Characterization of the structure and conformation of platelet-derived growth factor-BB (PDGF-BB) and proteinase-resistant mutants of PDGF-BB expressed in *Saccharomyces cerevisiae*

Stewart CRAIG,* John M. CLEMENTS,* Anne L. COOK,* David T. F. DRYDEN,† Dawn R. GREEN,* Karel HEREMANS,‡ Patricia M. KIRWIN,* Michael J. PRICE* and Anthony FALLON*

*British Bio-technology Ltd., Watlington Road, Cowley, Oxford OX4 5LY, U.K., †Department of Biochemistry and Genetics, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K., and ‡Laboratory of Chemical and Biological Dynamics, Department of Chemistry, Katholieke Universiteit Leuven, B-3001 Leuven, Belgium

A detailed biophysical study of the secondary and tertiary structures of recombinant platelet-derived growth factor (PDGF)-BB produced in yeast has been carried out. The secondary structure of the molecule is composed of 54 % β -sheet with less than 5 % ordered helix. The single tryptophan residue has been shown to be solvent-accessible; however, the ability of the side chain to rotate is severely restricted. The fluorescence emission is quenched at pH 7.0 and in the presence of high salt, but dequenched by titration to lower pH with a pK of 5.8. Two proteinase-resistant mutants of PDGF ([Ser²⁸]- and [Pro³²]-PDGF-BB) have also been characterized and shown to have secondary and tertiary structures indistinguishable from wild-type PDGF-BB. These are, therefore, suitable stable background molecules in which to carry out structure–activity–relationship studies on PDGF-BB.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a potent mitogen that has been implicated in wound repair and disease states such as inflammation and atherosclerosis (Ross *et al.*, 1986). Recombinant PDGF-BB, PDGF-AA and PDGF-AB have previously been prepared from a fusion protein expressed in *Escherichia coli* (Hoppe *et al.*, 1989, 1990). The expressed protein formed inclusion bodies, which were solubilized, and the protein was subsequently purified and allowed to refold/associate to active dimers. However, of the total expressed protein, only a very low percentage could be isolated using this system. Analysis of the recombinant dimers and authentic protein using c.d. suggested a low content of α -helix (< 10 %) and a high proportion of random coil (Vogel & Hoppe, 1989; Hoppe *et al.*, 1990). Despite the high level of unordered structure, the PDGF-BB molecule was shown to be rigid, with little conformational flexibility (Vogel & Hoppe, 1989).

A method of expressing and purifying recombinant PDGF-BB homodimer in yeast is described in the preceding paper (Cook *et al.*, 1991). Production of PDGF-BB in yeast is desirable because of the ease with which material can be purified and assayed for biological activity. However, expression levels of the mitogen are low, and a high proportion of the protein is cleaved internally after Arg-32 (Cook *et al.*, 1991). Site-directed mutagenesis has been used to produce two proteinase-resistant forms of the molecule ([Ser²⁸]- and [Pro³²]-PDGF-BB). Both mutants have the advantage of increased biological activities (attributable to the reduced cleavage of the molecule) (Cook *et al.*, 1991). It would be advantageous, therefore, to use these mutants as the background PDGF molecule in any mutagenesis study examining PDGF structure–activity relationships (SARs). However, it is first necessary to confirm that the engineered protein is correctly folded.

In the present study the secondary structure and tertiary conformation of recombinant PDGF-BB and proteinase-resistant

mutants, secreted from yeast, have been examined by using c.d. Raman spectroscopy, time-resolved fluorescence spectroscopy and steady-state fluorescence spectroscopy. Comparison of the conformational properties of PDGF-BB with those of the proteinase-resistant mutants verifies that these substituents have not affected the structure or folding of the molecule. Analysis of the far-u.v. c.d. spectra and the resonance Raman spectra has enabled a detailed determination of secondary-structure content.

