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Amino Acid Sequence and Posttranslational Modifications of Human Factor VII_a from Plasma and Transfected Baby Hamster Kidney Cells

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ABSTRACT: Blood coagulation factor VII is a vitamin K dependent glycoprotein which in its activated form, factor VII_a, participates in the coagulation process by activating factor X and/or factor IX in the presence of Ca^{2+} and tissue factor. Three types of potential posttranslational modifications exist in the human factor VII_a molecule, namely, 10 γ-carboxylated, N-terminally located glutamic acid residues, 1 β-hydroxylated aspartic acid residue, and 2 N-glycosylated asparagine residues. In the present study, the amino acid sequence and posttranslational modifications of recombinant factor VII_a as purified from the culture medium of a transfected baby hamster kidney cell line have been compared to human plasma factor VII_a. By use of HPLC, amino acid analysis, peptide mapping, and automated Edman degradations, the protein backbone of recombinant factor VII_a was found to be identical with human factor VII_a. Neither recombinant factor VII_a nor human plasma factor VII_a was found to contain β-hydroxyaspartic acid. In human plasma factor VII_a, the 10 N-terminally located glutamic acid residues were found to be fully γ-carboxylated whereas 9 full and 1 partial γ-carboxylated residues were found in the corresponding positions of the recombinant factor VII_a molecule. Asparagine residues 145 and 322 were found to be fully N-glycosylated in human plasma factor VII_a. In the recombinant factor VII_a, asparagine residue 322 was fully glycosylated whereas asparagine residue 145 was only partially (approximately 66%) glycosylated. Besides minor differences in the sialic acid and fucose contents, the overall carbohydrate compositions were nearly identical in recombinant factor VII_a and human plasma factor VII_a. These results show that factor VII_a as produced in the transfected baby hamster kidney cells is very similar to human plasma factor VII_a and that this cell line thus might represent an alternative source for human factor VII_a.

Factor VII is a vitamin K dependent glycoprotein which is synthesized by liver cells (Wion et al., 1985) and secreted into the blood. In its activated form (factor VII_a), the protein acts as a serine protease that participates in the extrinsic pathway of the blood coagulation leading to the formation of a fibrin clot (Davie et al., 1979). Factor VII is synthesized and secreted as a single-chain molecule (Kisiel & Davie, 1975; Radcliffe & Nemerson, 1975) consisting of 406 amino acid

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residues (Hagen et al., 1986). The activation of factor VII to factor VII_a involves the hydrolyses of a single peptide bond between Arg-152 and Ile-153, resulting in a two-chain molecule consisting of a light chain of 152 amino acid residues and a heavy chain of 254 amino acid residues held together by a single disulfide bond (Kisiel & McMullen, 1981; Hagen et al., 1986). By in vitro experiments, a number of activated coagulation proteins including factor X_a (Radcliffe & Nemerson, 1975), factor XII_a (Broze & Majerus, 1980), factor IX_a (Seligsohn et al., 1979), and factor II_a (Radcliffe & Nemerson, 1975) have been shown to activate factor VII to factor VII_a.

After the nucleotide sequencing of the cDNA for human factor VII, three types of posttranslational modifications have been thought to exist in the mature protein (Hagen et al., 1986). The N-terminal region of human factor VII contains 10 possible sites for γ-carboxylation of glutamic acid residues (Gla residues),[1] in that these residues are located in positions analogous to those of Gla residues in other vitamin K dependent proteins (Katayama et al., 1979).

The cDNA sequencing predicts an aspartic acid in position 63 of the human factor VII protein (Hagen et al., 1986), and by analogy to the bovine factor VII (McMullen et al., 1983), the bovine protein C (Drakenberg et al., 1983), the human factor IX (McMullen et al., 1983), and the human factor X (McMullen et al., 1983b), this residue might be β-hydroxylated.

The nucleotide sequence codes for an Asn-Ala-Ser sequence in positions 145–147 and an Asn-Ile-Thr sequence in positions 322–324, indicating N-glycosylation of Asn-145 and Asn-322 although Asn-X-Thr/Ser sequences do not necessarily imply N-glycosylation (Pohl et al., 1984).

Although human deficiencies in the factor VII_a activity are relatively rare (Roberts & Zeitler, 1982) hemophilia A patients with circulating factor VIII antibodies might benefit from an alternative factor VII_a treatment (Hedner & Kisiel, 1983). As factor VII is only present in small amounts in human plasma (approximately 0.5 mg/L), a mammalian expression system may be an alternative source which also eliminates potential risk of exposure of patients to viruses causing hepatitis and AIDS.

The present study was undertaken in order to characterize the three types of potential posttranslational modifications in factor VII_a purified from human plasma and to compare it to human factor VII_a isolated from a mammalian expression system.

Materials and Methods

Factor VII Purifications. A side fraction from the production of prothrombin complex (Heystek et al., 1973) was obtained from the Danish Serum Institute and used as starting material for the purification of plasma-derived human factor VII_a. The purification procedure includes anion-exchange chromatography using a $CaCl_2$-containing buffer as eluent (Broze & Majerus, 1981) followed by immunoabsorbent chromatography on a Sepharose 4B column to which a monoclonal antibody against human factor VII was coupled. The monoclonal antibody was obtained from Novo Biolabs, Denmark (Dr. J. Selmer). The antibody binds factor VII only in the presence of Ca^{2+}, which means that factor VII can be eluted from the column by buffers containing EDTA as also described for other Gla-containing proteins (Wakabayashi et al., 1986). The final purification was carried out by the use of two anion-exchange chromatography steps in which factor VII_a was eluted with buffers containing $CaCl_2$ and NaCl, respectively (Bjoern & Thim, 1986). When the above procedure is used, factor VII is activated to factor VII_a during the purification (Bjoern & Thim, 1986). Recombinant factor VII was obtained from a mammalian expression system. The culturing and transfection of BHK cells with human factor VII genomic material (Hagen et al., 1986) have been described in detail elsewhere (Berkner et al., 1986). The crude cell culture medium was filtered through a 0.65-μm filter. The clear solution was diluted with distilled water until a conductivity of 10 mS was reached. The pH of the solution was adjusted to 8.6 with 0.1 N NaOH. This solution was pumped onto a Mono Q column (Pharmacia Fine Chemicals) and r-factor VII_a was eluted with a $CaCl_2$-containing Tris-HCl buffer (Bjoern & Thim, 1986). The immunochromatographic step and the remaining part of the purification procedure were identical with those described above for the plasma-derived factor VII_a. The activation of r-factor VII to r-factor VII_a was achieved during the purification (Bjoern & Thim, 1986).

