enzyme: "lengthy and/or harsh" purification procedures result in the purification of green or yellow enzymes that are partially or completely devoid of the second chromophore but also have a substantial fraction of the flavin in fully oxidized form (Jorns et al., 1987). The photochemical method for obtaining SC†-enzyme is fully reproducible and yields an enzyme with essentially intact flavin chromophore. The availability of such an enzyme should help us discern in more detail the individual roles of the flavin and the second chromophores in the photosensitization reaction mediated by photolyase.

Registry No. FADH, 1910-41-4; DTT, 3483-12-3; NADH, 58-68-4; DNA photolyase, 37290-70-3; thymine dimer, 28806-14-6; cysteine, 52-90-4; 2-mercaptoethanol, 60-24-2; L-tryptophan, 73-22-3; L-histidine, 71-00-1; glycine, 56-40-6; L-methionine, 63-68-3; glycerol, 56-81-5; glycyl-L-tyrosine, 658-79-7.

References

Bensasson, R. V., & Land, E. J. (1971) *Trans. Faraday Soc. 67*, 1904.

Bensasson, R. V., Goldschmidt, C. R., Land, E. J., & Truscott, T. G. (1978) *Photochem. Photobiol. 28*, 277–281.

Ghisla, S., Massey, V., Lhoste, J. M., & Mayhew, S. G. (1974) *Biochemistry 13*, 589–597.

Heelis, P. F., & Sancar, A. (1986) *Biochemistry 25*, 8163–8166.

Jorns, M. S., Sancar, G. B., & Sancar, A. (1984) *Biochemistry 23*, 2673–2679.

Jorns, M. S., Sancar, G. B., & Sancar, A. (1985) *Biochemistry 24*, 1856–1861.

Jorns, M. S., Baldwin, E. T., Sancar, G. B., & Sancar, A. (1987) *J. Biol. Chem. 262*, 486–491.

Massey, V., & Palmer, G. (1966) *Biochemistry 5*, 3181–3189.

Medinger, T., & Wilkinson, F. (1965) *Trans. Faraday Soc. 61*, 3181–3189.

Sancar, A., & Sancar, G. B. (1984) *J. Mol. Biol. 172*, 223–227.

Sancar, A., Smith, F. W., & Sancar, A. (1984) *J. Biol. Chem. 259*, 6028–6032.

Sancar, G. B., Smith, F. W., Reid, R., Payne, G., Levy, M., & Sancer, A. (1987a) *J. Biol. Chem. 262*, 478–485.

Sancar, G. B., Jorns, M. S., Payne, G., Fluke, D. J., Rupert, C. S., & Sancar, A. (1987b) *J. Biol. Chem. 262*, 492–498.

Cleavage of Fibrin-Derived D-Dimer into Monomers by Endopeptidase from Puff Adder Venom (*Bitis arietans*) Acting at Cross-Linked Sites of the γ-Chain. Sequence of Carboxy-Terminal Cyanogen Bromide γ-Chain Fragments†

Langley Purves,*,‡ Maud Purves,‡ and Wolf Brandt§

Departments of Chemical Pathology and Biochemistry, University of Cape Town, Cape Town, Republic of South Africa

Received November 19, 1986; Revised Manuscript Received February 24, 1987

ABSTRACT: Puff adder venom contains a protease capable of cleaving the γ-chain of cross-linked D-dimer, derived from the plasmin digestion of fibrin, into apparently symmetrical monomers. The cross-linked γ-chains are separated in the process without apparent loss of mass and without loss of the substituent at the glutamine cross-link site, if fluorescent D-dimer (the lysine analogue dansylcadaverine used as substituent) is used as substrate [Purves, L. R., Purves, M., Lindsey, G. G., & Linton, N. J. (1986) *S. Afr. J. Sci. 82*, 30]. The γ-chain from puff adder venom digested D-monomer was isolated and cleaved by cyanogen bromide, and the carboxy-terminal peptide was isolated and sequenced. The carboxy-terminal peptide composition indicated a lower content of histidine, leucine, and glycine than expected. Manual microsequencing by gas-phase Edman degradation demonstrated that two amino-terminal ends were present. By use of the known sequence of the human fibrinogen γ-chain, the sequencing data could be resolved into a dipeptide cross-linked between lysine-406 and either glutamine-398 or -399 (residues 6 and 13 or 14 from the carboxy-terminal end of the γ-chain) with the loss of residues 401–404 that occur between the cross-link sites of both antiparallel cross-linked γ-chains. D-dimer is therefore separated into monomers by cleavage of the γ-chain between the cross-link sites. Two symmetrical fragments are produced consisting of a cross-linked dipeptide with the loss of four amino acids. The sequence is therefore equivalent to the original fibrinogen sequence

$$\begin{matrix} {}_{385}\text{KIIPFNRLTIGEG}(\text{Q}^{*}\text{Q}^{*})\text{H}_{400} \\ \text{X} \\ {}_{405}\text{A}(\text{K}^{*})\text{QAGDV}_{411} \end{matrix}$$

where *X* indicates a cross-link site. There is heterogeneous loss of ^{401}HLGG^{406} and possibly also ^{399}Q and ^{400}H in subfractions of the peptide. The predominant cross-linked glutamine appears to be residue 399, 13 residues from the carboxy-terminal end.