MATERIALS AND METHODS

All chemicals were AnalaR grade (BDH) unless otherwise stated. KI (AristaR) and Mes were from Sigma; Ultrapure acrylamide and Ultrapure guanidinium chloride (GdmCl) were from BRL. Protein samples were dissolved in 10 mM-acetic acid, pH 3.2 (BDH h.p.l.c. grade), or 10 mM-Mes/50 mM-NaCl, pH 6.2, made from h.p.l.c.-grade water (BDH). Samples for spectroscopy were filtered with a 0.22 μ m-pore-size Millex-GV filter unit (Millipore) before use.

Purification of recombinant wild-type and recombinant mutant PDGF

Wild-type and mutant PDGF-BB proteins were expressed in yeast and purified using the methods described in the preceding paper (Cook *et al.*, 1991). Sample purity was determined by SDS/PAGE analyses (Laemmli, 1970) and automated N-terminal Edman degradation using an Applied Biosystems 471A sequencer.

U.v.-absorbance measurements

All absorbance measurements were carried out using either a Cary-210 (Varian) or a PU-8740 (Pye-Unicam) spectrophotometer. The concentration of wild-type and proteinase-resistant proteins were determined using a calculated value for $A_{280, 1\text{ cm}}^{0.1\%}$ of 0.46.

Abbreviations used: DTT, dithiothreitol; GdmCl, guanidinium chloride; PDGF, platelet-derived growth factor; [Ser²⁸]PDGF-BB, platelet-derived growth factor-BB with Arg-28 $\rightarrow$ Ser substitution; [Pro³²]PDGF-BB, platelet-derived growth factor-BB with Arg-32 $\rightarrow$ Pro substitution.

C.d. spectra

C.d. spectra were measured by using a Jobin-Yvon Dichrograph IV constantly flushed with oxygen-free nitrogen. Spectra shown are averages of 4–16 scans with an average of an equal number of baseline scans subtracted. Samples in the near- and far-u.v. wavelength range were scanned at 5–10 nm/min with a 2 s time constant. Data collection and manipulations were carried out using a BBC+ microcomputer linked to the Dichrograph. The mean-residue M_r of PDGF-BB was calculated to be 111.8 using an M_r for the monomeric subunit of 12400. All c.d. data are expressed as molar ellipticities ($[\theta]$).

Fluorescence lifetime measurements

Fluorescence lifetimes and anisotropy were measured using an Edinburgh Instruments 199T lifetime fluorimeter. The excitation wavelength was set at 295 nm or 280 nm with a 20 nm vertically polarized bandwidth. Fluorescence emission was measured between 335 and 395 nm with a UG11 band-pass filter and WG355 cut-off filter. Polarization was controlled via a toggler, and the lamp pulse was measured with a Ludox scattering solution at 350 nm.

Steady-state fluorescence measurements

Steady-state fluorescence spectra and anisotropy measurements were made using a Perkin-Elmer LS-50 fluorimeter that was thermostatically controlled at $20 \pm 0.1^\circ\text{C}$ and fitted with polarization accessories.

Additions of acrylamide and KI quencher were made directly from concentrated stock solutions and mixed using the magnetic stirrer built into the turret. Sodium thiosulphate was added to KI solutions to prevent I_2 formation. Intensity measurements were adjusted for volume changes resulting from addition of quencher. Further corrections to emission intensities for the absorbance of acrylamide were made as described by Lakowicz (1983):

$$I_{\text{corr.}} = I_{\text{obs.}} \times \text{antilog}[(A_{\text{ex.}} + A_{\text{em.}})/2]$$

where $I_{\text{corr.}}$ and $I_{\text{obs.}}$ are the corrected and observed intensity at 347 nm, and $A_{\text{ex.}}$ and $A_{\text{em.}}$ are the absorbances of the solution at the excitation and emission wavelengths respectively.

Raman spectroscopy

Raman spectra were recorded with a Spex 1403 Raman spectrometer equipped with a cooled RCA C31034 photomultiplier. Excitation was provided by an Argon ion laser (Spectra Physics). The 488 nm line was used with a power of 100 mV on the sample. Spectra of freeze-dried samples were taken. A back-scattering geometry was used. An average of at least ten scans was collected for each spectrum. Data points were collected on a personal computer every 1 cm^{-1} with a spectral band-pass of 6 cm^{-1} .