Amino Acid Analysis. After hydrolysis in 6 M HCl at 110 °C in vacuum-sealed tubes for 24, 48, and 96 h, the samples (50 μg) were analyzed on a Beckman (Model 121 MB) automatic amino acid analyzer. Half-cystine was determined as the *S*-β-(4-pyridylethyl) derivative after reduction of the disulfide bonds by tributylphosphine (Rüegg & Rudinger, 1974) followed by coupling with 4-vinylpyridine (Friedman et al., 1970). Hydrolyses of 4-vinylpyridine-treated samples were performed by 4 M methanesulfonic acid or 3 M mercaptoethanesulfonic acid at 110 °C for 24 h as described above. Gla residues were converted to γ-methyleneglutamic acid (Wright et al., 1984), and such samples were hydrolyzed in 3 M mercaptoethanesulfonic acid at 110 °C for 24 h.

Separation of Light and Heavy Chains. Factor VII_a (about 5 mg) was dissolved in 1 mL of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 20 mM $CaCl_2$, and 4 M guanidine hydrochloride. The disulfide bonds were reduced by the addition of 10 mg of dithiothreitol and incubation at 25 °C for 20 h. The reduced molecule was derived by the addition of 35 μL of 4-vinylpyridine and incubation at 25 °C for 90 min. The pyridylethylated light and heavy chains were separated by injecting an aliquot of the reaction mixture (0.2 mL) onto a Vydac 214 TP54 column (250 × 4.6 mm) equilibrated at 30 °C at a flow rate of 1.5 mL/min with acetonitrile/water/trifluoroacetic acid (25.0:74.9:0.1). After 7 min of isocratic elution, the concentration of acetonitrile in the eluting solvent was raised to 60% (v/v) over 14 min using a linear gradient. The UV absorption was monitored at 256 nm (maximum absorbance for 4-pyridylethylated peptides).

Peptide Mapping. The pyridylethylated light chains of p-factor VII_a and r-factor VII_a (180 μg) were dissolved in 300 μL of 0.1 M Tris-HCl buffer (pH 7.5) and digested by the addition of 0.9 μg of trypsin (Novo Industri A/S) in 10 μL of water and incubation at 37 °C for 22 h. The reactions were stopped by the addition of 2 μL of 4 M HCl. The peptide mapping was carried out by the injection of digest mixture (50 μL) onto a Vydac 214 TP54 column (250 × 4.6 mm) equilibrated at 30 °C at a flow rate of 1.5 mL/min with acetonitrile/water/trifluoroacetic acid (6.0:93.9:0.1). After 4 min, the concentration of acetonitrile in the eluting solvent was raised to 15.5% (v/v) over 2 min and further to 38% (v/v) over 24 min. The pyridylethylated heavy chains (340 μg) were

[1] Abbreviations: Gla, γ-carboxyglutamic acid; BHA, β-hydroxyaspartic acid; BHK, baby hamster kidney; PE-Cys, pyridylethylated cysteine; FAB-MS, fast atom bombardment mass spectrometry; PTH, phenylthiohydantoin; p-factor VII_a, human plasma factor VII_a; r-factor VII_a, human recombinant factor VII_a; HIV, human immunodeficiency virus.

dissolved in 200 μL of 10 mM glycine–NaOH buffer (pH 10) and digested by the addition of 15 μg of trypsin in 15 μL of water and incubation at 37 °C for 24 h. The reactions were stopped by the addition of 4 μL of 4 M HCl. The peptide mapping of the heavy chains was carried out as described above except that the acetonitrile concentration was raised from 6% (v/v) to 48% (v/v) over 28 min.

Structural Characterizations. Amino acid sequence analysis was carried out by automated Edman degradation using an Applied Biosystems 470 A gas-phase sequencer (Thim et al., 1987). The detection limit for phenylthiohydantoin (PTH)-amino acids was 0.5 pmol. Analyses for β-hydroxyaspartic acid were carried out by amino acid analysis on the intact factor VII_a, the isolated light chain, and a fragment comprising amino acid residues 62–84 of the light chain. The latter fragment was prepared by digestion of the light chain with *Armillaria mellea* proteinase (Lewis et al., 1978) [enzyme to substrate ratio of 1:50 (w/w), pH 8.0, 37 °C, 16 h] followed by HPLC as described for peptide mapping of the light chain. A standard of DL-*threo*-β-hydroxyaspartic acid was obtained from Calbiochem. The C-terminal CNBr fragment of the heavy chain comprising residues 392–406 was prepared by dissolving 1 mg of factor VII_a in 0.5 mL of 70% (v/v) formic acid containing 50 μg of CNBr and incubation for 21 h at 25 °C followed by HPLC essentially as described for peptide mapping of the heavy chain.

Fast Atom Bombardment Mass Spectrometry. As an alternative method for analysis of β-hydroxyaspartic acid, the light-chain fragment comprising residues 62–84 was digested with chymotrypsin [enzyme to substrate ratio of 1:50 (w/w), pH 8.4, 37 °C, 4 h]. The digest was analyzed by positive-ion fast atom bombardment mass spectrometry by M-Scan Ltd., U.K.

Carbohydrate Analysis. Samples of p-factor VII_a and r-factor VII_a were desalted prior to analysis. This was achieved by gel filtration through a column holding Trisacryl GFO5 (Réactifs, IBF) equilibrated and eluted with 1% (w/v) NH_4HCO_3, pH 8.0. The void volume fractions containing factor VII_a were combined, and lyophilization was performed by vacuum centrifugation followed by reconstitution in 0.1% trifluoroacetic acid. Factor VII_a concentrations were determined by amino acid analysis. The analysis of the composition of the carbohydrate moieties was carried out as a modification of previously published procedures (Chaplin, 1982; Jentoft, 1985). Dry methanolic hydrogen chloride (0.63 M) was prepared by adding 4.65 mL of acetyl chloride to 100 mL of dry methanol (cooled on dry ice). A cabinet flushed with dry, oxygen-free nitrogen was used for all reagent manipulations. Evaporations were obtained by vacuum centrifugation with subsequent bleeding with dry, oxygen-free nitrogen.

