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Purification and partial characterization of rat factor D

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Rat factor D has been purified to homogeneity (10559-fold) from serum by chromatography on CM-Sepharose Fast Flow, phenyl-Sepharose CL-4B and Mono S and has been shown to resemble its human and mouse counterparts both in substrate specificity and in its susceptibility to inhibition by the organophosphorous inhibitor di-isopropyl-fluorophosphate. The rat enzyme, however, is heavily glycosylated and binds to wheat-germ lectin–Sepharose 6MB and 5-hydroxytryptamine–agarose, but not to concanavalin A–Sepharose 4B. All of the carbohydrate chains are *N*-linked. Enzymic removal of this carbohydrate decreased the M_r by approx. 15000. The deglycosylated rat enzyme had the same mobility as native human factor D on SDS/PAGE, corresponding to an M_r of 24500. *N*-Terminal sequence analysis of the first 30 amino acids of rat factor D highlighted the sequence similarity with human factor D (> 76%) and, in particular, with mouse adipsin (> 93%).

INTRODUCTION

The complement system can be activated by two distinct routes, the classical and the alternative pathways. The formation of alternative pathway C3 convertase is regulated by the activity of factor D, an unusual tryptic-like serine protease of high specificity that cleaves a single Arg–Lys bond in factor B when the latter is bound to C3b. Human factor D is the smallest of the complement components (M_r 24000) and has been fairly well characterized [1,2]. Guinea pig [3] and horse [4] factor D are similar, having M_r values of about 22000, but bovine factor D [4] was reported to be smaller, at M_r 15000. Mouse adipsin, which is secreted from 3T3 cells in two forms (M_r 37000 and 44000), has now been identified as mouse factor D [5,6]. In this study rat factor D has been purified to homogeneity, partially characterized and compared with the mouse and human enzymes.

MATERIALS AND METHODS

Materials

Human factor B, cobra venom factor and goat anti-(human factor B) were purchased from Calbiochem, and human factor D was obtained from Quidel. Rabbit anti-(goat IgG) alkaline phosphatase conjugate, 5-hydroxytryptamine–agarose, *N*-acetyl-D-glucosamine, methyl α-D-mannopyranoside and neuraminidase were from Sigma; glycopeptidase F and glycan detection kit were from Boehringer Mannheim, and peptide-4-methyl-coumaryl-7-amide (MCA) substrates were from Peninsula Laboratories. Nitrocellulose and silver stain were purchased from Bio-Rad, polyvinylidene difluoride (PVDF; Immobilon) membranes were from Millipore, and rat serum was from Serotec. Wheat-germ lectin–Sepharose 6MB, phenyl-Sepharose CL-4B, CM-Sepharose Fast Flow and concanavalin A (Con A)–Sepharose 4B were from Pharmacia.

Purification

Rat serum (50 ml) after dialysis (10 mM-sodium acetate buffer/0.15 M-NaCl, pH 6.0) and filtering (Whatman GF/C) was loaded on to an equilibrated CM-Sepharose Fast Flow column (2.5 cm × 19.5 cm, flow rate 100 ml/h) and washed with dialysis buffer. Eluted activity was concentrated (Amicon PM10), dialysed (10 mM-sodium phosphate buffer, pH 6.8) and an equal volume (60 ml) of 10 mM-sodium phosphate buffer/3 M-$(NH_4)_2SO_4$, pH 6.8, was added prior to loading (120 ml) on to a phenyl-Sepharose CL-4B column (2.5 cm × 8.0 cm, flow rate 80 ml/h) equilibrated in 10 mM-sodium phosphate buffer/1.5 M-$(NH_4)_2SO_4$, pH 6.8. The column was washed with equilibration buffer (80 ml) followed by a gradient (200 ml total) from equilibration buffer to 10 mM-sodium phosphate buffer, pH 6.8. Active fractions were pooled, concentrated, dialysed (10 mM-sodium acetate buffer/0.1 M-NaCl, pH 6.0) and loaded (6 ml) on to a second CM-Sepharose Fast Flow column (1.5 cm × 9.0 cm, flow rate 30 ml/h) that had been equilibrated in dialysis buffer. After an equilibration buffer wash (40 ml), enzyme activity was eluted by a gradient (80 ml total) from equilibration buffer to 10 mM-sodium acetate buffer/0.15 M-NaCl, pH 6.0. Factor D activity was pooled, concentrated and dialysed (10 mM-sodium acetate buffer/0.1 M-NaCl, pH 6.0) before loading (0.6 ml) on to a Mono S (HR5/5) column (flow rate 60 ml/h) equilibrated in dialysis buffer. After an equilibration buffer wash (7 ml), activity was eluted by a linear gradient (50 ml total) from equilibration buffer to 10 mM-sodium acetate buffer/0.2 M-NaCl, pH 6.0. Excluding the chromatography on Mono S, all manipulations were undertaken at 4 °C.