Water-band and aromatic-side-chain vibrations were subtracted to minimize background contributions to the amide-I intensity distribution. A singular value analysis for solving least-squares problems was used for the data (Williams, 1983, 1986). In all, 15 data points between 1630 and 1700 cm^{-1} were used to represent the measured spectrum. These points are then fitted by a superimposition of spectra for 15 proteins of known three-dimensional structure. For the reference proteins, the percentage of secondary-structure classes was assigned from X-ray data as described by Levitt & Greer (1977). Reference data for proteins 1–10, 12 and 15–18 in Table 2 of Williams (1983) were taken from Table 5 of Williams (1983). The fit determines the weight of the individual reference proteins in the measured spectrum. By summing the fractions of secondary structure in the known proteins the structure of the protein under study can be estimated.

RESULTS AND DISCUSSION

Far-u.v. c.d.

The far-u.v. c.d. spectra of PDGF-BB in its native, unfolded and reduced forms are shown in Fig. 1. The spectra have a minimum centred around 201 nm and a pronounced shoulder at 218 nm. The shape of the spectra is similar to that published previously, but the intensity is lower (Vogel & Hoppe, 1989; Hoppe *et al.*, 1990). The spectrum suggests a high content of irregular structure, with little evidence of helical motif. Comparison of native PDGF-BB with the unfolded molecule shows an increase in the content of unordered structure on unfolding/reducing the protein. Fig. 2 shows the far-u.v. spectra of the proteinase-resistant molecules [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB, which are of similar shape to, but of higher intensity than, wild-type PDGF-BB. The [Ser²⁸]PDGF-BB molecule displays the most intense minimum of the two mutants.

PDGF-BB and [Ser²⁸]PDGF-BB display identical spectra when measured in 10 mM-acetic acid, pH 3.2, or 10 mM-Mes/50 mM-NaCl, pH 6.2 (results not shown). No pH-induced secondary-structure transitions are observed for the protein in this range, and hence its helical content is not a function of pH.

Raman spectroscopy

In order to confirm the secondary-structure content calculated from c.d., the samples were also examined by using Raman spectroscopy (as described in the Materials and methods section). The method used distinguishes between β -sheet, ordered and disordered helix, in addition to β -turn and undefined structure classes. The results of the Raman analysis are shown in Table 1 and are compared with the estimates of secondary structure

Fig. 1. Far-u.v. c.d. spectra of native (—) PDGF-BB and unfolded/reduced (dots) PDGF-BB

Spectra are an average of eight scans with the baseline subtracted. The protein concentration was 0.676 mg/ml and 0.181 mg/ml in 10 mM-acetic acid, pH 3.2, and 7.8 M-GdmCl/10 mM-DTT, pH 8.8, respectively. Spectra were recorded at 20°C with a 0.01 cm -path-length cell and 2 nm bandwidth.

Fig. 2. Far-u.v. c.d. spectra of wild-type (dots), [Ser²⁸]- and [Pro³²]-PDGF-BB

Spectra are an average of eight scans with the baseline subtracted. Protein concentration for wild-type, [Ser²⁸]- and [Pro³²]-PDGF-BB was 0.676, 0.876 and 0.248 mg/ml in 10 mM-acetic acid, pH 3.2, respectively. Spectra were recorded at 20 °C with a 0.01 cm-path-length cell and 2 nm bandwidth.

obtained from CONTIN analysis (Provencher & Gloeckner, 1981; Provencher, 1982) of the far-u.v. c.d. spectra. The disordered helix class is a combination of non-hydrogen-bonded helical residues, 3_{10} and α_{II} residues (Williams, 1986). The CONTIN analysis recognizes only helix and sheet, but does not distinguish β -turns or other structural classes. These are therefore referred to as 'undefined'.

The Raman data correlate very closely with the secondary-structure estimates obtained from the far-u.v. c.d. spectra (Table 1). The antiparallel β -sheet content (51 %) is higher than that previously reported, but is consistent with the rigidity observed by the same authors (Vogel & Hoppe, 1989). The helical content is calculated by both methods to be 4–5 % (plus some disordered helical residues). In a continuous sequence this would correspond

to four to six residues which, including 'disordered' end residues, would suggest a total helical length of, perhaps, eight residues.

