First determination of the secondary structure of purified factor VIII light chain

Nicolas BIHOREAU,*§ Marie-Pierre FONTAINE-AUPART,† Annick LEHEGARAT,* Michel DESMADRIL‡ and Jeannine M. YON†

*T.M. Innovation (Centre National de Transfusion Sanguine Institut Mérieux), 3 Avenue des Tropiques, F-91943, Les Ulis, †Laboratoire de Photophysique Moléculaire, Université de Paris-Sud, F-91405, Orsay Cedex and ‡Laboratoire d'Enzymologie Physico-Chimique et Moléculaire, Unité de Recherche du CNRS, Associée à l'Université Paris-Sud, F-91405, Orsay Cedex, France

The first analysis of the secondary structure of human factor VIII light chain was performed by c.d. spectroscopy. The purification process described in this paper allowed us to obtain the large amounts of purified factor VIII light chains required for c.d. experiments. Since this 80 kDa protein is non-covalently associated with a heavy chain to form the active molecule, isolated factor VIII light chains were obtained after immunoadsorption and dissociation of the immobilized active complexes by EDTA. Furthermore, factor VIII light chains were discriminated from the residual active complexes and the free heavy chains by a final ion-exchange-chromatography step. This f.p.l.c. analysis showed that factor VIII light chains were less electronegative than the active complexes. The results of conformational analysis by c.d. show that the protein possesses a high degree of regular secondary structure (58 %) with approx. 22 % of α -helix and 36 % of β -strand structures. The protein was completely unfolded by 3 M-guanidine hydrochloride. The results obtained from the analysis of c.d. spectra were compared with those predicted from three different statistical methods based on amino-acid sequence. The secondary structure information obtained from these methods was in good agreement with the c.d. results. These results were comparable with the secondary structure prediction of ceruloplasmin, a protein known to show sequence identity to factor VIII.

INTRODUCTION

Factor VIII is a plasma glycoprotein whose deficiency or absence causes a bleeding disorder: haemophilia A. It acts, in its activated form, as a cofactor of factor IXa in the proteolysis of factor X to factor Xa in the presence of phospholipids and calcium (Kane & Davie, 1988). The factor VIII gene has been cloned and the sequence of the mature protein, consisting of 2332 amino acids with a molecular mass of 330 kDa, was deduced (Toole *et al.*, 1984; Gitschier *et al.*, 1984; Wood *et al.*, 1984; Vehar *et al.*, 1984). Factor VIII circulates as a series of active heterodimers consisting of an 80 kDa light chain bound through a metal ion to heavy chains of various sizes (90–210 kDa). The loss of activity is concomitant with the dissociation of the heavy-light chain complex by EDTA (Fass *et al.*, 1982; Hultin, 1985; Eaton *et al.*, 1986; Fay, 1988; Nordfang & Ezban, 1988) indicating that the association of the two chains is indispensable for functioning of factor VIII.

Until now, only the primary structure of the factor VIII light chain was known. This polypeptide chain is composed of 684 amino acids. According to Vehar *et al.* (1984), factor VIII light chain contains two domains: a unique A domain of 371 amino acids and a duplicated C domain of 153 and 160 amino acids that possess 40 % internal sequence similarity. These domains are arranged as follows: A₃-C₁-C₂ (Vehar *et al.*, 1984). Furthermore, factor VIII light chain possesses at its N-terminus the major binding site of von Willebrand factor (vWF) (Foster *et al.*, 1988; Pittman & Kaufman, 1989), another glycoprotein which stabilizes factor VIII in plasma.

Despite the great number of reports about the factor VIII light chain structure-function relationship, nothing is known about the three-dimensional structure of the protein. In this paper we

present the first structural data about the native factor VIII light chain in solution using c.d. spectroscopy. This structural analysis needed large amounts of factor VIII light chains which were obtained by an immunopurification process. Purification of factor VIII light chain for analytical studies has been previously described by Nordfang *et al.* (1987). The authors showed that purified factor VIII light chains were slightly contaminated with heavy chains and vWF. Thus, we characterized the purified factor VIII light chain and identified the contaminants by f.p.l.c.