Covalent stabilization of the fibrin clot is brought about by the formation of γ-(ε-glutamyllysine) cross-links between antiparallel γ-chains of fibrin (Lorand, 1983) by the enzyme "transglutaminase"—factor XIIIa of the coagulation cascade (EC 2.3.2.13, glutaminyl-peptide glutamyltransferase). The amino acid sequence of the γ-chain is highly conserved, permitting hybrid cross-links of the γ-chains of fibrin from different species, e.g., bovine and human (Chen & Doolittle, 1971). The cross-link has been assigned to sites lysine-406 and glutamine-398 (6 and 14 residues from the carboxy-ter-

† This research was supported by grants from the South African Medical Research Council and the University of Cape Town Staff Research Fund from the Nellie Atkinson and Bekker bequests.

* Address correspondence to this author.

‡ Department of Chemical Pathology.

§ Department of Biochemistry.

0006-2960/87/0426-4640$01.50/0

minal end) of reciprocal chains, 7 residues apart (Chen & Doolittle, 1971; Sharp et al., 1972; Doolittle, 1973). The cross-link site was assigned by locating [^{14}C]glycine ethyl ester substituted on fibrin by transglutaminase (Chen & Doolittle, 1970). The assumption made was that this occurred at the natural γ-chain cross-link site.

Since the actual cross-link sites have not yet been demonstrated by direct sequencing of cross-linked γ-chains, it is possible that both glutamines at sites 398 and 399 could be the target for transglutaminase activity with two cross-links being present as well as a substituent, [^{14}C]glycine ethyl ester or dansylcadaverine, substituted on the remaining glutamine (residue 13 or 14 from the carboxy-terminal end). If this were so, then symmetric products would always be produced whether cleavage took place at the isopeptide bond or through endopeptidase activity between the cross-linked sites.

D-dimer can also be separated into monomers when the molecule is rendered susceptible to further plasmin action by the removal of calcium. Under these conditions two smaller D-monomers with shortened γ-chains and a peptide containing the cross-link together with fluorescent dansylcadaverine are produced (Purves & Lindsey, 1978).

Puff adder venom cleaves the di-γ-chain of D-dimer, resulting in two symmetrical D-monomers. There is no apparent reduction in size of the γ-chain, although the β-chain is hydrolyzed at a slower rate, yielding a smaller β-chain and hence a smaller D-monomer. The cleavage of the di-γ-chain precedes and is therefore independent of the progressive reduction in size of the β-chain (Purves et al., 1986). The possibility that the β-chain cleavage is due to the presence of another enzyme has not been excluded.

In this paper we report the isolation and sequencing of the carboxy-terminal cyanogen bromide fragment of the γ-chain from puff adder venom cleaved D-dimer derived from fibrin (D-PAV).[1] From the known sequence of the human fibrinogen γ-chain (Henschen et al., 1983), we are able to define the site of cleavage of the di-γ-chain of covalently cross-linked fibrin by the puff adder venom protease.

Experimental Procedures

Materials. The following materials were obtained from the sources cited: fibrinogen and plasminogen from Sigma; lyophilized puff adder venom from the Transvaal Snake Park, Half-Way House, South Africa. Fluorescent and nonfluorescent D-dimers were prepared as described (Purves & Lindsey, 1978; Purves et al., 1980). DEAE-Sepharose CL-6B and Sephadex G50 were obtained from Pharmacia; cyanogen bromide and iodoacetamide were from Eastman; Picotag reagents were from Waters. Sequencing reagents and solvents were Pierce Sequanal grade. Polyacrylamide gel electrophoresis in the presence of 1% SDS (SDS–PAGE) was carried out in 6–8% or 4–20% density gradient slab gels and stained with Coomassie Blue (Sigma). Protein concentrations were estimated with the Coomassie Blue dye reagent (Bio-Rad). Puff adder venom protease used in this work was partially purified as described (Purves et al., 1986).

Preparation of D-PAV Monomer. Purified puff adder venom (5 mg) was added to a mixture of 500 mg of nonfluorescent D-dimer and 5 mg of fluorescent D-dimer in a buffer: 0.1 M Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $ZnCl_2$. The fluorescent D-dimer was added to facilitate location of the γ-chain. The digestion was carried out for 18 h at 37 °C and monitored by SDS–PAGE in 4–20% gels. The reaction was stopped by addition of 5 mM EDTA (final concentration).

The digest was dialyzed against a 10 mM Tris, pH 8.6, buffer and applied to a DEAE-cellulose (Whatman) column (20 × 2 cm) equilibrated with the same buffer, and the protein eluted with a gradient of 0–0.3 M NaCl. A pool was made of the major fluorescent peak.

Isolation of γ- and β-Chains from D-PAV Monomer. The D-PAV monomer (345 mg) was made 6 M with respect to guanidinium chloride and reduced and alkylated (Doolittle et al., 1977b). The solution was desalted on a Sephadex G25 column equilibrated with a 10 mM Tris, pH 7.5, buffer containing 8 M urea. The reduced and alkylated γ- and β-chains were separated on a DEAE-Sepharose CL-6B column (20 × 2 cm) equilibrated with a 10 mM Tris, pH 7.5, buffer containing 8 M urea and eluted with a gradient of 0–0.3 M NaCl (Figure 1). Aliquots were analyzed by PAGE with a 5% acetic acid buffer, pH 4.0, containing 2.5 M urea. The pools from the major peaks were exhaustively dialyzed against distilled water and lyophilized. The yield of γ-chain was 100 mg.

