding base pair (Teitelbaum & Englander, 1975), and this makes them potentially very useful in monitoring nucleic acid dynamics by NMR. In order to accurately interpret helix–coil dynamics at various positions in a nucleic acid such as tRNA, one obviously needs to be able to assign each low-field imino proton resonance to its specific base pair. However, imino proton assignments have previously been made by a variety of indirect methods, often with conflicting results, and this has complicated the interpretation of dynamic studies on these molecules. Earlier indirect assignment strategies include hairpin fragment studies (Lightfoot et al., 1973), empirically calibrated ring-current shift estimates (Shulman et al., 1973; Kearns, 1976; Reid et al., 1979), chemical modification (Salemink et al., 1977; Hurd & Reid, 1979), theoretical ring-current shift calculations (Robillard et al., 1976), and paramagnetic ion binding (Hurd et al., 1979), all of which require prior assumptions about the solution structure of the molecule. In a somewhat different approach, the first resonances to melt upon raising the temperature of the tRNA were assigned to tertiary base pairs on the unproven assumption that tertiary folding is the most labile part of the tRNA solution structure (Hilbers & Shulman, 1974; Romer & Varadi, 1977).

The development of FTNMR in H_2O solution and the application of double-resonance methods to exchangeable protons by Redfield and colleagues (Redfield, 1978; Johnston & Redfield, 1977) ushered in a new era of nucleic acid research in which imino protons could be directly assigned by means of dipolar proton–proton coupling, i.e., the nuclear Overhauser effect or NOE. In this technique the resonance of interest is selectively saturated; surrounding protons within 4–5 Å become partially cross-saturated and appear as smaller peaks in the difference spectrum. With this technique Redfield and coworkers originally identified the two strongly coupled imino protons within a GU wobble base pair (Johnston & Redfield, 1978) and subsequently identified the imino protons of the two intervening Watson–Crick neighbors between the two wobble pairs in the dihydrouridine helix of $tRNA^{Asp}$ (Roy & Redfield, 1981). Sequential NOE connectivities have been used to assign the imino protons of consecutive Watson–Crick pairs in entire helices in various tRNAs (Hare & Reid, 1982a,b; Heerschap et al., 1982); extension of this technique has led to the complete assignment of all imino protons in *Escherichia coli* isoleucine tRNA and valine tRNA (Hare, 1983) and in yeast phenylalanine tRNA (Roy & Redfield, 1983; Heerschap et al., 1983a,b). However, Heerschap et al. differ from Roy and

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* Address correspondence to this author at the Department of Chemistry.

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; tRNA, transfer ribonucleic acid; T, ribothymidine; D, dihydrouridine; FTNMR, Fourier-transform nuclear magnetic resonance.

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