The c.d. study was extended using empirical prediction methods. Considering the sequence similarity between factor VIII and ceruloplasmin light chains (Kane & Davie, 1986), the secondary structure of the two proteins were found to be similar.

MATERIALS AND METHODS

Assays

Factor VIII activity and von Willebrand antigen were determined as previously described (Bihoreau *et al.*, 1989).

Protein concentrations were determined by a modified Schaffner & Weismann method (1973) using serum albumin (Sigma) as the standard. Molar concentrations of factor VIII light chains were calculated using a molecular mass of 80 kDa. Specific activity was defined as the ratio of the coagulant activity to the protein concentration.

Immunopurification of factor VIII

Factor VIII was immunopurified from commercial concentrates using a monoclonal anti-(factor VIII light chain) antibody (GAG1-463-A8) as described previously (Croissant *et al.*, 1986).

The immunopurified factor VIII was subsequently charac-

Abbreviation used: vWF, von Willebrand factor.

§ To whom correspondence should be addressed.

terized by f.p.l.c. using the mono-Q column and identified by SDS/PAGE analysis.

Effect of EDTA on factor VIII

Factor VIII dissociation was performed using purified heavy-light chain complexes eluted from f.p.l.c., and incubated (v/v) at room temperature with a 20 mM-Bis-Tris/150 mM-NaCl/10 mM-CaCl₂ buffer, pH 7.2 (referred to as buffer A), containing 100 mM-EDTA. The dissociation kinetics were followed over 24 h by the measurement of coagulant activity. After total inactivation, factor VIII was re-injected on to the mono-Q column under the same conditions.

Purification of factor VIII light chain

Large amounts of factor VIII light chains were purified for c.d. analysis. Immunopurified factor VIII was used as the starting material and applied to the immunosorbent containing the anti-(light chain) monoclonal antibody. Immobilized factor VIII was mixed and stirred at room temperature for 3 h with buffer B (20 mM-imidazole, 100 mM-lysine, 150 mM-NaCl), pH 6.8, containing 100 mM-EDTA to dissociate the active complexes. Every hour, a control aliquot of the liquid phase was analysed by SDS/PAGE. The immunosorbent was then washed with buffer B containing 350 mM-CaCl₂. The factor VIII light chains were eluted using buffer B containing 50% (v/v) ethylene glycol, and adjusted to pH 7.5. Ethylene glycol removal and buffer changing were carried out by gel filtration using a Sephadex G-25 column equilibrated with buffer A. The immunopurified factor VIII light chains were subjected to f.p.l.c. and eluted from the Mono-Q anion-exchange column under the conditions described previously (Bihoreau *et al.*, 1989). For c.d. measurements, purified light chains were desalted with a 20 mM-phosphate buffer, pH 7.2 (buffer C).

C.d.

The c.d. spectra of factor VIII light chains were recorded at 22 °C with a Jobin Yvon Mark V micrograph interfaced to an Apple 2 microcomputer for data acquisition. The c.d. scale was calibrated with a solution of isoandrosterone at 1.23 mg/ml ($\Delta\epsilon = 3.31 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 304 nm). The spectra were recorded at a scanning rate of 0.1 nm/s (between 185 and 250 nm) and with a difference dichroic absorbance sensitivity (ΔA) of 2×10^{-6} .

Three scans were performed for each sample and every spectrum was corrected for the baseline contribution of the phosphate buffer. ΔA at each wavelength was averaged over the three spectra before being converted into the differential absorption coefficient $\Delta\epsilon \text{ (M}^{-1} \cdot \text{cm}^{-1})$ by the equation:

$$\Delta\epsilon = \frac{\Delta A}{l \times c \times N}$$

where l is the cell pathlength in cm and c the protein concentration in M. For factor VIII light chain the number of peptide bonds, N , was 683.

The content of regular secondary structures in the factor VIII light chain was estimated over the wavelengths 185–250 nm, according to a computation method derived from Chang *et al.* (1978) implemented on a Hewlett-Packard microcomputer. The reference data set for the analysis was based on the known structures of 16 proteins belonging to the different classes of Levitt & Chothia (1976).