Cyanogen Bromide Digestion of the γ-Chain of D-PAV Monomer. Lyophilized γ-chain (30 mg) was dissolved in 70% formic acid (5 mg/mL). Cyanogen bromide was added (10 mg/mL), and the reaction vial was kept under nitrogen for 18 h at room temperature. The solution was thereafter diluted 10 times with distilled water and lyophilized. The dried peptides were dissolved in 10% acetic acid and molecular sieved on a Sephadex G50 column (200 x 1 cm) with 10% acetic acid (Figure 2).

The fluorescence in the fractions was detected after evaporation of the acid solvent by vacuum centrifugation and addition of 200 μL of 0.1% NH_4HCO_3. The fluorescent peak was pooled and lyophilized.

Isolation of the Carboxy-Terminal Peptide from the γ-Chain of D-PAV Monomer. The lyophilized peptides were redissolved in 0.1% NH_4HCO_3 or 0.1% TFA and separated by high-performance liquid chromatography (HPLC).

In order to visualize the fluorescent peptide (added at 1% to facilitate location of the γ-chain), one HPLC separation used an alkaline ammonium bicarbonate buffer system with an acetonitrile gradient to elute from a RadialPak C_8 column (Figure 3). The fluorescent peptide was not expected to elute at the same position as the main component of the unsubstituted peptide. The relevant peaks were pooled, and a portion of the pool was run on an acid HPLC system (Figure 4).

Peptide Hydrolysis and Amino Acid Composition. The Picotag (Waters) method was used to hydrolyze aliquots of the dried peptide (18 h at 105 °C under nitrogen with gas-phase 6 N HCl hydrolysis). The Waters system for PITC-amino acid derivatization and separation was used. The carboxy-terminal peptide could be readily identified by a characteristic signature, viz., the absence of serine. The yield of carboxy-terminal peptide was calculated on the basis of the lysine content (Table I).

Manual Gas-Phase Microsequencing by Edman Degradation. The method used will be published in greater detail (W. F. Brandt and G. Frank, unpublished method). Oxygen scavengers and dithiothreitol were not added to reagents, but these were always freshly opened and stored at −40 °C under nitrogen.

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PAV, puff adder venom; D-PAV, puff adder venom cleaved D-dimer; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; DEAE, diethylaminoethyl; DP, dipeptide; HPLC, high-performance liquid chromatography.

Preparation of Glass Paper Filter. A disk of Whatman GF/C paper, 0.8-cm diameter, was inserted into a 2-cm glass tube with a drawn-out end. The filter was washed with 100 μL of TFA, followed by four additions of 100 μL of butyl chloride. This was repeated twice, and the glass filter was dried in vacuo at 50 °C for 15 min. Polybrene (Pierce), 10 μL of a 50 mg/mL solution in water, was added to the center of the filter, and the filter was then dried in vacuo for 15 min. The tube containing the filter was supported on glass tubing and placed together with a tube containing 0.5 mL of TFA in a 50-mL reaction bottle with a tap permitting evacuation and nitrogen replacement. The Picotag (Waters) vessels were convenient for this purpose. A blank cycle was performed by exposing the filter to TFA vapor at 50 °C for 15 min. Thereafter the filter was washed with 3 × 200 μL additions of ethyl acetate and then 2 × 200 μL additions of butyl chloride.

Addition of Sample. The filter was dried in vacuo, and the peptide, 5 nmol in 10 μL of 50% TFA in water, was added accurately to the center of the filter and then dried in vacuo.

Coupling Reaction. The microliters of 5% PITC in heptane was added to the center of the filter. A tube containing 0.5 mL of 5% triethylamine was also added to the reaction vessel that was then flushed with nitrogen and kept at 50 °C for 40 min. The filter was dried in vacuo, washed with additions of 2 × 200 μL of heptane followed by 2 × 200 μL of ethyl acetate, and then dried in vacuo again (adequate drying was assessed by a pressure less than 50 millitorr).

Cleavage Reaction. A tube containing 0.5 mL of TFA was added to the reaction vessel and flushed briefly with nitrogen. After exposure of TFA vapor at 50 °C for 15 min the filter was dried briefly (to 200 millitorr) and extracted with 3 × 200 μL washes of butyl chloride collected into a 10 × 75 mm tube (previously washed with TFA). The filter was dried in vacuo and was then ready for the next cycle of the coupling reaction. The washings were dried under a stream of nitrogen at 50 °C.

Conversion Reaction. The dried thiazolinone was converted to the phenylthiohydantoin (PTH) derivative by the addition of 20 μL of 50% TFA in water to the sample, and, together with a tube containing 0.5 mL of 50% TFA in water, heated at 90 °C for 10 min in the reaction vessel. The tube was then dried in vacuo, and the contents were analyzed by HPLC.

Automated Gas-Phase Microsequencing. A method was used in which an automated spinning-cup sequencer was converted into a vapor- (gas) phase sequencer (Brandt et al., 1984).