Secondary-structure predictions have previously been reported for PDGF-BB along with a prototype model for the protein structure (Vogel & Hoppe, 1989). Predictions using the Genetics Computer Group (University of Wisconsin) and (D. Brown, personal communication) the Leeds Suite (University of Leeds) software packages estimate the structural content to be around 30 % sheet and 45 % helix. The content of α -helix has obviously been over-estimated by the predictive methods used.

In the prototype model of PDGF structure (Vogel & Hoppe, 1989), it is proposed that the only helical motif lies within the sequence 91–104. The results from this study indicate that the highest prediction is for a helix to form between residues 88 and 107. Within that predicted helical region, an apolar heptad (i/i + 3) periodicity (Parry, 1982; Cohen & Parry, 1986) involving Thr-88, Leu-91, Leu-95, Lys-98 (permitted owing to the long carbon chain), Val-102 and Ala-105 is evident. The presence of this apolar repeat is usually (> 80 % cases) indicative of interactions between helices with the interface of interaction along the apolar stripe. If the helical motif were formed by this sequence region, then any interaction between helices could only occur by intermolecular association. It is possible, therefore, that this region of the molecule forms part of a structured interface in the PDGF-BB dimer. Further support for this theory is provided by the fact that deletion of Cys-99 (Vogel & Hoppe, 1989) or mutation of Lys-98 to Leu (Murray & Kelly, 1988) appears to prevent dimer formation. However, the measured helical content is too low to form this highly predicted helical region. An eight-residue helical segment would be too small to form the entire dimer interface, but could form three apolar contacts of a heptad repeat and provide some stability to the interaction. Confirmation of this theory must await a detailed analysis of three-dimensional structure.

As detailed above, the proteinase-resistant [Ser²⁸]- and [Pro³²]-PDGF-BB molecules display more intense c.d. spectra than do the wild-type (Figs. 1 and 2). The analysis of the [Pro³²]-PDGF-BB far-u.v. c.d. spectrum does not recognize any helix; however, the Raman data do identify this motif. The high correlation between the Raman analysis data for wild-type, [Ser²⁸]- and [Pro³²]-PDGF-BB plus the agreement with the c.d. data for wild-type and [Ser²⁸]- molecules suggests that CONTIN has failed to estimate accurately the small percentage of helix present in [Pro³²]-PDGF-BB. Analysis of the c.d. and Raman spectra (Table 1) for the proteins therefore confirms that they have the same secondary-structure content as the wild-type molecule. Recombinant PDGF-B exhibits a complex glycosylation profile (Settineri *et al.*, 1990). Examination of the published data suggests

Table 1. Comparison of secondary-structure estimates for PDGF obtained by c.d. and Raman spectroscopy

The far-u.v. c.d. was measured by using a 0.1 mm path length and a 2 nm bandwidth at 20 °C. The Raman data were measured and analysed as described in the Materials and methods section. The S.E.M. for all the data is 1–3 %. Abbreviation: n.a., not applicable.

Structure	PDGF ... Method ...	Percentage of molecule in that structure					
		PDGF-BB		[Ser ²⁸]PDGF		[Pro ³²]PDGF	
		Raman	C.d.	Raman	C.d.	Raman	C.d.
Ordered helix		4.1	4.0	5.3	4.0	5.1	0
Disordered helix		7.2	n.a.	7.7	n.a.	7.8	n.a.
β -Sheet		56.4	51.0	54.9	49.0	54.6	54.0
β -Turn		20.4	n.a.	20.2	n.a.	20.3	n.a.
Undefined		11.5	45.0	11.5	47.0	11.6	46.0

that substitutions at positions 28 and 32 would not affect the extent of glycosylation and potentially, therefore, modify the spectral properties of the proteins. The observed variation in far-u.v. c.d. spectra may result from the degree of internal proteolytic cleavage reported for the wild-type molecule (Cook *et al.*, 1991). Despite the spectral intensity difference, the mutations Arg-28 → Ser and Arg-32 → Pro, which prevent internal proteolytic cleavage, do not appear to perturb the folding of secondary structure in PDGF-BB.