The c.d. experiments performed in the far-u.v. region used protein solutions diluted in phosphate buffer. Coating of factor VIII on to glass surfaces has already been reported (Chuen-Shang *et al.*, 1989). Since such a possibility may decrease the c.d. intensity, spectra of factor VIII light chains were recorded at

different protein concentrations in quartz rectangular cells (Hellma) of various pathlengths: 2 and 10 $\mu\text{g/ml}$ with pathlengths of 10 mm, 5 and 20 $\mu\text{g/ml}$ in pathlengths of 2 mm (the absorption coefficient of factor VIII light chain at 280 nm was estimated to be $120000 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Furthermore, the c.d. spectra were recorded in coated cells using dimethyldichlorosilane solution (from BDH).

Estimation of secondary structures by prediction methods

To predict the secondary structure of the human factor VIII light chain, we used the prediction methods of Chou & Fasman (1978) [in a modified version implemented by Ralph *et al.* (1987)], of Garnier (Gibrat *et al.*, 1987), and of Garnier *et al.* (1978). All the algorithms were based on the amino-acid sequence of the protein previously reported by Vehar *et al.* (1984).

The method of Chou & Fasman was provided by BISANCE (Base Informatique de Séquences de biomolécules pour les Chercheurs Européens) (which is implemented at the CITI 2 computing centre on a DPS 8 Honeywell Bull). In the implementation of Ralph *et al.* (1987), the nucleation region is four-residues-long for α -helix and three-residues-long for β -strands. Moreover, the propagation of a helix is carried from the 'highest seed' between two terminator regions. The default parameters were used to increase the accuracy of the predictions.

The method of Garnier (Gibrat *et al.*, 1987) was used as implemented in the commercial package PC-GENE (which runs on IBM-compatible microcomputers). The method is based on information theory (Biou *et al.*, 1988). To predict the conformational state (α -helix, β -strand, or coil) of the j th residue of a sequence, the eight neighbouring residues on either side are taken into account. In the case of the human factor VIII light chain, all decision constants were set to zero; this means that the prediction is made with no *a priori* knowledge of the contents in terms of α -helix and β -structure.

The method of Garnier *et al.* is also based on information theory but uses residue-pair information instead of single-residue information. In this implementation of the method no decision constants are involved. This program is available from BISANCE.

RESULTS

Identification of factor VIII light chain

Fig. 1 represents the f.p.l.c. characterization of the immunopurified factor VIII. As previously reported (Bihoreau *et al.*, 1991), the factor VIII active complexes ranging from 210–80 to 90–80 kDa were eluted between 500 and 550 mM-NaCl (Fig. 1, peaks 2–4). F.p.l.c. analysis also shows an inactive peak eluted from the Mono-Q column at 380 mM-NaCl (Fig. 1, peak 1) clearly separated from those of the active complexes of factor VIII. Peak 1 (Fig. 1), characterized by SDS/PAGE as polypeptides of 80 kDa, corresponded to factor VIII light chain.

The effect of EDTA was studied to verify that these polypeptides of 80 kDa were generated from the active complexes upon dissociation. The different active complexes of factor VIII (Fig. 1, fractions 36–50) were mixed (v/v) with 100 mM-EDTA buffer. Fig. 2(a) shows that this treatment resulted in a decrease of the coagulant activity to 66% after 2 h. After 24 h, when only 2% of the initial activity remained, inactive factor VIII was subjected to the anion-exchange column. Fig. 2(b) represents the chromatographic pattern before and after EDTA treatment. After the action of EDTA, we observed the disappearance of peak 2 correlated with the loss of coagulant activity (Fig. 2, inset) and the generation of an inactive peak (Fig. 2b, peak 1). These inactive fractions, eluting at 380 mM-NaCl, corresponded to the

Fig. 1. F.p.l.c. and SDS/PAGE characterization of immunopurified plasma-derived factor VIII