High-Performance Liquid Chromatography. A Waters system was used throughout with wavelengths of 214 or 254 nm used for peptide detection together with fluorescence detection at alkaline pH. PTH-amino acids were detected at 254 nm. Injections were manual, and column temperature was maintained at 45 °C. Flow rate was 1 mL/min. Peptides were separated by using two systems: (1) A 5 μBondapak C_{18} (Waters) cartridge (8 mm × 10 cm) in a radial compression system was used. The column was equilibrated with 0.1% NH_4HCO_3 and the sample eluted with a linear gradient up to 25% acetonitrile over 40 min and then up to 40% over 10 min. (2) An Ultrapore RPSC (Beckman) C_3 column (4.6 × 75 mm) was equilibrated with 0.1% TFA in water containing 10% acetonitrile and the sample eluted with a linear gradient over 40 min up to 40% acetonitrile in 0.1% TFA.

Amino acid composition was performed by using the complete Picotag (Waters) system with gas-phase 6 N HCl hydrolysis of samples and PITC derivatization and separation of the thiazolinone derivatives on the Picotag-selected C_{18} column with commercial reagents. PTH-amino acids were separated by using two systems: (1) A Novapak C_{18} (Waters) column (3.9 mm × 15 cm) was used. The column was equilibrated with 5% tetrahydrofuran (Merck) containing 30 mL of 3 M sodium acetate, pH 3.8, and 7 mL of 3 M sodium acetate, pH 4.6, per liter. The sample was eluted with linear gradients of acetonitrile: 5% over 2 min, 30% in 20 min, isocratic at 30% for 2 min, and 70% over 5 min. (2) The same column was used with equilibration with Picotag buffer A followed by elution of the sample with a linear gradient over 20 min up to 30% acetonitrile in water. Picotag buffer A consists of 0.14 M sodium acetate containing 0.5 mL of triethylamine/L titrated to pH 6.40 with glacial acetic acid, and 6% acetonitrile is added before use.

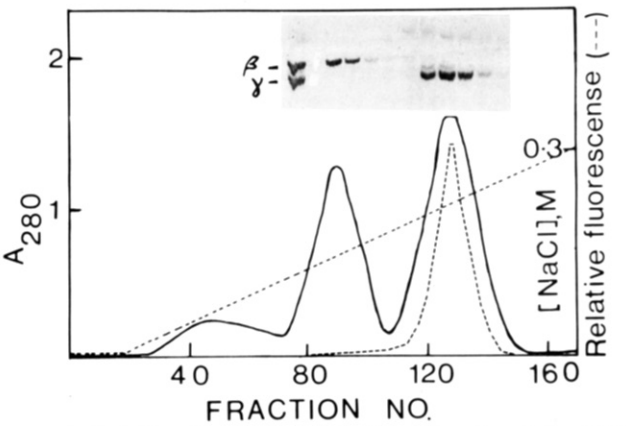

FIGURE 1: Isolation of reduced and alkylated β- and γ-chains (345 mg) on a DEAE-Sepharose CL-6B column (2 × 20 cm) equilibrated with 10 mM Tris buffer, pH 7.5, containing 8 M urea. Flow rate 34 mL/h. Fraction volume 2.25 mL. Absorbance 280 nm (—). Fluorescence in arbitrary units (---), excitation 340 nm, and emission 520 nm (from the addition of 1% dansylcadaverine-substituted D-dimer to the starting material in the preparation of D-PAV monomer). γ-chains were pooled as indicated. (Inset) PAGE of indicated fractions in an acetic acid buffer containing 2.5 M urea.

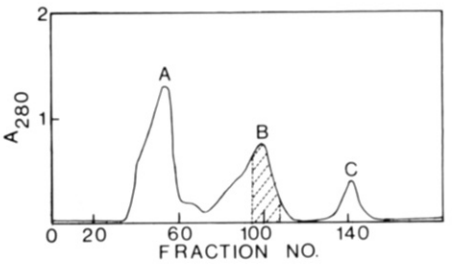

FIGURE 2: Separation of cyanogen bromide peptides of the γ-chain of D-PAV monomer (30 mg) on a Sephadex G50 column (1 × 200 cm) in 10% acetic acid. Flow rate 10 mL/h. Fraction volume 1.0 mL. The hatched area indicates the fluorescent fractions that were pooled (see Experimental Procedures).

RESULTS

The D-PAV monomer from the DEAE-cellulose column was collected by pooling the fluorescent samples (due to the initial addition of 1% fluorescent D-dimer). After reduction and alkylation, the β- and γ-chains were clearly separated by chromatography as shown by acetic acid/urea–PAGE (Figure 1). After cyanogen bromide cleavage of the purified γ-chain, the peptides were separated by molecular sieving. The lyophilized fractions, made alkaline with ammonium bicarbonate, showed that the fluorescence was located at one end of the β peak (Figure 2). This distribution conformed with the expectation of a carboxy-terminal peptide, 2864 daltons, with the other peptides having sizes of 19 284, 4875, 4854, 3891,