Near-u.v. c.d.

In the 250–320 nm c.d. range, tertiary-structure data can be obtained from the environment of aromatic amino acids. This technique provides a useful index for the correct association of secondary-structure units into the native conformation (see, e.g., Craig *et al.*, 1985). PDGF-BB contains no tyrosine residues, a single tryptophan residue and three phenylalanine residues. The near-u.v. c.d. spectra of wild-type, [Ser²⁸]- and [Pro³²]-PDGF-BB (not shown) are of low intensity (Fig. 3). Some phenylalanine fine structure is evident, but no strong aromatic bands arising from Trp-40 are present. The higher-wavelength band may be due to disulphide-bond contribution to the c.d. spectrum. Given that no other contributions from PDGF-BB are likely to cancel out the longer-wavelength ¹L_p bands (Strickland, 1974), tryptophan 40 is not, therefore, in an asymmetric environment.

Steady-state fluorescence emission and anisotropy

In the absence of a suitable aromatic c.d. probe of tertiary structure, the fluorescence emission spectra of wild-type, [Ser²⁸]- and [Pro³²]-PDGF-BB have been investigated as an alternative

Fig. 3. Near-u.v. c.d. spectra of PDGF-BB (—) and [Ser²⁸]PDGF-BB (dots).

Spectra are an average of four scans with the baseline subtracted. Protein concentrations for wild-type and [Ser²⁸]PDGF-BB were between 0.15 and 0.9 mg/ml in 10 mM-acetic acid, pH 3.2. Spectra were recorded at 20 °C with a 1 cm path length and a 1 nm bandwidth.

Fig. 4. Steady-state fluorescence emission spectra of wild-type [Ser²⁸]- and [Pro³²]-PDGF-BB.

Spectra are displayed with baselines subtracted; values were calculated as relative fluorescence. The protein concentration was 0.154–0.165 mg/ml in 10 mM-acetic acid, pH 3.2. The sample at 20 °C was excited at 280 nm with a 2.5 nm bandwidth and 1 cm path length, and the intensity of emission was measured at 2.5 nm bandwidth.

conformational probe (Fig. 4). All three proteins have the same emission intensity and maximum at 347 nm, which is characteristic of a tryptophan residue in a polar environment. Comparison with *N*-acetyltryptophan amide under the same conditions and concentration indicates that the fluorescence emission is quenched by 50%. Chemical quenching of fluorescence has been examined using acrylamide and KI. Analysis of the data using modified Stern–Volmer plots (Lehrer, 1971) yields (from the y -intercept) fractional accessibilities of Trp-40 to both I[−] and acrylamide of 1.0, indicating a surface exposed location (not shown).

Anisotropy measurements of proteins provide dynamic information on fluorescent chromophores from the rate of rotation of the chromophore relative to the whole molecule rotating in solution. A surface-exposed tryptophan residue would be expected to display rapid motion compared with the tumbling of the whole molecule in solution. Steady-state anisotropy measurements (Table 2) demonstrate identical environments for Trp-40 in wild-type, [Ser²⁸]- and [Pro³²]-PDGF-BB. However, the rotation of this aromatic residue appears to be relatively slow compared with the freely moving *N*-acetyltryptophan amide. These combined results demonstrate that the single tryptophan residue in PDGF-BB is in a surface-exposed position, but that,

Table 2. Steady-state fluorescence-anisotropy data for PDGF

Anisotropy was measured at 347 nm (5 nm slit) with sample excitation at 280 nm (5 nm slit) using a 1 cm path length at 20 °C. Samples were dissolved in 10 mM-acetic acid, pH 3.2, except where described in the text.