Factor VII_a (2.5–3 nmol) or standard carbohydrates (5–20 nmol, Sigma) were dried together with *meso*-inositol (approximately 5 nmol, Fluka) in 300-μL screw-topped vials (Pierce Chemical Co.). Dry methanol HCl (40 μL) was added together with methyl acetate (10 μL), and the samples were incubated at 70 °C for 16 h. After the samples were cooled, 2-methyl-2-propanol (10 μL) was added, and the solvents were evaporated. Dry methanol (50 μL), pyridine (5 μL), and acetic anhydride (5 μL) were added successively with intermediate mixing. Re-N-acetylation of the amino sugars was obtained by incubation at room temperature for 60 min before evaporation. In order to eliminate the possible *O*-acetyl groups (Reinhold, 1972), a second, mild methanolysis was performed at 70 °C for 60 min with dry methanol (60 μL), dry methanolic HCl (10 μL, 0.63 M), and methyl acetate (10 μL). After evaporation, perbenzoylation of methyl glycosides was achieved by overnight incubation at 37 °C with 100 μL of a freshly prepared solution of 10% (w/v) benzoic anhydride and 5% (w/v) (dimethylamino)pyridine in pyridine. Chromatography was carried out on 5-μL samples using HPLC equipped with a column switching valve. Sample cleanup was achieved on a Hewlett-Packard ODS (C18) cartridge (2.1 × 20 mm, 5 μm) equilibrated with 35% (v/v) acetonitrile at 50 °C. After 2 min of elution at 1 mL/min, the effluent of the sample cleanup cartridge was diverted to the analytical column (3.9 × 150 mm, Waters Nova-pak C-18, 4 μm), and elution with 35% (v/v) acetonitrile was maintained for an additional 3 min. At this time, a linear gradient of acetonitrile (1%/min) was started, and at 40 min, the elution was fixed at 70% (v/v) acetonitrile until the end of the chromatography at 50 min. The sugar derivatives were detected by the UV absorbance of the benzoyl group at 230 nm, and the retention times were within the range from 20 to 50 min. In addition, *N*-acetylneuraminic acid was determined by a colorimetric method (Massamiri et al., 1978). No de-O-acetylation of samples was performed prior to analysis, for which reason only *N*-acetylneuraminic acid, but not 9-O- and 8-O-acetylated forms, was determined. Human α-1-acid glycoprotein (Sigma) was used as standard assuming a value of 9.48% (w/w) of sialic acid (Massamiri et al., 1978). For the calculation of moles of sialic acid per mole of factor VII_a protein, molecular weights of 309 and 45 514 were used for *N*-acetylneuraminic acid and non-glycosylated factor VII_a, respectively.

RESULTS

Purification of Factor VII_a and Separation of Light and Heavy Chains. With the use of the four-step purification procedure, an overall yield of p-factor VII_a as well as r-factor VII_a of approximately 40% was obtained. A part of the loss during the purification was caused by the generation of factor VII_a degradation products. Two types of degradation products have been identified, namely, factor VII_a which has lost the N-terminal Gla region and factor VII_a which has been hydrolyzed at different sites in the heavy chain. The des-Gla factor VII_a degradation product was removed in the anionic exchange chromatography steps whereas some of the heavy-chain degradation products copurify with intact factor VII_a. These heavy-chain degradation products, which constitute 4–6% of the intact factor VII_a, also coelute with intact factor VII_a in the HPLC chromatograms shown for the purified p-factor VII_a and r-factor VII_a (Figure 1, panels A and B). The HPLC elution profiles of the reduced and pyridylethylated p-factor VII_a and r-factor VII_a are shown in Figure 1 (panels C and D). The elution position of the light chain of p-factor VII_a (14.53 min) is identical with the elution position of the light chain of r-factor VII_a (14.50 min); the same applies to the elution positions of the two heavy chains (19.84 and 19.88 min for the plasma and recombinant material, respectively). The other peaks in the chromatograms represent degradation products of the heavy chain. The pattern of these heavy-chain fragments is similar in the two preparations (Figure 1, panels C and D), indicating that p-factor VII_a and r-factor VII_a are equally exposed to degradation in the heavy chain during the purification.

Primary Structure. The results from the amino acid analysis are summarized in Table I. The Glx content (Glu + Gln + Gla) was found to be 48.8 in the p-factor VII_a compared to a theoretical value of 47 deduced from cDNA. Besides this minor difference, the amino acid compositions of p-factor VII_a and r-factor VII_a are in good agreement with the amino acid composition as deduced from cDNA se-

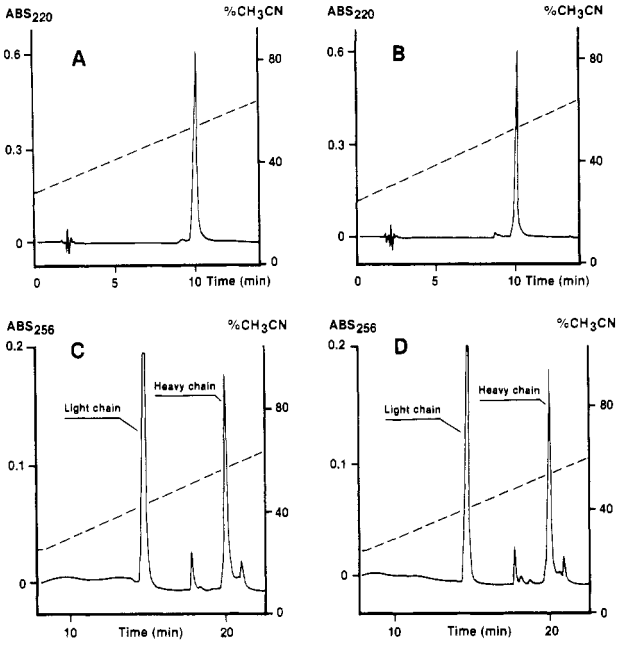

FIGURE 1: Reverse-phase HPLC chromatograms of p-factor VII_a (panel A), r-factor VII_a (panel B), pyridylethylated light and heavy chains of p-factor VII_a (panel C), and r-factor VII_a (panel D).

quencing (Hagen et al., 1986).