Assays

Factor D activity throughout the purification was monitored by haemolytic assay using rabbit erythrocytes and factor-D-depleted human serum. The latter was prepared by chromatographing human serum (50 ml) on CM-Sepharose Fast Flow (100 ml) equilibrated in 50 mM-sodium phosphate buffer/0.2 M-NaCl, pH 6.0. Unbound protein was pooled, concentrated back to the original serum volume and made up to 8 mM-EGTA and 2 mM-$MgCl_2$. The haemolytic assay comprised, in a total volume of 150 μl, rabbit erythrocytes (10^7 cells in 100 μl of 5 mM-Veronal-buffered saline/12 mM-$MgCl_2$, pH 7.4), factor-D-depleted serum (25 μl) and enzyme sample (25 μl). After incubation (37 °C, 1 h) the reaction was terminated by the addition of 1 ml of 5 mM-Veronal-buffered saline/10 mM-EDTA, pH 7.4

Abbreviations used: DFP, di-isopropylfluorophosphate; MCA, 4-methylcoumaryl-7-amide; PTH, phenylthiohydantoin; Boc, t-butyloxycarbonyl; PVDF, polyvinylidene difluoride; Con A, concanavalin A.

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Table 1. Summary of rat factor D purification

1 unit of enzyme activity is defined as that amount of factor D giving 10% lysis in the standard haemolytic assay described in the Materials and methods section.

Purification step	Volume (ml)	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Yield (%)	Purification (fold)
1. Serum	50	6275	10300	1.64	100	1
2. CM-Sepharose Fast Flow	120	81.6	9710	119	94.3	72.6
3. Phenyl-Sepharose	6.5	2.79	3470	1244	33.7	758.5
4. CM-Sepharose Fast Flow	0.6	0.268	1700	6343	16.5	3867.7
5. Mono S	1.0	0.041	710	17317	6.9	10559.1

(ice cold). After centrifugation (500 *g*, 10 min) the absorbance of the supernatants was measured at 412 nm and the activity expressed as a percentage of that in a lysed control (100 μl of erythrocytes plus 50 μl of water incubated as above; reaction stopped with 1 ml of water).

Factor D activity was measured in a pure system by incubation for 2 h at 37 °C in 60 μl of 5 mM-Veronal-buffered saline/12 mM-$MgCl_2$, pH 7.4, with human factor B (5 μg) and cobra venom factor (1 μg). Di-isopropylfluorophosphate (DFP; 1–20 mM) was included during inhibitor studies. The reaction was stopped with SDS/PAGE sample buffer (no reducing agent), the incubates were electrophoresed [7] and the Ba and Bb products were detected by Western blot [8].

Cleavage of Boc-Leu-Lys-Arg-MCA and Boc-Leu-Gly-Arg-MCA (0.2 mM) by rat factor D (1 μg) was monitored by measuring the generation (37 °C, 1 h) in 75 μl of 10 mM-sodium phosphate buffer/140 mM-NaCl/12 mM-$MgCl_2$, pH 7.4, of 7-amino-4-methylcoumarin in a Perkin–Elmer LS-5B luminescence spectrometer ($\lambda_{ex.}$ 380 nm, $\lambda_{em.}$ 460 nm).