Table I

(A) Amino Acid Composition of Isolated Peptide (Peak A, Figure 3)

amino acids	corrected[a] and normalized		probable no.[d]	expected no.[d]	difference
N + D	2.07[b]	2.96[c]	2	2	
Q + E	2.25	3.21	2, 3	4	−1, −2
S	0	0	0	0	
G	1.90	2.71	2, 3	5	−2, −3
H	0.34	0.48	0, 1	2	−1, −2
R	1.05	1.40	1	1	
T	0.86	1.22	1	1	
A	1.76	2.5	2	2	
P	1	1.40	1	1	
V	0.70	1	1	1	
I	1.60	2.28	2, 3	3	−1
L	0.73	1.04	1	2	−1
F	1.05	1.50	1	1	
K	1.70	2.42	2	2	

(B) Carboxy-Terminal Cyanogen Bromide Peptide of the Human Fibrinogen γ-Chain[e]

Sequence	K	I	I	P	F	N	R	L	T	I	G	E	G	Q*	Q*	H	H	L
Losses	(i)													(q	h)	H	L	

Sequence	G	G	A	K*	Q	A	G	D	V	
Losses	G	G								

[a] Corrected for recovery of hydrolyzed amino acids standards. [b] Normalized to one residue, P. [c] Normalized to one residue, V. [d] The values of probable and expected are given as residues per mole. [e] The known sequence is aligned against the missing residues. i, h, and q represent probable extra losses in a subfraction of the peptide. * represent cross-link sites, either Q or K. The ratio of I to L was always 2:1. Partial hydrolysis of II could be responsible for low yield.

3047, 1214, 615, 589, and 188 daltons. The dansyl-cadaverine-substituted peptide was clearly separated from the main unsubstituted peptide(s) (Figure 3) in HPLC under alkaline conditions using a RadialPak (Waters) C_8 column. The precise molecular constitution of the fluorescent peptide has not been studied further yet except to note that two main species appeared to be present as is the case with the unsubstituted peptides. The amino acid composition of the HPLC fractions showed that peaks A and B (Figure 3) both had the hallmarks of the carboxy-terminal peptide, viz., no serine, equimolar valine, phenylalanine, proline, and threonine, and

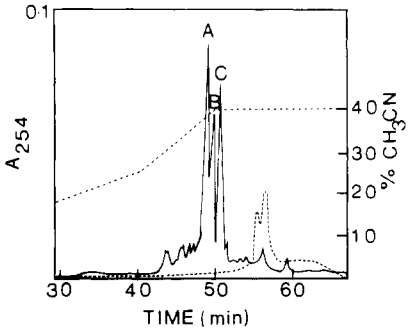

FIGURE 3: HPLC separation of carboxy-terminal peptides of the γ-chain of D-PAV monomer from pooled fractions (Figure 2) using a RadialPak C_8 (Waters) column. The mobile phase was 0.1% NH_4HCO_3 containing 5% acetonitrile, and the sample was eluted with an acetonitrile gradient up to 40%. Flow rate 1 mL/min. Absorption 254 nm (—) and fluorescence in arbitrary units (- - -) (Waters detector). Peaks A and B were both shown to be carboxy-terminal peptides on the basis of amino acid composition (see Results).

more isoleucine than leucine (Table I, B). There were minor differences in amino acid composition between peaks A and B. HPLC of individual peaks in an acid system again yielded two peaks. Peaks A and B were therefore pooled and run on an acid HPLC system, and this yielded two overlapping peaks (Figure 4, peaks D and E) with similar amino acid composition and sequences.

The amino acid composition (Table I, A) showed that certain amino acids were at lower levels than expected—notably leucine, glycine, histidine, glutamine, and possibly isoleucine. With hindsight, these amino acids, with the exception of isoleucine, occur as a group and could have been removed by exopeptidase activity after a chain cleavage (Table I, B). The deficient isoleucine could be due to incomplete hydrolysis of the two adjacent isoleucine residues in positions 2 and 3 of the peptide. Subsequently, the expected three isoleucine residues appeared in the sequence (Table II).

The manual gas-phase sequencing procedure proved to be very effective with five cycles per 8-h working day being achieved (multiple samples can be sequenced simultaneously). Since the squence of the γ-chain is known, identities were not

Table II: Gas-Phase Edman Degradation Microsequencing[a]

	manual						automated			
cycle	residue 1		carry-over	residue 2		carry-over	residue 1		residue 2	
1	K	8.0	0	A	4.0	0	K	9.6	A	12.0
2	I	7.0	?				I			
3	I	6.0	2.0	Q	2.0	0.2	I	13.0	Q	6.1 (E 4.0)
4	P	5.0	0	A	5.0	0	P	9.3	A	11.0
5	F	4.0	1.0	G	4.0	1.0	F	11.0	G	10.6
6	N + D	8.0	1.5	(D)			N + D	3.4	(D)	
7	(R)[b]	?	0	V	4.0	0.2	R[c]	1.6	V[d]	2.9
8	L	5.5	1.0				L	7.7		
9	T[e]	4.0	2.0 (0.2)				T[f]	3.6		
10	I	2.2	0.2				I	6.3		
11	G	2.0	?				G	6.5		
12	E	2.0	?				E	6.0		
13	G	1.0	?				G	5.2		
14	Q[g]	1.2	?	DP[h]	3.0	?	Q	0.1 (E 2.0)		
15	Q	0.2	0.1	DP	4.0	?	Q[i]	1.0 (E 1.0)		
16	H	1.0	? (0.05)	DP	3.0	0.2	*j*			
17	(H)	0.05								
18										