Figure 2 shows the separations of peptides obtained by trypsin digestion of the pyridylethylated light chain of p-factor VII_a (panel A) and r-factor VII_a (panel C). As can be seen from these chromatograms, all major peaks have practically identical chromatographic behavior. The solubility of the pyridylethylated heavy chain was rather low at neutral pH, and therefore it was not possible to digest this peptide at pH 7.5. By increasing the pH to 10, the solubility increased significantly, and the digestion of the heavy chain was carried out with trypsin (4% w/w) at this high pH value. The peptide maps obtained from the heavy chains of p-factor VII_a and r-factor VII_a are shown in Figure 2 (panels B and D).

Table I: Amino Acid Composition of Human Plasma Factor VII_a and Recombinant Factor VII_a

	p-factor VII_a	r-factor VII_a	deduced from cDNA[a]
Asx	33.1	32.3	33[b]
Thr[d]	21.9	21.9	22
Ser[d]	28.9	29.1	29
Glx	48.8	47.3	47[c]
Pro[e]	20.9	20.8	21
Gly	37.5	35.9	37
Ala	21.3	21.1	21
Val	27.9	27.5	27
Met	4.5	3.9	4
Ile	15.5	15.2	17
Leu	38.0	38.0	38
Tyr[e]	11.9	11.8	12
Phe	13.1	13.2	13
Lys	17.0	17.4	17
His	12.1	11.7	12
Trp[e]	8.3	7.1	8
Arg	23.7	23.9	24
PE-Cys[e]	23.7	23.6	24

[a] Hagen et al. (1986). [b] Including one possible β-hydroxyaspartic acid. [c] Including 10 possible Gla residues. [d] Determined by extrapolation to zero time of hydrolysis. [e] Determined by hydrolysis in 4 M methanesulfonic acid.

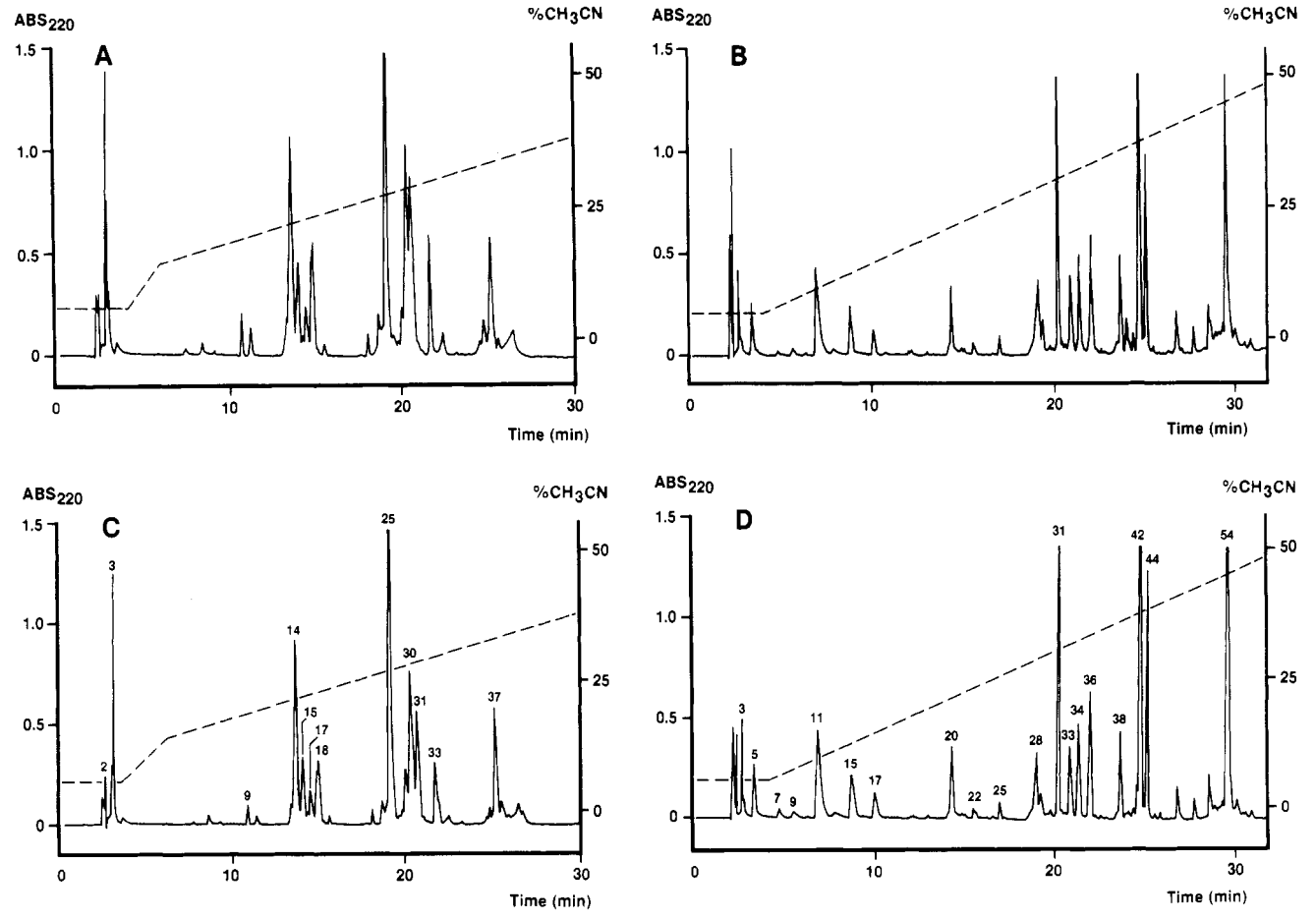

FIGURE 2: Tryptic peptide maps of light and heavy chains of p-factor VII_a (panels A and B) and r-factor VII_a (panels C and D). Fractions corresponding to the individual absorbance peaks were collected as indicated. The peptides identified in the fractions are given in Figure 3.