The chromatographic pattern of factor VIII was obtained on a Mono-Q column (HR 5/5). Factor VIII was applied to the column at a flow rate of 0.5 ml/min. Following the final injection, the column was washed with 10 ml of equilibration buffer (20 mM-Bistris, 0.15 M-NaCl, 10 mM-CaCl₂, pH 7.2) and a gradient of 0.15–1 M-NaCl (—) was initiated. Fractions were collected at 1 min intervals and assayed for clotting activity (represented by the histogram). Absorbance was measured at 280 nm as a continuous tracing. The fractions obtained by f.p.l.c. separation were analysed by SDS/PAGE. Aliquots of each fraction were submitted to electrophoresis using a 6–12% (w/v) gradient gel, then the gel was silver stained. Lanes 1, 2, 3 and 4 correspond to peaks 1, 2, 3 and 4 of the f.p.l.c. pattern respectively.

factor VIII light chain of molecular mass 80 kDa, as previously identified (Fig. 1, peak 1).

Purification of factor VIII light chain for c.d. analysis

Immunopurified factor VIII (specific activity = 5000 units/mg of protein) was adsorbed on the anti-(light chain) immunosorbent. After 2.5 h, only 5% of the initial coagulant activity remained in the non-adsorbed fraction, indicating that the majority of the active factor VIII was bound to the immunosorbent. This immobilized factor VIII was dissolved with EDTA buffer for 3 h. The coagulant activity of this eluted product represented only 7% of the initial activity. The normal yield of elution, obtained without previous dissociation of factor VIII by EDTA, was 70% (Bihoreau *et al.*, 1989).

This product was then subjected to f.p.l.c. (Fig. 3). Compared with Fig. 1, we noted in Fig. 3 the decrease of peak 2 and the significant increase of peak 1. All the residual clotting activity was found in a minor peak, eluting from the Mono-Q column at 500 mM-NaCl (Fig. 3a, peak 2) and corresponding to active factor VIII complexes of molecular mass 185–80 to 115–80 kDa (Fig. 3b). We also observed that polypeptides corresponding to factor VIII heavy chains were not adsorbed on the anion-exchange column (Fig. 3b, n.a.). The major inactive peak (Fig. 3a, peak 1) was eluted at 380 mM-NaCl. Characterization of the corresponding fractions by SDS/PAGE showed that they contained polypeptides of molecular mass 80 kDa, identified previously as factor VIII light chains (Fig. 3b).

The content of von Willebrand antigen was evaluated in the starting material and in the different eluted fractions of the f.p.l.c. analysis. The ratio between vWF and factor VIII was 200 (w/w) in the commercial concentrates, while it was only 0.05 (w/w) in the fractions corresponding to factor VIII light chains eluted in peak 1 of the f.p.l.c. (Fig. 3a).

Fig. 2. Effect of EDTA on purified factor VIII

Purified factor VIII fractions (peaks 2, 3 and 4, Fig. 1), obtained after f.p.l.c. were mixed with EDTA. The kinetics of inactivation are illustrated in (a). The activity of factor VIII was measured by the 'one-stage clotting' assay at different time intervals for 24 h in the presence (■) and absence (□) of chelating agent. (b) shows f.p.l.c. characterization of inactive factor VIII after EDTA treatment. After 24 h, factor VIII with EDTA (●) or without EDTA (—) was applied to the Mono-Q column in the same conditions as mentioned in the legend of Fig. 1. The activity was measured in the different eluted fractions in the presence (■) and absence (□) of EDTA (inset).

Secondary structure analysis

C.d. analysis. The c.d. spectrum of human factor VIII light chain is represented in Fig. 4. The c.d. spectra were not dependent on protein concentration at concentrations of 2, 5, 10, 20 μ g/ml, in coated or uncoated cells, indicating that factor VIII light chains were not adsorbed on to the surface of the glass cells.