[a] The values in manual sequencing are relative peak areas. The repetitive yield was about 85%. Values in automated sequencing are in nanomoles. Carry-over refers to residues found in the subsequent cycle where it can be identified as such; ? indicates where this cannot be done for technical reasons of peak separation or a repeated residue. [b] R expected but three smaller peaks found instead. [c] R identified as a small peak. [d] V, low yield of this carboxy-terminal residue. [e] T only present as dehydrothreonine and carried over for three cycles. [f] T identified as a triplet, the extra two peaks being dehydroalanine adducts with dithiothreitol. [g] Q only a single major peak present. [h] DP indicates presence of peak eluting at 1.5 min and not present in prior cycles. The early eluting DP peak could not be separated in the automated method. [i] Q identifiable above carry-over. [j] No residue seen.

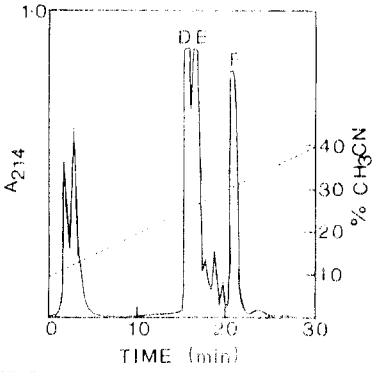

FIGURE 4: HPLC separation of pooled carboxy-terminal peptides (peaks A and B, Figure 3) on an Ultrapore RPSC (Beckman) C_3 column. The mobile phase was 0.1% TFA containing 10% acetonitrile, and the peptides were eluted with a linear acetonitrile gradient over 30 min from 10% to 40%. Peaks D and E were both shown to be carboxy-terminal peptides on the basis of amino acid composition. (Two peaks were also seen when either peak A or B was applied separately—see text for comments on heterogeneity.)

confirmed by back-hydrolysis, but several residues were checked on a different PTH analysis system. Arginine (residue 7) was not identifiable as a single peak, and threonine was present only as very hydrophobic dehydrothreonine with carry-over for two cycles. At residue 14, glutamine was found as well as a new peak (Table II, DP) that eluted very early. A smaller amount of glutamine, possibly carried over, was found at residue 15 together with the early peak in larger amounts. The exact constitution of this early peak could not be ascertained, but the synthetic dipeptide γ-L-glutamyl-ε-L-lysine, when subjected to the gas-phase procedure, eluted in a similar position albeit with a few unidentified minor peaks. The di-PTH derivative of the dipeptide made by a solution method, however, yielded material eluting near to lysine but distinct from it and on hydrolysis in 6 N HCl (gas phase) and rederivatization with PITC and conversion to the PTH derivative yielded glutamic acid and lysine. The differences have not been investigated further. Histidine was present in substoichiometric amounts (Table I), suggesting a degree of heterogeneity. The manual sequence showed histidine to be present at residue 16 and possibly also at position 17. However, carry-over could not be excluded as the histidine was the terminal residue at either position 16 or 17.

The carboxy-terminal amino acid of the γ-chain, i.e., valine, was seen in good yield despite its terminal position but occurred at cycle 7. The first cycle and the third to seventh cycles showed the presence of two residues. The second cycle contained only a single residue. The eighth to thirteenth cycles showed a clear-cut single residue and were all easily identified. Glutamine and a putative dipeptide (DP) were present at cycles 14 and 15 but with glutamine predominant at cycle 14 and the putative dipeptide predominant at cycle 15.

Automated sequencing of the carboxy-terminal peptide gave essentially similar results. Threonine was seen as a characteristic triplet, and arginine and valine gave low yields at cycle 7. At cycle 14, glutamic acid and small amounts of glutamine were found. Glutamine and possibly carried-over glutamic acid were seen at cycle 15. The system did not permit the visualization of very early eluting material. Histidine was not seen at all. The glutamine expected at cycle 3 was found partly as glutamic acid, and this degradation could be expected to increase with each cycle, perhaps accounting for glutamic acid instead of glutamine as the main component at cycle 14. (The glutamic acid is derived from the chemical deamidation of glutamine and is not the result of isopeptide bond hydrolysis although partial enzymatic deamidation or transamidation, e.g., by transglutaminase, cannot be excluded).

By use of the known sequence of the human fibrinogen γ-chain, the sequencing data could be resolved into a dipeptide cross-linked at lysine-406 and either glutamine-398 or -399 (residues 6 and 13 or 14 from the carboxy-terminal end of the γ-chain) with the loss of residues 401–404 that occur between the cross-link sites of both antiparallel cross-linked γ-chains. This effectively cleaves the D-dimer into two monomers and the cross-linked di-γ-chain into two symmetrical fragments consisting of a cross-linked dipeptide with the loss of four amino acids.

The sequence is therefore equivalent to the original fibrinogen sequence

$$^{385}\text{KIIPFNRLTIGEG(Q}^{*}\text{Q}^{*})\text{H}_{400}$$
$$\times$$
$$^{405}\text{A(K}^{*})\text{QAGDV}_{411}$$

where *X* indicates a cross-link site. There is heterogeneous loss of ^{401}HLGG^{406} and possibly also ^{399}Q and ^{400}H in subfractions of the peptide.