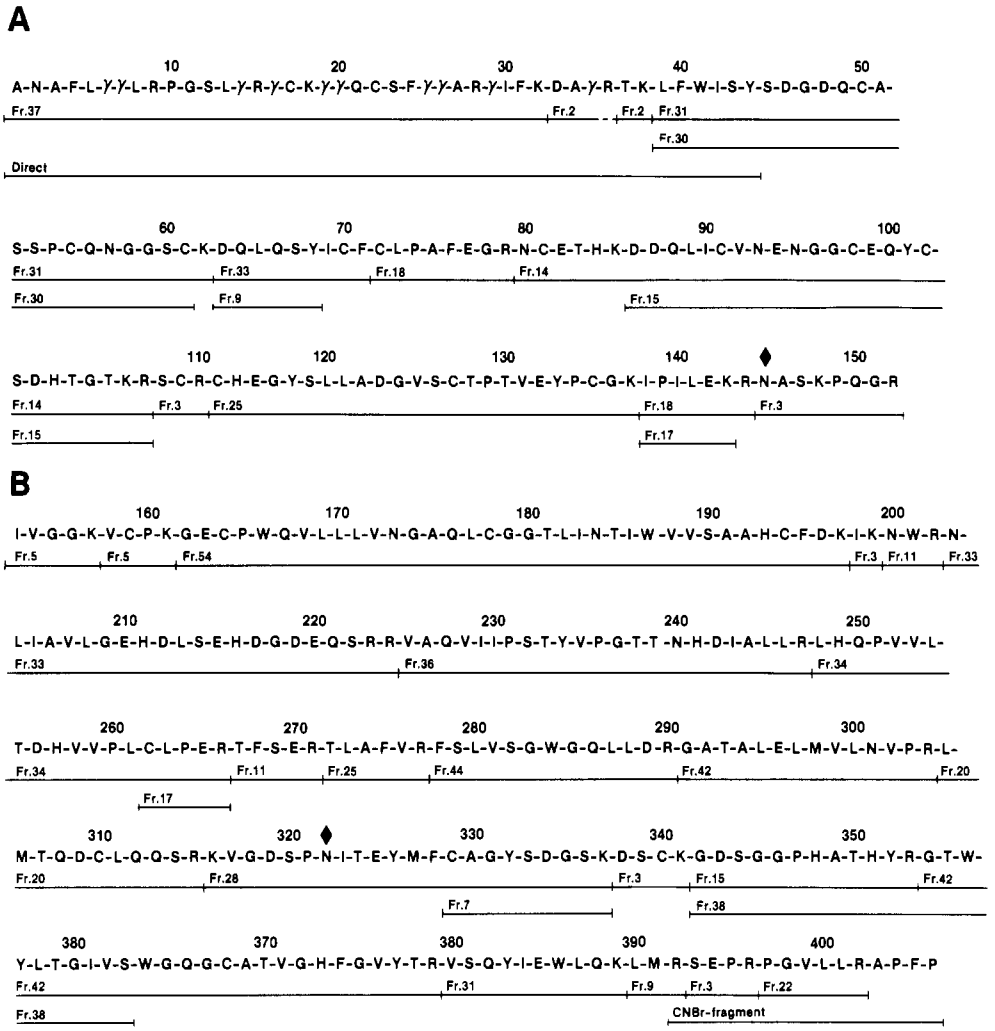

FIGURE 3: Amino acid sequence of light chain (panel A) and the heavy chain (panel B) of human factor VII$_a$ as deduced from cDNA (Hagen et al., 1986). The numbers refer to the HPLC fraction number from which the peptides were isolated (Figure 2). The solid lines indicate regions of peptides proven by amino acid sequence analysis. Potential N-glycosylation sites are marked by (♦). The C-terminal CNBr fragment was isolated as described under Materials and Methods.

The N-terminal sequencing of the native r-factor VII$_a$ and p-factor VII$_a$ showed the presence of two phenylthiohydantoin-amino acids in each degradation cycle arising from the N-terminal of the light chain (Ala-Asn-Ala-etc.) and the N-terminal of the heavy chain (Ile-Val-Gly-etc.), respectively. As judged from the sequencing data, the two chains were present in equimolar amounts. No sequences corresponding to factor VII$_a$ with parts of the prepro-leader sequence still on (Hagen et al., 1986) could be identified either in p-factor VII$_a$ or in r-factor VII$_a$. This indicates that complete processing between the prepro-leader sequence and the mature factor VII has been achieved also for r-factor VII$_a$ isolated from the mammalian expression system. Within the detection limit of the sequence analysis, it is also concluded that the conversion of factor VII to factor VII$_a$ [hydrolysis of the peptide bond Arg_{152}–Ile_{153} (Kisiel & McMullen, 1981)] has been achieved with a high degree of specificity.

The N-terminal sequence analyses of the pyridylethylated light chain of p-factor VII$_a$ and r-factor VII$_a$ are shown in Table II. Unambiguous assignation of the first 44 residues, including the PE-Cys in positions 17 and 22 but excluding the 10 Gla residues, was possible. The tryptic maps of the pyridylethylated light and heavy chains of p-factor VII$_a$ and r-factor VII are shown in Figure 2. All fractions corresponding to the individual absorbance peaks were collected manually as indicated in Figure 2 and subjected to amino acid sequence analysis. The results are summarized in Figure 3. As can be seen from this figure, the trypsin used was most probably contaminated with chymotrypsin since several fragments have an aromatic amino acid as the C-terminal residue. By combining the sequence analysis on the intact light chain (Table II) and the tryptic fragments, peptide fragments covering the entire light chain were obtained (Figure 3, panel A). Tryptic fragments covering the entire heavy chain, except the four last residues, were likewise obtained. The C-terminal tryptic peptide (Ala-Pro-Phe-Pro) was not identified in the analyzed fractions from the peptide maps. This peptide is probably eluted at the beginning of the chromatogram, but due to the large amount of buffer salts from the digest mixture also eluting at this position (e.g., glycine), it is not possible to sequence peptides in these fractions. The C-terminal part of the sequence of the heavy chain has therefore been elucidated by sequencing the CNBr fragment comprising residues 392–406 (Figure 3, panel B). By combining the above results based on digestion with a single enzyme, peptide fragments covering the entire factor VII$_a$ molecule have been sequenced. Although no overlapping peptide fragments have been isolated, the amino acid sequence of the tryptic fragments confirms the structure of human factor VII$_a$ as deduced from cDNA sequencing (Hagen et al., 1986).