Glycosylation

The ability of purified rat factor D to bind lectins and 5-hydroxytryptamine was assessed as follows. Columns (3–4 ml) containing either wheat-germ lectin–Sepharose 6MB or Con A–Sepharose 4B were equilibrated in 50 mM-sodium phosphate buffer/0.2 M-NaCl, pH 7.0. The columns were then loaded with factor D (13 μg) and washed with equilibration buffer (9–12 ml) and then with either equilibration buffer containing 0.5 M-*N*-acetyl-D-glucosamine (wheat-germ) or 0.5 M-methyl α-D-mannopyranoside (Con A). The proteins α_2-macroglobulin (wheat-germ) and transferrin (Con A), known to bind these lectins, were used as positive controls. The 5-hydroxytryptamine–agarose column (3 ml) was equilibrated in 2 mM-sodium phosphate buffer, pH 6.8, before loading the enzyme (13 μg). The column was washed successively with equilibration buffer, 50 mM- and 100 mM-sodium phosphate buffer, pH 6.8, 100 mM-sodium phosphate buffer/0.25 M-NaCl, pH 6.8, and finally 200 mM-sodium formate buffer, pH 3.0. In this case fetuin and asialofetuin were used as positive and negative control proteins respectively.

Clostridium perfringens neuraminidase (1 μg) was used to desialylate rat factor D (20 °C, 20 h; 5 μg of factor D in 30 μl of 50 mM-sodium acetate buffer, pH 5.0). *Flavobacterium meningosepticum* glycopeptidase F (2 munits) was employed to deglycosylate rat factor D (37 °C, 20 h; 5 μg of factor D in 30 μl of 100 mM-sodium phosphate buffer/0.5% Triton X-100/10 mM-dithiothreitol/10 mM-EDTA, pH 7.2). In both cases the reactions were stopped with SDS/PAGE sample buffer [7] followed by electrophoresis [7] and blotting on to nitrocellulose [8]. Carbohydrate was then detected using a glycan staining kit according to the manufacturer's instructions [9].

Sequencing

Both glycosylated and deglycosylated rat factor D, after SDS/PAGE (12% gel), were transferred to a PVDF membrane [10], and the bands were excised and exposed to automated Edman degradation on an Applied Biosystems 477A sequencer equipped with an on-line 120A phenylthiohydantoin (PTH) analyser.

Protein determination

Protein was measured by the method of Bradford [11], using BSA as standard.

RESULTS AND DISCUSSION

Factor D was purified to apparent homogeneity from rat serum using two cation exchangers and one hydrophobic affinity chromatography step. Table 1 shows the overall purification (10559-fold), which resulted in 41 μg of pure protein with an activity yield of 6.9%. This is probably an underestimate of yield and fold purification, as the crude factor D fractions will contain interfering complement components capable of giving an anomalously high factor D activity in the haemolytic assay.

The elution profile from the first CM-Sepharose Fast Flow column is shown in Fig. 1(*a*). In order to effect good separation of the factor D activity from the bulk of eluted protein, using isocratic development, it was absolutely essential to ensure that the sample was exactly at pH 6.0 prior to loading; separation diminished as the sample pH increased above 6.0. This chromatography step resulted in an activity yield of 94% and removed about 98% of the extraneous protein and is, therefore, an extremely efficient step. Similarly, chromatography on phenyl-Sepharose CL-4B (Fig. 1*b*) removed 96% of the contaminating protein from the CM-Sepharose Fast Flow-purified sample and afforded another 10-fold purification of factor D. The activity is eluted from this column in a concentrated form which permits sufficient dilution (hence no dialysis required) of the enzyme activity, prior to assay, to negate the effects that $(NH_4)_2SO_4$ has on the haemolysis of red blood cells. Re-chromatography of rat factor D on CM-Sepharose Fast Flow in the same buffer as the first column, but with a lower (from 0.15 M to 0.1 M) NaCl concentration, ensured retention on the cation exchanger and enabled development of the column by gradient elution (Fig. 1*c*). Although considerable (90%) extraneous protein was removed from the sample by this step, only a 5-fold purification was observed. The final purification step involved f.p.l.c. on Mono S (Fig. 1*d*). Factor D activity was well resolved, eluting as a sharp peak early in the NaCl gradient. Fig. 2 clearly shows the homogeneity of the final rat factor D sample on SDS/PAGE and also highlights the efficacy of the Mono S step in removing several major contaminating species. Further refinement of this final step could, perhaps, enable chromatography of the post-