A degree of heterogeneity seems to exist in two respects: (1) The cross-linked glutamine would appear to be either residue 14 or 15 with 15 predominating. (2) The loss of carboxy-terminal amino acids may include both histidines and possibly even a glutamine (residues 17, 18, and 16 of the peptide). There may also be deamidation of some of the glutamines near the cross-link site. However, the amino-terminal residue of the secondary sequence is unequivocally alanine with the following lysine not appearing in the second cycle and presumably still cross-linked to the glutamines at either site 15 or 16.

The recovery of carboxy-terminal peptide, quantitated by the lysine content of peaks A and B (Figure 3), accounted for 60% of the expected yield from the D-dimer used as starting material. Considering that only central components of peaks, e.g., as indicated by fluorescence, were collected, the slightly heterogeneous carboxy-terminal peptides probably represented the major component, and the existence of other variants was unlikely.

Discussion

The carboxy-terminal end of the γ-chain has a number of defined sites participating in physiological reactions. The conformation of the carboxy-terminal end of the γ-chain is depedent on bound calcium (Haverkate & Timan, 1977; Purves & Lindsey, 1978). Removal of this calcium exposes more sites for plasmin cleavage with release of a peptide containing the cross-links between the γ-chains and therefore separation of the D-dimer into monomers. The requirement for calcium removal probably indicates that it is physiologically irrelevant. Fibrinolysis in vivo is achieved by plasminolysis at defined sites, releasing the D and E domains but not affecting any D domains cross-linked by transglutaminase.

The only enzyme we have found capable of separating the D-dimer into monomers is found in the venom of several Crotalidae and Viperidae, notably the puff adder (*Bitis arietans*) (Purves et al., 1986). Since the cleavage yielded apparently symmetrical monomers without γ-chain shortening or loss of substituted lysine analogues, e.g., dansylcadaverine, the simplest explanation would have been an isopeptide bond cleavage, especially if, as has been assumed, one of the cross-link sites is always blocked by any substituent (Table III).

Our results provide the explanation for the ability of puff adder venom protease to produce cleavage of D-dimer into

Table III: Cross-Link Configurations of the Human Di-γ-Chain Carboxy-Terminal CNBr Peptide and the Mode of Puff Adder Venom Cleavage[a]

(A) accepted configuration	:--------------14-------6-----c X X c-----6- -----14--------------:	two cross-links
(B) substitution by, e.g., dansylcadaverine (*)	:--------------14-------6-----c X c-----6-----13-14--------------: (*) *	one cross-link
(C) cleavage at the cross-link site by PAV	:--------------14--c n-6-----c X (γ − 4) c-----6-n c--14---------------:	asymmetric fragments, 7-residue difference f(γ − 11)
(D) cleavage at a single cross-link site	:--------------14------6-----c X * (γ + 7) c-----6-n c--14---------------:	asymmetric fragments, 18-residue difference f(γ − 11)
(E) cross-link not inhibited by a substitution on adjacent residues	* :--------------14-13-----6-----c X X c-----6-----13-14----------------: *	
(F) alternate cross-link site	* :--------------14-13-----6-----c X X c------6-----13-14-------------: *	
(G) symmetrical cleavage products if model E or F appertains	* :--------------14-13-c n-6-----c X X c-----6-n c-13-14--------------: *	

[a] The diagrams represent the cross-linked carboxy-terminal end of the γ-chain fragments after cyanogen bromide cleavage: (:) residue after cyanogen bromide cleavage, (n) amino-terminal residue, (c) carboxy-terminal residue, (f) fluorescent substituent if dansylcadaverine is used as the lysine analogue for transglutaminase activity, (X), site of isopeptide cross-link, (*) substituent, and (γ − 4, γ − 11, γ + 7) peptides differing from the γ-chain residue number.

Table IV: Comparison of Carboxy-Terminal Sequences at Cross-Link Sites of Rat,[a] Bovine,[b] and Human[c] γ^A-Chains[d]

						*	‡	‡			‡	‡		*					
rat	S	I	G	D	G	Q	Q	H	H	M	G	G	S	K	Q	V	G	D	M
bovine	A	I	G	Q	G	Q	Q	H	Q	L	G	G	A	K	Q	A	G	D	V
human	T	I	G	Q	G	Q	Q	H	H	L	G	G_Λ	A	K	Q	A	G	D	V
						14	13	12	11	10	9	8	7	6	5	4	3	2	1
						*								*					

[a] Hommandberg et al., 1985. [b] Chen & Doolittle, 1971. [c] Henschen et al., 1983. [d] * indicate cross-link sites. The numbering begins at the carboxy-terminal end of the γ-chain. Λ indicates the cleavage site in the human γ-chain. ‡ indicate possible consensus for puff adder venom protease specificity.

monomers. The presence of the cross-links between the γ-chains of D-dimer provides a potential new conformation for selective γ-chain cleavage. The cleavage occurs between the cross-link sites, i.e., between the lysine and glutamine acids 6 and 14 (or 13) residues from the carboxy-terminal end of the γ^A-chain (Table III). Symmetrical products would be produced if *two* cross-links were present, irrespective of whether the cross-link site is at either glutamine residue (Table III, E and F). However, the presence of only *one* cross-link due to a substitution should lead to assymmetrical products (Table III, D) with differences of as much as 18 residues. If both chains are nevertheless cleaved, a smaller difference of 7 residues between substituted and doubly cross-linked chains would be found (Table III, C).