γ-Carboxylations. By homology to other coagulation proteins, it has been suggested that human factor VII$_a$ contains

Table II: Automated Edman Degradation of the Pyridylethylated Light Chain of p-Factor VII_a and r-Factor VII_a

cycle	PTH-amino acid	p-factor VII_a yield (nmol)[a]	r-factor VII_a yield (nmol)[a]
1	Ala	4.8	12.2
2	Asn	4.9	8.4
3	Ala	5.5	10.9
4	Phe	4.4	9.5
5	Leu	5.1	9.5
6	(Gla)		
7	(Gla)		
8	Leu	4.1	8.6
9	Arg	1.3	2.1
10	Pro	2.3	6.9
11	Gly	2.2	5.5
12	Ser	0.6	2.0
13	Leu	2.2	6.0
14	(Gla)		
15	Arg	1.1	1.9
16	(Gla)		
17	PE-Cys	1.5	3.5
18	Lys	2.6	4.3
19	(Gla)		
20	(Gla)		
21	Gln	1.7	3.5
22	PE-Cys	1.1	2.5
23	Ser	0.4	0.9
24	Phe	1.7	2.2
25	(Gla)		
26	(Gla)		
27	Ala	1.3	2.3
28	Arg	0.8	1.4
29	(Gla)		
30	Ile	0.9	2.0
31	Phe	1.0	2.0
32	Lys	1.4	2.5
33	Asp	0.6	1.7
34	Ala	0.9	1.8
35	(Gla)		
36	Arg	0.4	0.9
37	Thr	0.3	0.3
38	Lys	0.6	1.2
39	Leu	0.9	1.4
40	Phe	0.6	1.0
41	Trp	0.2	0.2
42	Ile	1.7	1.1
43	Ser	0.4	0.4
44	Tyr	0.6	0.9

[a] Average repetitive yield: 93.0% (p-factor VII_a), 93.7% (r-factor VII_a).

10 Gla residues (Hagen et al., 1986). The Gla content of p-factor VII and r-factor VII_a was determined by amino acid analysis as the difference between the Glx content (Glu + Gln + Gla) in the native factor VII_a and the Glx content (Glu + Gln) in the γ-methyleneglutamic acid derived factor VII_a. A value of 10.1 mol of Gla/mol of protein was found for r-factor VII_a (47.3 − 37.2) and 11.0 (48.8 − 37.8) was found for p-factor VII_a. The presence of Gla residues in the sequence does not give rise to any detectable PTH-Gla using the normal Edman degradation method; however, a smaller amount of PTH-Glu was always detectable in cycles corresponding to the Gla residues. This is probably due to some degree of decarboxylation of Gla residues during normal sequencing. The actual amounts of glutamic acid in these cycles were compared to the expected amount of glutamic acid if the particular residues were not γ-carboxylated. The calculation of the expected amounts is based on the repetitive yields stated in Table II. Such calculation was made for the light chains of both p-factor VII_a and r-factor VII_a. As can be seen from these results (Table III), the amounts of PTH-Glu found in the Edman cycles corresponding to Gla residues are from 0 to 8.3% except for Gla residue 10 (position 35 of the light chain) in r-factor VII_a, in which the amount of PTH-Glu is 56%. It cannot be excluded that this residue is more sensitive to decarboxylation during sequencing than the other nine Gla residues. It is, however, more likely that this residue is not fully γ-carboxylated in the r-factor VII_a molecule. From the sequencing results, it is concluded that the Glu residues in positions 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35 of p-factor VII_a are fully γ-carboxylated; the same applied to the Glu residues of r-factor VII_a except for residue 35 which seems to be only half γ-carboxylated.

β-Hydroxylation. The cDNA sequencing predicts an aspartic acid in position 63 of the human factor VII sequence (Hagen et al., 1986), and by homology to bovine factor VII (McMullen et al., 1983a) and other vitamin K dependent coagulation proteins (Drakenberg et al., 1983; McMullen et al., 1983a,b), this residue might be β-hydroxylated. A standard of DL-*threo*-β-hydroxyaspartic acid was hydrolyzed under the same conditions as for factor VII_a, and by amino acid analysis, it eluted as two characteristic peaks in front of aspartic acid (McMullen, 1983a). In the hydrolyzed samples of p-factor VII_a as well as r-factor VII_a, no peaks corresponding to the elution positions of the hydrolyzed β-hydroxyaspartic acid standard were found. The light chain and the fragment comprising residues 62–84 of the light chain were also submitted to amino acid analysis. None of these samples originating from p-factor VII_a as well as r-factor VII_a were found to contain β-hydroxyaspartic acid. Two peptide fragments containing residue 63 as the N-terminal amino acid were isolated from the tryptic digest of the light chain (Figure 3, panel A). By sequencing these peptides originating from p-factor VII_a as well as r-factor VII_a, this residue was found

Table III: Amount of Glutamic Acid in Edman Degradation Cycles Corresponding to the Positions of Gla Residues Compared to the Expected Amount of Glutamic Acid If the Particular Residues Were Not γ-Carboxylated[a]

	human plasma factor VII_a			recombinant factor VII_a		
residue	amount of Glu (pmol)	expected amount of Glu[b] (pmol)	actual/expected (%)	amount of Glu (pmol)	expected amount of Glu[c] (pmol)	actual/expected (%)
6	71	4252	1.7	72	8256	0.9
7	67	4141	1.6	73	7736	0.9
14	82	2492	3.3	143	4906	2.9
16	33	2155	1.5	108	4307	2.5
17	125	1733	7.2	0	3543	0
20	0	1612	0	104	3320	3.1
25	32	1121	2.9	0	2398	0
26	49	1043	4.7	21	2247	0.9
29	15	839	1.8	131	1848	7.0
35	45	543	8.3	700	1251	56.0

[a] Data taken from the sequence analysis of the light chain of p-factor VII_a and r-factor VII_a (Table II). [b] Calculation based on the following data: amount applied, 8.5 nmol; initial yield, 75%; average repetitive yield, 93.0%. [c] Calculation based on the following data: amount applied, 13.6 nmol; initial yield, 75%; average repetitive yield, 93.7%.