The c.d. spectrum of factor VIII light chain is characterized by a major negative band at 215 nm with a $\Delta\epsilon$ value of $-3.3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and a positive band near 195 nm with a $\Delta\epsilon$ value of $1.8 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This spectrum exhibited features expected for proteins containing a substantial amount of regular structures (Greenfield & Fasman, 1969). The following relationship was used to analyse the c.d. spectra:

$$\Delta\epsilon = f_{\alpha}(\Delta\epsilon)_{\alpha} + f_{\beta}(\Delta\epsilon)_{\beta} + f_c(\Delta\epsilon)_c$$

where f_{α} , f_{β} , f_c are the fractions of helices, β -strands and irregular structures respectively. From the spectra recorded over the wavelength range 185–250 nm, a structural content of 22% α -helix, 36% β -structures and 42% irregular structures (Table 1) was found for factor VIII light chain.

Fig. 3. F.p.l.c. (a) and SDS/PAGE (b) characterization of immunopurified factor VIII light chain

(a) The chromatographic pattern of immunopurified factor VIII light chains was obtained on a Mono-Q column (HR 5/5) using the same conditions described in Fig. 1. The activity measured in the different eluted fractions was represented by the histogram. (b) The non-adsorbed fraction (n.a.) on the Mono-Q and the peaks 1 and 2 of the f.p.l.c. analysis were characterized by electrophoresis as described in Fig. 1. The corresponding densitogram was represented with the molecular mass of each fragment in kDa.

Fig. 4. C.d. spectra of the purified factor VIII light chain

A, Spectrum of the protein in 20 mM-phosphate buffer at pH 7 and 25 °C. Protein concentration was 10 µg/ml, pathlength 10 mm. B, Spectrum of the protein in the same buffer containing 3 M-guanidine hydrochloride.

The helix content was also estimated from the $\Delta\epsilon$ value at 222 nm by the equation described by Lin *et al.* (1989). The amount of α -helix calculated for the factor VIII light chain was

Table 1. Secondary structure results obtained by c.d. and prediction methods

Secondary structure	Secondary structure present (%)			
	C.d.	Prediction method		
		Garnier	Chou & Fasman	Garnier <i>et al.</i>
α -Helix	22	29	34	30
β -Sheet	36	23	31	19
Unordered structure	42	48	35	51

22%, which is consistent with the result obtained using the curve-fitting procedure.

The purified factor VIII light chain was readily denatured by 3 M-guanidine hydrochloride. The c.d. spectrum obtained corresponds to a completely unfolded protein (Fig. 4).

Secondary structure predictions. The results of three statistical methods are presented in Table 1. Both experimental c.d. results and secondary structural predictions revealed that factor VIII light chain was highly structured with about 60% of regular structures (α -helices and β -strands).

The three methods gave approx. the same prediction for α -helix content (31%). The good agreement in the α -helix predictions contrasted with the dispersion in the predictions of the percentage of β -strand structures. The methods of Garnier (Gibrat *et al.* 1987) and Garnier *et al.* (1978) gave similar predictions for β -structure content (19 and 23% respectively). The method of Chou & Fasman (1978) predicted a significantly higher β -strand content (31%).

DISCUSSION

Until now, only the amino-acid sequence of the factor VIII light chain has been known. No information is available concerning the conformation of the protein. In this paper we describe a purification procedure used to obtain sufficient amounts of factor VIII light chain to perform the first conformational analysis of the unproteolysed 80 kDa polypeptide by c.d. spectroscopy. From knowledge of the primary structure of this protein, its secondary structure was also estimated by statistical prediction methods and compared with the c.d. results.

This structural study required a previous characterization of factor VIII light chains. We showed that factor VIII light chains of molecular mass 80 kDa can be separated from the active factor VIII complexes using a Mono-Q column. Factor VIII light chains were eluted from this anion-exchange column at 380 mM-NaCl. The active complexes of factor VIII were better retained by this column, suggesting that the global charge of the free light chain is more electropositive. This result is consistent with previous work by Eaton *et al.* (1986), who established that the isoelectric points of factor VIII light chains and of active complexes were 6.5 and 6 respectively.

The heavy and light chains of factor VIII can be dissociated by chelating the metal ion (Fass *et al.*, 1982). After EDTA treatment, we noted that the loss of coagulant activity was correlated with the disappearance of the active factor VIII complexes characterized by f.p.l.c. and the concomitant increase of the peak eluted at 380 mM-NaCl identified previously. These results demonstrate that the 80 kDa polypeptides originated from the dissociation of the active complexes of factor VIII.