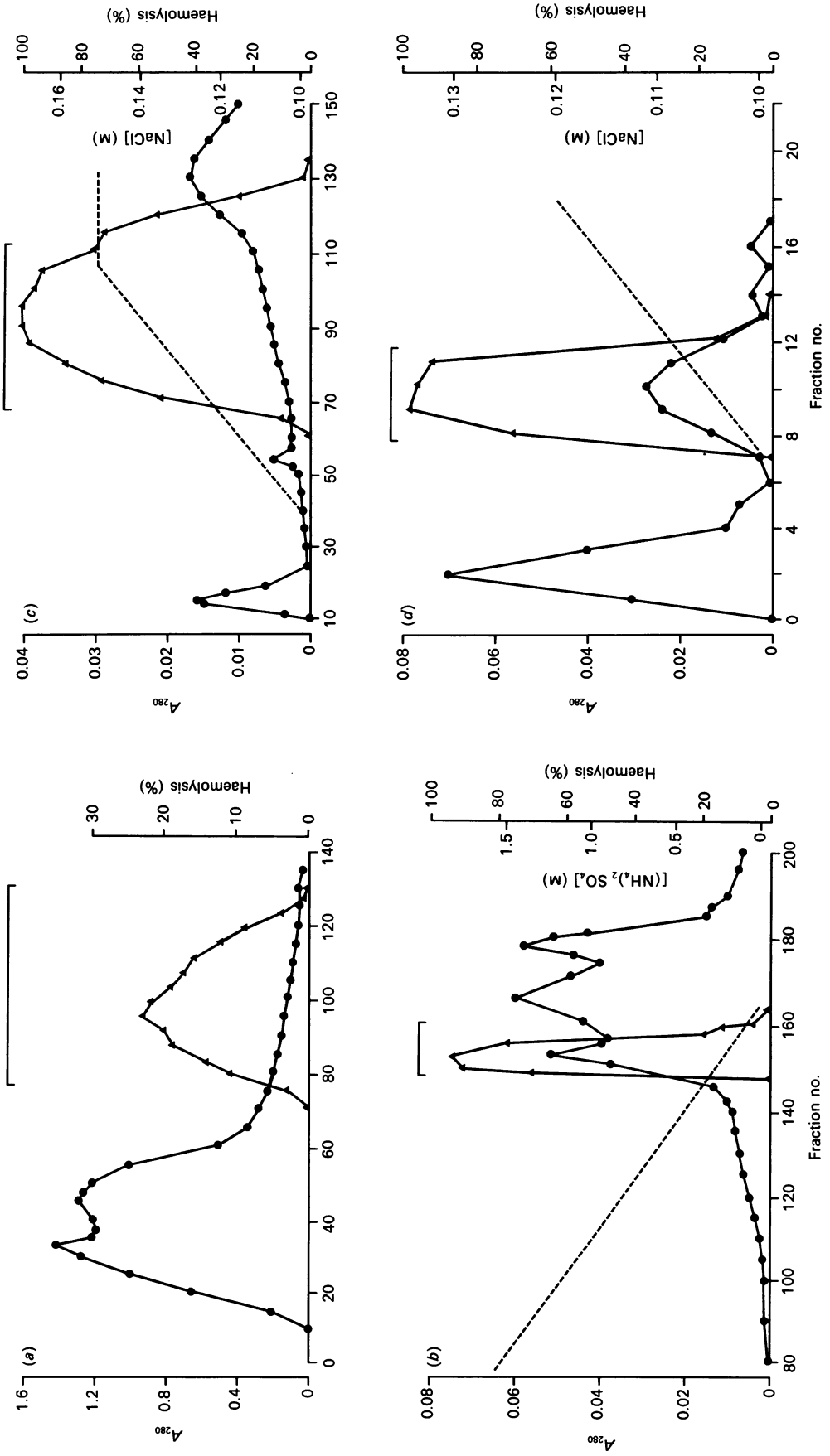

Fig. 1. Elution profiles throughout the purification of rat factor D

(*a*) First CM-Sepharose Fast Flow. The column (2.5 cm × 19.5 cm) was equilibrated with 10 mM-sodium acetate buffer/0.15 M-NaCl, pH 6.0 (flow rate 100 ml/h), before loading rat serum (50 ml) that had previously been dialysed into equilibration buffer. The column was developed in the same buffer (fraction size 2.4 ml). (*b*) Phenyl-Sepharose CL-4B. Sample (120 ml) in 10 mM-sodium phosphate buffer/1.5 M-$(NH_4)_2SO_4$, pH 6.8, was loaded on to the column (2.5 cm × 8.0 cm, flow rate 80 ml/h) previously equilibrated in the same buffer. Following a wash (80 ml) with equilibration buffer the column was developed with a gradient (200 ml total) from equilibration buffer to 10 mM-sodium phosphate buffer, pH 6.8 (fraction size 2.4 ml). (*c*) Second CM-Sepharose Fast Flow. Sample (6 ml) in 10 mM-sodium acetate buffer/0.1 M-NaCl, pH 6.0, was loaded on to a column (1.5 cm × 9.0 cm, flow rate 30 ml/h) that had been equilibrated in the same buffer. Following an equilibration buffer wash (40 ml), activity was