Table IV: Signals Observed in the Positive-Ion FAB-MS Spectrum of the Chymotryptic Digest of Peptide Fragment Comprising Residues 62–84 of Plasma Factor VII_a

m/z[a]	peptide
487	Ile-PECys-Phe
655	PECys-Leu-Pro-Ala-Phe
737	Ser-Tyr-Ile-PECys-Phe
881	Lys-Asp-Gln-Leu-Gln-Ser-Tyr
882	Lys-Asp-Glx-Leu-Glx-Ser-Tyr[b]
997	PECys-Leu-Pro-Ala-Phe-Glu-Gly-Arg-Asn-PECys
1686	PECys-Leu-Pro-Ala-Phe-Glu-Gly-Arg-Asn-PECys-Glu-Thr-His

[a] This corresponds to the protonated molecular ion, $(M + H)^+$. [b] This peptide corresponds to the monodesamido form of the peptide with the m/z of 881.

to be PTH-Asp, and no sign of PTH-β-hydroxy-Asp was found. To further analyze the possible presence of β-hydroxyaspartic acid in position 63, the *Armillaria mellea* protease fragment comprising residues 62–84 was isolated from the light chain of p-factor VII_a and subjected to positive-ion fast atom bombardment mass spectrometry analysis. Prior to this analysis, the peptide was digested with chymotrypsin. The signals observed in the positive-ion FAB-MS spectrum are given in Table IV. The signals observed in the spectrum result in the complete mapping of the peptide from residues 62 to 84. A strong signal was observed at m/z 881 corresponding to the sequence Lys-Asp-Gln-Leu-Gln-Ser-Tyr. The signal was accompanied by a signal at m/z 882 which indicates that one of the Gln residues may have been partially deamidated. Only a very weak signal of approximately background height was observed at m/z 897 which would have been anticipated to be strong if the peptide Lys-BHA-Gln-Leu-Gln-Ser-Tyr was present. From the above results, it is concluded that neither p-factor VII_a nor r-factor VII_a contains β-hydroxyaspartic acid.

Glycosylations. Two potential carbohydrate attachment sites comprising residues 145–147 (Asn-Ala-Ser) and residues 322–324 (Asn-Ile-Thr) exist in the factor VII_a molecule (Hagen et al., 1986). The amino acid sequencing of the peptide comprising residues 145–152 of the light chain (Figure 3, panel A) gives rise to the PTH derivative of residue 145 in Edman degradation cycle 1. By sequencing the peptide originating from r-factor VII_a, this cycle was found to contain PTH derivatives eluting at the positions of PTH-Asp (802 pmol) and PTH-Asn (374 pmol). If residue 145 was not glycosylated, a total amount of PTH-Asp and PTH-Asn of approximately 3500 pmol would be expected. Thus, the sequencing results indicate that Asn-145 is partially (approximately 66%) glycosylated. A corresponding sequence analysis of p-factor VII_a showed no sign of PTH-Asp or PTH-Asn in this Edman degradation cycle, indicating that p-factor VII_a is fully glycosylated at Asn-145. The sequencing of the peptide comprising residues 316–337 of the heavy chain (Figure 3, panel B) gives rise to the Edman degradation product of Asn-322 in cycle 7. This cycle was blank, and no sign of PTH-Asp or PTH-Asn was seen in peptides originating from p-factor VII_a as well as from r-factor VII_a, indicating that Asn-322 is fully glycosylated in both proteins.

The carbohydrate moieties of p-factor VII_a as well as r-factor VII_a are composed of *N*-acetylglucosamine, mannose, galactose, fucose, and sialic acid (Table V). Some minor quantitative differences were seen in the two types of factor VII_a molecules, the most pronounced difference being a somewhat higher fucose content and a lower sialic acid content of r-factor VII_a as compared to p-factor VII_a. However, both types of molecules contain the same type of carbohydrates,

Table V: Carbohydrate Composition of Plasma Factor VII_a and Recombinant Factor VII_a

	residue[a] (mol/mol of factor VII_a)	
	p-factor VII_a	r-factor VII_a
Fuc[b]	1.8	3.0
Man[b]	6.1	6.1
Gal[b]	5.5	4.2
GlcNAc[b]	6.9	7.1
NeuAc[b]	7.4	5.2
NeuAc[c]	4.9	3.9

[a] Abbreviations: Fuc, fucose; Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid (sialic acid). [b] Determined by HPLC. [c] Determined by the assay of Massamiri et al. (1978).

and the minor quantitative differences may reflect carbohydrate heterogeneity in both types of factor VII_a molecules.

Discussion

One of the major challenges in hemophilia care is the treatment of hemophilia patients having developed antibodies against factor VIII/IX. One possibility is to use so-called "factor VIII bypassing activity" products containing a mixture of several activated coagulation factors (VII_a, IX_a, and X_a). However, of these, factor VII_a seems to be the most attractive one since it is hemostatically active only after having complexed with tissue factor exposed at the site of injury. Therefore, injected factor VII_a should not cause a systemic activation of the coagulation cascade but only induce local hemostasis at the site of injury. The experience in a few patients seems to confirm this hypothesis (Hedner & Kisiel, 1983). Plasma contains only trace amounts of factor VII, and consequently, it meets with substantial difficulties to prepare sufficient amounts of purified factor VII_a from human plasma. Furthermore, all products prepared from human plasma bear the risk of transmitting viruses such as hepatitis virus and HIV. Therefore, factor VII_a has been expressed in a mammalian cell system and purified from the culture media. However, to be able to substitute r-factor VII_a for p-factor VII_a in the clinic, it has to be shown that r-factor VII_a has the same biological activity as p-factor VII_a. A prerequisite for a normal activity is a molecule with normal posttranslational modifications. Thus, an intact Gla region is necessary for the adsorption of the coagulation protein to phospholipid surfaces (Borowski et al., 1985).

In the present study, human factor VII_a isolated from plasma and from the culture medium of transfected baby hamster kidney cells has been compared. The two types of molecules were found to have identical chromatographic behavior on reverse-phase HPLC both in their native form (Figure 1, panels A and B) and as reduced and pyridylethylated light and heavy chains (Figure 1, panels C and D). Previous study has shown that human plasma (Broze & Majerus, 1982) as well as the culture medium of transfected BHK cells (Berkner et al., 1986) contains the single-chain form of factor VII. This and other studies (Radcliffe & Nemerson, 1975; Bjoern & Thim, 1986) have shown that a complete activation of factor VII to factor VII_a can be accomplished by the use of anion-exchange chromatographic steps in the purification scheme. The mechanism of this activation is unknown. The degree of activation seems, at least to some extent, to depend upon the amount of factor VII loaded per volume of gel material. Single-chain factor VII has some proteolytic activity itself [approximately 4% of that of factor VII_a (Broze & Majerus, 1982)], and autoactivation in the presence of positively charged groups may be the mechanism of activation.