The analysis of the secondary structure of factor VIII light chain by c.d. required about 20–30 µg of totally purified protein.

Fig. 5. Empirical secondary structure of factor VIII light chains

Predictions by the methods of (1) Chou & Fasman, (2) Garnier and (3) Garnier *et al.* Key to shading: ■, α -helix; ▒, β -strand; □, non-periodical structures.

The 80 kDa polypeptides obtained by immunopurification and f.p.l.c. were sufficiently concentrated to carry out c.d. measurements. Furthermore, the immunopurification method allowed us to dissociate the vWF from factor VIII light chains and the ion-exchange-chromatography process separated the 80 kDa polypeptide from the free heavy chains.

The secondary structure of factor VIII light chain was evaluated experimentally by c.d. measurements. These experiments were validated first by the reproducibility of the results at different sample dilutions and by the loss of protein folding after guanidine hydrochloride treatment. The analysis of the c.d. spectrum of the factor VIII light chain by the curve-fitting procedure was only based on the α , β and coil conformations. The accuracy of the c.d. assessment of secondary structure of an unknown protein was intrinsically unreliable, whatever the decomposition method used. Since β -structures have a much broader range of conformation than α -helices, the c.d. spectra of β -structures were expected to be more variable than those of α -helices. Moreover, the quality of the deconvolution depends on the quality of the results, below 200 nm, mainly concerning the β -structure (Johnson, 1990). The poor signal/noise ratio observed below 200 nm allowed us to obtain good accuracy for α -helix content only.

The present experimental results indicated that human factor VIII light chain contains 42% irregular structure, 36% β -strands, and 22% α -helix. Using the equation reported by Lin *et al.* (1989), we have obtained the same helical content directly from the c.d. spectrum. The secondary structure of the factor VIII light chain, which emphasizes the preponderance of β -strands and irregular structure compared with the relatively low α -helix content, is in general agreement with earlier results obtained for ceruloplasmin (Noyer & Putnam, 1981; Ortel *et al.*, 1984), an homologous factor VIII protein (Kane & Davie, 1986).

The secondary structure of factor VIII light chain was also evaluated from the primary sequence of the protein by three theoretical methods. The mean computer predictions were as follows: 55% of ordered structures with 31% for α -helix and 24% for β -structure. The comparison of these theoretical results with c.d. results indicates differences in α -helix (29%) and β -strands (33%) content. These discrepancies were consistent with the validity of the prediction methods estimated at 60% (Biou *et al.*, 1988). The same discrepancies have been observed in different proteins in work by other authors (Lin *et al.*, 1989). Furthermore Busetta & Hospital (1982) have shown that proteins with a mixed-type (α/β) structure have worse prediction levels than those with a single type of secondary structure (all α -helix or all β -structure). This observation applies to factor VIII light chain.

Nevertheless, theoretical results gave the same percentage of ordered structures (55%) as experimental results.

In Fig. 5, we noted that a mixed-type (α/β) distribution was present along the three-domain (A_3 , C_1 , C_2) factor VIII light chain. Furthermore, we have determined the α/β ratio which was approx. the same for the homologous domains C_1 and C_2 (respectively 0.5 and 0.8) and 2.2 for the A_3 domain. The preponderance of the β -structure in the C domains and of the α -helix in the A domain could be a secondary structural characteristic of the factor VIII light chain.

Fig. 5 also showed that the three different methods predicted only the presence of α -helix or irregular structures on the N-terminal region of the factor VIII light chain (39 amino acids). This N-terminal region which contains the vWF-binding site should be exposed to the exterior of the protein. So, the binding *in vivo* of vWF, to stabilize factor VIII, could prevent the denaturation of the unstable N-terminal α -helix of the light chain. Further studies are necessary to confirm this hypothesis.

We thank Mrs. M. Pellán for typing the manuscript.