Sample	Anisotropy
<i>N</i> -Acetyltryptophan amide	0.005 ± 0.002
PDGF-BB	
Folded	0.084 ± 0.001
Unfolded (U ₁)	0.064 ± 0.007
Unfolded/reduced (U ₂)	0.029 ± 0.007
[Ser ²⁸]PDGF-BB	0.086 ± 0.002
[Pro ³²]PDGF-BB	0.085 ± 0.002
[Ser ²⁸]PDGF-BB (2.4 M-NaCl)	0.114 ± 0.006

Fig. 5. Steady-state fluorescence emission spectra of PDGF-BB at different stages of denaturation and reduction

Spectra are displayed with baselines subtracted; values were calculated as relative fluorescence. Samples at 20 °C were excited at 280 nm with a 2.5 nm bandwidth and 1 cm path length, and the intensity of emission was measured at 2.5 nm bandwidth. Sample concentration was 0.165–0.181 mg/ml under the following conditions: N, native PDGF-BB in 10 mM-acetic acid, pH 3.2; U_1 , PDGF-BB in 7.8 M-GdmCl/10 mM-DTT/10 mM-acetic acid, pH 3.2; U_x , PDGF-BB in 7.8 M-GdmCl/10 mM-DTT/10 mM-acetic acid, pH adjusted to 9.2, reduction time 10 min; U_y , PDGF-BB in 7.8 M-GdmCl/10 mM-DTT/10 mM-acetic acid, pH 9.2, reduction time 90 min; U_z , PDGF-BB in 7.8 M-GdmCl/10 mM-DTT/10 mM-acetic acid, pH 9.2, reduction time 180 min. All reductions were carried out at 20 °C.

since the rotation of the Trp-40 side chain is quite slow, steric hindrance is obviously present.

Exposure of the protein to 7.5 M-GdmCl/10 mM-DTT, pH 3.2, results in the disruption of non-covalent interactions in the protein domain (designated 'state U_1 '). The low pH will not, however, lead to efficient disulphide-bond reduction. Under these conditions, wild-type (and [Ser²⁸]PDGF-BB) show a dequenching of Trp-40 fluorescence and an emission maximum wavelength red shift from 347 nm to 351 nm (Fig. 5). On increasing the solution pH of U_1 to 9.2 (to enable disulphide-bond reduction), a rapid (1–10 min) decrease in emission intensity is observed with a further red shift of intensity maximum to 356 nm (state U_x). The intensity subsequently decreases more slowly over a further 1.5–2 h (U_y , U_z). Far-u.v. c.d. studies under these conditions (S. Craig, unpublished work) demonstrate a loss of secondary structure in U_1 with no further changes being observed on increasing the pH to state U_z .

These results suggest that two different phenomena are being observed:

- Movement of a quenching group relative to Trp-40 when the non-covalent interactions are broken (in U_1).
- Complete unfolding of the molecule when the disulphide bonds are broken (state U_z).

This situation is similar to that observed for interleukin-1 β (Craig *et al.*, 1987), in which exposure to denaturant caused a charged group to move away immediately (< 1 s) from the tryptophan, resulting in dequenching of emission. This was followed by a first-order decrease in fluorescence intensity as the protein unfolds (rate of unfolding > 10 min) with a red shift in emission maximum.

Steady-state-anisotropy measurements (Table 2) confirm that, in 7.5 M-GdmCl/10 mM-DTT/10 mM-acetic acid, pH 3.2, Trp-40 rotates slightly faster than in the native state. On increasing the pH with resulting disulphide-bond reduction, Trp-40 becomes much more mobile. The 'tight' disulphide-bonded central region of PDGF probably maintains a chain restriction around Trp-40

even in high denaturant which is only relaxed on reduction when this residue is exposed in the fully unfolded state of the protein.

Fluorescence-lifetime measurements

The single tryptophan residue in PDGF-BB (measured at pH 3.2) exhibits the complex fluorescence-lifetime decay curve normally associated with this type of aromatic group. A three-component fit was found to be the best solution to the decay data. The three different fluorescence lifetimes (decay rate constants) and amplitudes measured (Table 3) show only very minor changes on pH titration (S. Craig, unpublished work).