eluted with a gradient (80 ml total) from equilibration buffer to 10 mM-sodium acetate buffer/0.15 M-NaCl, pH 6.0 (fraction size 1.2 ml). (*d*) Mono S. Sample (0.6 ml) in 10 mM-sodium acetate buffer/0.1 M-NaCl, pH 6.0, was loaded on to a column (HR5/5, flow rate 60 ml/h) previously equilibrated in the same buffer. Following a wash (7 ml) with equilibration buffer, activity was eluted with a linear gradient (50 ml total) from equilibration buffer to 10 mM-sodium acetate buffer/0.2 M-NaCl, pH 6.0 (fraction size 1 ml). ●, A_{280}; ▲, factor D activity; ---, salt gradients. Fractions enclosed within the braces were pooled at each stage.

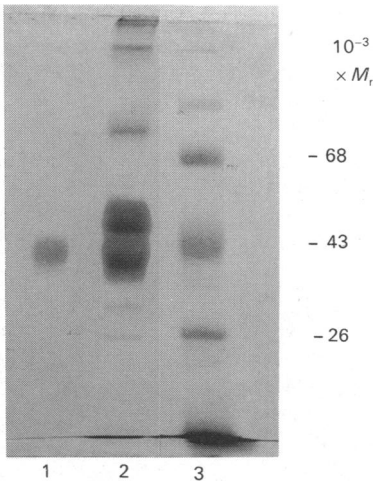

Fig. 2. SDS/PAGE of rat factor D before and after purification on Mono S

Samples of rat factor D, from steps 4 (pre-Mono S) and 5 (post-Mono S) of the purification protocol shown in Table 1, were electrophoresed after denaturing in SDS/PAGE buffer [7]. Protein was visualized by silver staining according to the manufacturer's instructions (Bio-Rad). Track 1, post-Mono S (0.6 μg); track 2, pre-Mono S (4.5 μg); track 3, protein standards.