REFERENCES

- Bihoreau, N., Sauger, A., Van de Pol, H. & Yon, J. (1989) *Eur. J. Biochem.* **185**, 111–118
- Bihoreau, N., Paolantonacci, P., Bardelle, C., Fontaine-Aupart, M. P., Krishnan, S., Yon, J. & Romet-Lemonne, J. L. (1991) *Biochem. J.* **277**, 23–31
- Biou, V., Gibrat, J. F., Levin, J. M., Robson, B. & Garnier, J. (1988) *Protein Eng.* **2**, 185–191
- Busetta, B. & Hospital, M. (1982) *Biochim. Biophys. Acta* **701**, 11–18
- Chang, C. T., Wu, C. S. C. & Yang, J. T. (1978) *Anal. Biochem.* **91**, 13–31
- Chou, P. Y. & Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251–276
- Chuen-Shang, C., Wu, G. & Che, C. (1989) *Anal. Biochem.* **177**, 178–182
- Croissant, M. P., Van de Pol, H., Lee, H. H. & Allain, J. P. (1986) *Thromb. Haemostasis* **56**, 271–276
- Eaton, D., Rodriguez, H. & Vehar, G. A. (1986) *Biochemistry* **25**, 505–512
- Fass, D. N., Knutson, G. J. & Katzmman, J. A. (1982) *Blood* **59**, 594–600
- Fay, P. J. (1988) *Arch. Biochim. Biophys.* **262**, 525–531
- Foster, P. A., Fulcher, C. A., Houghten, R. A. & Zimmerman, T. S. (1988) *J. Biol. Chem.* **263**, 5230–5234
- Garnier, J., Osguthorpe, T. J. J. & Robson, B. (1978) *J. Mol. Biol.* **120**, 97–120
- Gibrat, J. F., Garnier, J. & Robson, B. (1987) *J. Mol. Biol.* **198**, 425–443
- Gitschier, J., Wood, W. I., Goralka, J. M., Wien, K. L., Chen, E. Y., Eaton, D. H., Vehar, G. A., Capon, D. J. & Lawn, R. M. (1984) *Nature (London)* **312**, 326–330
- Greenfield, N. & Fasman, G. D. (1969) *Biochemistry* **8**, 4108–4116
- Hultin, M. B. (1985) *Blood* **66**, 53–58
- Johnson, W. C. (1990) *Proteins* **7**, 205–214
- Kane, W. H. & Davie, E. W. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6800–6804
- Kane, W. H. & Davie, E. W. (1988) *Blood* **71**, 539–555
- Levitt, M. & Chothia, C. (1976) *Nature (London)* **261**, 552–558
- Lin, T. H., Quinn, T. P., Grandgenett, D. S. & Walsh, M. (1989) *Proteins* **5**, 156–165
- Nordfang, O. & Ezban, M. (1988) *J. Biol. Chem.* **263**, 1115–1118
- Nordfang, O., Ezban, M. & Hansen, J. J. (1987) *Thromb. Haemostasis* **58**, 1043–1048
- Noyer, M. S. & Putnam, F. (1981) *Biochemistry* **20**, 3536–3542
- Ortel, T., Takahashi, N. S. & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4761–4765
- Pittman, D. D. & Kaufman, R. J. (1989) *Thromb. Haemostasis* **61**, 161–165
- Ralph, W. W., Webster, T. & Smith, T. F. (1987) *Comput. Appl. Biosci.* **3**, 211–216
- Schaffner, W. & Weissmann, C. (1973) *Anal. Biochem.* **56**, 502–514
- Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Beucker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N. & Hewick, R. M. (1984) *Nature (London)* **312**, 343–347

Vehar, G. A., Keyt, B., Eaton, D. L., Rodriguez, H. R., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M. & Capon, D. J. (1984) *Nature* (London) **312**, 337–342

Wood, W. I., Capon, D. J., Simonsen, C. C., Eaton, D. L., Gitschier, J., Keyt, B., Seeburg, P. H., Smith, D. H., Hellingshead, P., Wien, K. L., Delwart, E., Tuddenham, E. G. D., Vehar, G. A. & Lawn, R. M. (1984) *Nature* (London) **312**, 330–337

Received 30 December 1991/14 May 1992; accepted 29 May 1992