Both wild-type and [Ser²⁸]PDGF-BB exhibit identical increases in the degree of quenching of steady-state fluorescence emission on elevating the pH of the solution (Fig. 6). The decrease in emission intensity occurs in a transition with pK_a 5.8, and only a small decrease in quenching is observed over the pH 8–9 range. No changes in the wavelength of maximum emission (λ_{max}) occur, and it is suggested that the increased quenching occurs as a result of changes in the spatial location of a neighbouring side chain that is determined by a group (possibly either a carboxy or His-94) with pK_a 5.8. The absence of change in the fluorescence

Table 3. Fluorescence-lifetime analysis of PDGF-BB

Protein was dissolved at 0.26 mg/ml in 10 mM-acetic acid, pH 3.2. Lifetime measurements were carried out as described in the Materials and methods section, with excitation at 280 nm using a 1 cm path length at 20 °C.

Component	Rate constant (ns ⁻¹)	Amplitude (%)
t_1	1.125 ± 0.043	39.0
t_2	2.649 ± 0.081	1.0
t_3	5.301 ± 0.103	10.0

Fig. 6. Effect of pH on the steady-state fluorescence-emission intensity of PDGF-BB

Protein initially at 0.05 mg/ml in 10 mM-acetic acid, pH 3.2, was titrated with small amounts of HCl and NaOH at 20 °C. The protein was excited at 280 nm with a 5 nm bandwidth, and the intensity of emission at 347 nm was measured with a 5 nm bandwidth and a 1 cm-path-length cell. Superimposed on the data is the theoretical transition curve (pK_a = 5.8) calculated from the titration of [Ser²⁸]PDGF-BB fluorescence.

Fig. 7. Effect of NaCl on the fluorescence emission spectrum of [Ser²⁸]PDGF-BB

Spectra have baselines subtracted; values were calculated as relative fluorescence. The protein concentration was 0.0497 and 0.0551 mg/ml in the presence and absence of 2.4 M-NaCl in 10 mM-acetic acid, pH 3.2. The sample at 20 °C was excited at 280 nm with a 2.5 nm bandwidth and 1 cm path length and the intensity of emission was measured at 2.5 nm bandwidth.

lifetime parameters in this pH range implies a static quenching mechanism.

This conclusion is reinforced by the effects observed on exposing [Ser²⁸]PDGF-BB to high-salt conditions (Fig. 7), whereupon Trp-40 appears to be buried back into the protein matrix. The blue shift in emission maximum, increased quenching and increased rotational restriction (Table 2) all indicate shielding of the ring from solvent. This shielding effect could occur as a result of the rearrangement of side chains in response to the change in solvation at the surface.

Complex effects in the environment of Trp-40 are therefore occurring as a result of titration of different groups in the molecule. Anisotropy-lifetime-decay measurements at pH 3.2, 8.3 and 9.7 all give rate constants of > 6–12 ns. This decay constant indicates that Trp-40 is rotating very slowly, confirming the steady-state-anisotropy data. The slow rotation of Trp-40 is not affected by titration of the quenching group.

Conclusions

The secondary-structure content of recombinant PDGF-BB has now been determined accurately by two independent techniques. Of the total structure, 54% has been shown to be anti-parallel β -sheet, with only 5% ordered α -helical structure. These results are in marked contrast with the predicted values of around 30% sheet and 45% helix. The level of β -sheet determined by these methods is also higher than that previously reported (Vogel & Hoppe, 1989) for authentic- and *E. coli*-derived recombinant PDGF-BB, estimated from single-wavelength analysis of c.d. data.

PDGF is known to be a rigid molecule (Vogel & Hoppe, 1989). We have now demonstrated that a surface-exposed residue in the structural core region is severely restricted in its movement. Complex ionic interactions affect the environment of this tryptophan residue without disturbing the rotational inhibition. The steric hindrance to ring motion probably resides in a local chain conformation caused by a disulphide pairing. It seems likely that the very stable structural core of the protein arises from tightly folded anti-parallel β -sheets locked with disulphide bonds.

The [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB mutants (in which the B-chain residues are replaced by those in PDGF-AA) have the same secondary structure and tertiary conformation as wild-

type PDGF-BB. These proteinase-resistant proteins can therefore replace wild-type PDGF-BB as a model for structure-activity-relationship studies involving site-directed mutagenesis. This structural characterization also serves as a baseline for comparison of mutants which exhibit modified biological profiles.

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