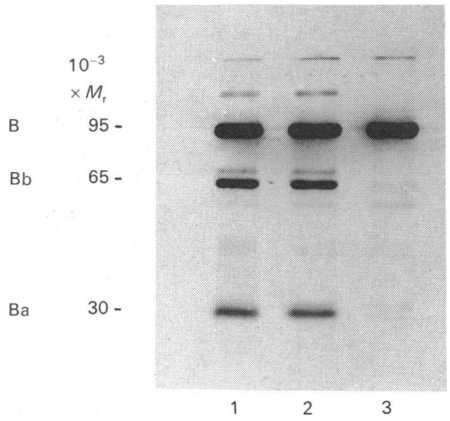

Fig. 3. Cleavage of human factor B by rat and human factor D

Factor D (1 μg) was incubated (2 h, 37 °C) with human factor B (5 μg) and cobra venom factor (1 μg) in 5 mM-Veronal-buffered saline/12 mM-$MgCl_2$, pH 7.4 (60 μl). The incubates were electrophoresed after terminating the reactions with SDS/PAGE sample buffer [7]. Bands corresponding to factor B and fragments Ba and Bb were detected by Western blotting with goat anti-(human factor B) and rabbit anti-(goat IgG)–alkaline phosphatase conjugate. Track 1, factor B plus rat factor D; track 2, factor B plus human factor D; track 3, factor B alone.

phenyl-Sepharose sample directly on Mono S, hence reducing the overall protocol to just three column steps.

Activity throughout the purification was monitored by measuring the haemolysis of rabbit erythrocytes by factor-D-depleted human serum after reconstitution with the rat enzyme, so it was imperative to show that the rat enzyme was capable of cleaving the physiological substrate, factor B, in a pure system. Both the rat and the human factor D appear to cleave the same scissile bond in human factor B substrate, as indicated by the identical mobility, determined by SDS/PAGE, of the Ba and Bb fragments produced by each enzyme (Fig. 3). Mouse adipsin has been reported to cleave the same bond [6].

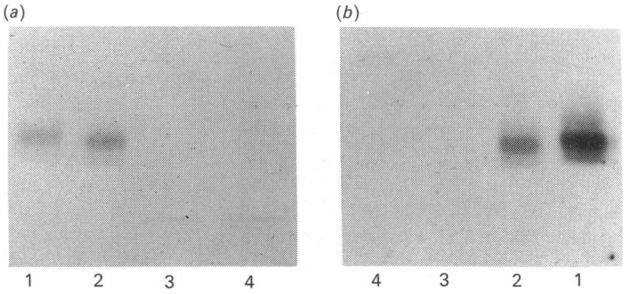

Fig. 4. SDS/PAGE of neuraminidase-treated factor D

Factor D (5 μg) was incubated (20 h, 20 °C) with neuraminidase (1 μg) in 50 mM-sodium acetate buffer, pH 5.0 (30 μl). Reactions were stopped with SDS/PAGE sample buffer and electrophoresed [7] on duplicate gels. One gel was silver-stained for protein (*a*) and the other was blotted on to nitrocellulose for carbohydrate detection with a glycan staining kit (*b*). Tracks: 1, rat factor D; 2, rat factor D plus neuraminidase; 3, human factor D; 4, human factor D plus neuraminidase.

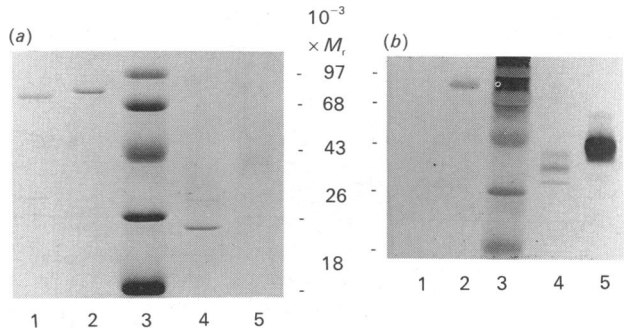

Fig. 5. SDS/PAGE of glycopeptidase F-treated rat factor D

Factor D (5 μg) was incubated (20 h, 37 °C) with glycopeptidase F (2 munits) in 100 mM-sodium phosphate buffer/0.5% Triton X-100/10 mM-dithiothreitol/10 mM-EDTA, pH 7.2 (30 μl). Samples were subsequently treated as described in the legend to Fig. 4. (*a*) Protein stain, (*b*) glycan stain. Deglycosylation of transferrin was used as a positive control. Tracks: 1, transferrin plus glycopeptidase F; 2, transferrin; 3, protein standards; 4, rat factor D plus glycopeptidase F; 5, rat factor D. The band evident in tracks 1 and 4 of gel (*a*) corresponds to glycopeptidase F.