The isolated light and heavy chains of p-factor VII_a and r-factor VII_a show identical tryptic maps (Figure 2). Peptide fragments obtained mainly from the tryptic digests of the light and heavy chains have been isolated and subjected to automated Edman degradation. Although a series of other peptide fragments is needed to fully prove the sequence of factor VII_a on amino acid level, the peptide isolated and sequenced in the present study covers the entire sequence (Figure 3) and confirms the amino acid sequence as deduced from cDNA (Hagen et al., 1986).

The Gla content of p-factor VII_a and r-factor VII_a was determined by amino acid analysis as the difference between the content of Glu, Gln, and Gla in the native molecule and the Glu and Gln content in the γ-methyleneglutamic acid derived molecule, and thus the calculated value is encumbered with a relatively large degree of uncertainty. The calculated value of 11 Gla per molecule in the p-factor VII_a thus may be the result of an overestimation of Gln + Glu + Gla in the native molecule in which this value was determined to 48.8 residues per mole (theoretical value: 47 residues per mole). The amino acid sequence analysis on the isolated light chains of p-factor VII_a and r-factor VII_a indicated that the 10 N-terminally located glutamic acid residues were fully γ-carboxylated except for Glu residue 10 (amino acid residue 35) in the r-factor VII_a molecule (Table III). In the Edman degradation products from this particular cycle, a considerable amount of PTH-Glu was found corresponding to approximately half-γ-carboxylation of this residue (Table III). The small amount of PTH-Glu observed in the other cycles corresponding to the positions of Gla residues probably represents decarboxylated Gla residues created during the sequencing.

On the basis of previous results on human and bovine factor IX (McMullen et al., 1983a), bovine factor VII (McMullen et al., 1983a), human factor X (McMullen et al., 1983b), and bovine protein C (Drakenburg et al., 1983), it would be expected that residue 63 of human factor VII_a was a β-hydroxylated aspartic acid. By amino acid analysis (Table I), sequence analysis (Figure 3), and positive-ion FAB-MS analysis (Table IV), this residue was shown to be aspartic acid in the human factor VII_a molecule. This result is unexpected since residue 63 is placed in the epidermal growth factor domain of human factor VII_a which in other homologous proteins results in β-hydroxylation (Stenflo & Öhlin, 1987). The function of β-hydroxylated aspartic acid in vitamin K dependent proteins is unknown. It has been proposed that factor IX binds Fe^{3+} with physiologically significant affinity and that the β-hydroxyaspartic acid residue in factor IX is a chelator for the bound metal (Fowler et al., 1986).

The results from the sequence analysis show that the two potential N-glycosylation sites (Asn-145 and Asn-322) are fully glycosylated in p-factor VII_a in that no PTH-Asp or PTH-Asn was observed in the Edman degradation products from these residues. In the corresponding sample from r-factor VII_a, Asn-322 was found to be fully glycosylated whereas Asn-145 was found to be only partially (approximately 66%) glycosylated. The reduced sialic acid content in r-factor VII as compared to p-factor VII (Table V) may be a result of the partial glycosylation of Asn-145 in the r-factor VII_a molecule.

The carbohydrate moieties of p-factor VII_a are composed of *N*-acetylglucosamine, mannose, galactose, fucose, and sialic acid. This composition matches the components of asparagine-linked oligosaccharides of the complex type, which has been found in the vitamin K dependent coagulation factors X, II, and IX of bovine origin (Mizuochi et al., 1979, 1980, 1983). Further analysis is in progress in order to characterize the structure of the carbohydrate moieties of factor VII_a. The carbohydrate composition of r-factor VII_a is similar to that of p-factor VII_a (Table V), and these findings are in agreement with the previously published glycosylation capability of BHK cells (Yamashita et al., 1984; Pierce et al., 1986).

In summary, the present study has elucidated some minor differences between plasma-derived factor VII_a and r-factor VII_a expressed by BHK cells as for the degree of γ-carboxylation and glycosylation. However, these dissimilarities may not be of any major importance with regard to the biological activity of r-factor VII_a since preliminary in vitro studies have demonstrated a similar ability of r-factor VII_a and p-factor VII_a to normalize the prolonged activated partial thromboplastin time (APTT) in hemophilia A and B plasma (Hedner et al., 1987). Whether they will be of any significance in a clinical setting will be explored within the near future.

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Endogenous Phosphorylation of Basic Protein in Myelin of Varying Degrees of Compaction†

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ABSTRACT: Fractions containing myelin of varying degrees of compaction were prepared from human white matter. Protein kinase activity in these fractions was measured by using both endogenous and exogenous myelin basic protein (MBP) as substrates. In both cases, less compact myelin fractions possessed higher levels of protein kinase activity than the compact myelin fraction. In addition, the specific activity of phosphorylated basic protein was greater in the loosely compacted fractions than in compact multilamellar myelin. When basic protein in compact myelin or the myelin fractions was phosphorylated by the endogenous kinase, approximately 70% of the [^{32}P]phosphate was incorporated at a single site, identified as Ser-102. The remaining 30% was found in three other minor sites. Electron microscopy of less compact myelin showed it was composed of fewer lamellae which correlated with a relative decrease in the proportion of cationic charge isomers (microheteromers) when MBP was subjected to gel electrophoresis at alkaline pH. The shift in charge microheterogeneity of basic protein to the less cationic isomers in the less compact myelin fractions correlated with an increase in protein kinase activity and a greater specific activity of phosphorylated basic protein.

Myelin, the multilamellar structure which surrounds the axons, has an important role in impulse transmission. However, myelin may exist in various levels of compaction possibly reflecting different functions in various parts of the central nervous system. The isolation and characterization of several less compact myelin-containing fractions prepared from human white matter were reported recently (Cruz & Moscarello, 1985). The myelin membranes contained in these fractions appeared to be morphologically and biochemically distinct from each other and from compact myelin. The compact myelin fraction contained the characteristic multilamellar structures whereas the denser fractions contained less compacted myelin with fewer lamellae. Similar results were obtained with myelin subfractions prepared from rodent brain (Matthieu & Waehneldt, 1978; McIntyre et al., 1978; Pereyra & Braun, 1983). Although the factors responsible for the differences in myelin compaction are not known, it has been suggested that charge microheterogeneity of myelin basic protein (MBP)[1] may play an important role in this process (Boggs et al., 1982). The isolation of several charge isomers (components; Chou et al., 1976) of MBP has permitted us to study the effect of small changes in charge on the ability of the protein to interact with, and organize, lipid bilayers.

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[1] Abbreviations: MBP, myelin basic protein; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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