The poor turnover of synthetic substrate analogues by human factor D has been documented [12] and probably emphasizes the importance of the conformational change induced at the catalytic centre of factor D by the natural substrate factor B when the latter is complexed to C3b. In our studies rat factor D appears to behave similarly, in that the turnover of two synthetic substrates containing arginyl-MCA was barely detectable, and was certainly insignificant when compared with the $k_{cat.}$ observed for other tryptic enzymes. Mouse adipsin, at approx. 3 times the concentration of rat factor D used in this study, and over a 16 h incubation period, has been reported to show very low, but significant, activity against one of these substrates which was inhibited by 10 mM-DFP [6]. Human factor D is inhibited only by low millimolar (10 mM) concentrations of DFP [1], and rat factor D in our studies responded to inhibition by DFP in a similar manner; 1 mM-DFP had no effect, whereas 10 mM-DFP totally inhibited the formation of products Ba and Bb when analysed by Western blots following SDS/PAGE.

The poor resolution of rat factor D on gel filtration and the heterogeneity observed on native PAGE and SDS/PAGE (M_r 36000–45000) suggested that the enzyme might be a glyco-

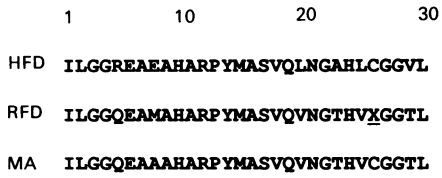

Fig. 6. *N*-Terminal sequence identity

Alignment of mouse adipsin (MA), human (HFD) and rat (RFD) factor D is shown. Residue 26 in RFD is underlined.

protein. This was confirmed by the ability of the enzyme to bind specifically to wheat-germ lectin–Sepharose 6MB. Interestingly the enzyme did not bind to Con A–Sepharose 4B under conditions where transferrin would. This latter result contrasts with those reported [6] for adipsin protein expressed in Sf9 insect cells or CHO cells, which could bind Con A–Sepharose 4B, albeit under different buffer conditions. Rat factor D bound (eluted with 100 mM-sodium phosphate buffer) to 5-hydroxytryptamine–agarose under conditions where asialofetuin did not bind (eluted in 2 mM buffer) but fetuin did bind (eluted with formate buffer), suggesting the presence of sialic acid residues [13,14].

The contribution of sialic acid to the observed heterogeneity was determined by treating the enzyme with neuraminidase (Fig. 4). It can be seen that neuraminidase treatment decreased both the glycan staining and the M_r (by approx. 2000), but not the heterogeneity. Human factor D did not change in size on similar treatment, nor did it stain for glycan.

Removal of the *N*-linked carbohydrate from rat factor D by treatment with glycopeptidase F is shown in Fig. 5. The gel stained for protein highlights the sharpening of the diffuse zone into a very tight well resolved band, concomitant with a remarkable drop in apparent M_r from 39000 to 24500. No discernible difference in M_r was observed with human factor D under identical conditions. The gel stained for glycan showed that the diffuse zone at M_r 39000 had disappeared, with only a few deglycosylated intermediates apparent. The deglycosylated species at M_r 24500, observed with protein stain, was undetectable with glycan stain, suggesting that all of the carbohydrate attached was *N*-linked and that 24500 is the M_r of the unglycosylated rat factor D polypeptide chain. (The deglycosylated intermediates were absent in incubations exposed to prolonged digestion.) A similar finding has been reported upon digestion of mouse adipsin with endoglycosidase F, the species at M_r 37000 and 44000 being converted to a single form of M_r 25500 [5].

Both native and deglycosylated rat factor D were partially sequenced and the 30 *N*-terminal residues were compared by FASTA program [15] searches of the NBRF protein sequence database. Fig. 6 shows that rat factor D has greater than 93% identity with mouse adipsin and greater than 76% identity with human factor D over the first 30 residues. No PTH derivative was identified in cycle 21 of the glycosylated rat factor D; however, aspartic acid was identified in this position in the deglycosylated enzyme. This corresponds to asparagine in the native rat factor D (which is deamidated during glycopeptidase F-mediated deglycosylation). The residue in position 26, although not identified, is probably cysteine.

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