The natural (unsubstituted) cross-linked γ-chains described in this paper appear to have both possible cross-links present. The recovery of the cross-linked dipeptide was at least 60% of that expected, which implies that the predominant species was analyzed. The original assignment of the cross-link site was at glutamine 14 from the carboxy-terminal end, based on [^{14}C]glycine ethyl ester substitution by transglutaminase, and the lesser amount of substitution at glutamine 13 was attributed to the small size of the nonnatural substrate. Model building also suggested that the glutamine, 14 residues from the carboxy-terminal end, was advantageously positioned on the α-helix, being in the same sector as the lysine, 6 residues from the carboxy-terminal end (Doolittle et al., 1972). However, labeling with a substituent is not the same as cross-linking.

Our data suggest that there is cross-linking at either glutamine, with perhaps the glutamine at position 13 from the carboxy-terminal end (residue 399) being predominant. This leaves open the possibility that *both* substitution *and* cross-linking could occur on the same molecule. This would simplify explanations for the symmetry of substituted products after puff adder venom protease cleavage (Table III, G). These questions will have to be resolved by direct sequencing of the substituted chains cleaved by puff adder venom protease.

Alanine (residue 419) was consistently found at the newly generated amino-terminal end, but the carboxy-terminal end was heterogeneous to a certain extent (e.g., substoichiometric amounts of histidine). This is an adequate explanation for the failure of previous attempts to determine the carboxy-terminal sequence by direct methods, e.g., carboxypeptidase Y and tritium labeling (data not shown). The proximity of the cross-link site to the heterogeneous carboxy-terminal residues

is another perturbing factor.

Since bovine and rat D-dimers are also susceptible (data not shown), there is an indication of a possible consensus (Table IV) of either diglycyl or histidylglutamyl residues and the presence of the cross-link. The possibility that the site-specific endopeptidase is contaminated by an exopeptidase that generates the heterogeneous ends cannot be excluded at present (Purves et al., 1986). Since the presence of the cross-links provides the specificity necessary for γ-chain cleavage by puff adder venom protease, this suggests that the fibrinogen conformation is sufficiently altered so that new epitopes might be created and raises the possibility that monoclonal antibodies could be produced with specificity for cross-linked fibrin by using puff adder venom cleaved peptides as antigen.

The manual gas-phase microsequencing technique used in this paper is very suitable for sequences of up to at least 20 residues, with a repetitive yield in this study of about 85%. The ability to detect carboxy-terminal residues was superior to an automated technique and had the advantage of demonstrating an unusual early peak, the putative cross-linked dipeptide. Carry-over from one cycle to the next is greater than with automated sequencing and cannot be appreciably reduced by extensive washing; however, it is not problematic except with repeated residues. The instrumental requirements are minimal, and this technique can be recommended for sequencing proteins blotted onto glass paper (W. F. Brandt and G. Frank, unpublished method).

References

Brandt, W. F., Alk, H., Chauhan, M., & von Holt, C. (1984) *FEBS Lett. 174*, 228.

Chen, R., & Doolittle, R. F. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 472.

Chen, R., & Doolittle, R. F. (1971) *Biochemistry 10*, 4486.

Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1.

Doolittle, R. F., Cassman, K. G., Chen, R., Sharp, J. J., & Wooding, G. L. (1972) *Ann. N.Y. Acad. Sci. 202*, 114.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., & Takagi, T. (1977a) *Biochemistry 16*, 1710.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., & Friezner, S. J. (1977b) *Biochemistry 16*, 1715.

Haverkate, F., & Timan, G. (1977) *Thromb. Res. 10*, 803.

Henschen, A., Lottspeich, F., Kehl, M., & Southern, C. (1983) *Ann. N.Y. Acad. Sci. 408*, 28.

Hommandberg, G. A., Evans, D. B., Kane, C. M., & Mosesson, M. (1985) *Thromb. Res. 39*, 263.

Lorand, L. (1983) *Ann. N.Y. Acad. Sci. 408*, 226.

Purves, L. R., & Lindsey, G. G. (1978) *S. Afr. J. Sci. 74*, 202.

Purves, L. R., Lindsey, G. G., & Franks, J. J. (1980) *Biochemistry 19*, 4051.

Purves, L. R., Purves, M., Lindsey, G. G., & Linton, N. J. (1986) *S. Afr. J. Sci. 82*, 30.

Sharp, J. J., Cassman, K. G., & Doolittle, R. F. (1972) *FEBS Lett. 25*, 334.

Interaction between Adenovirus DNA-Binding Protein and Single-Stranded Polynucleotides Studied by Circular Dichroism and Ultraviolet Absorption†

Herbert van Amerongen,*,‡ Rienk van Grondelle,‡ and Peter C. van der Vliet§

Department of Biophysics, Physics Laboratory of the Free University, 1081 HV Amsterdam, The Netherlands, and Laboratory for Physiological Chemistry, State University of Utrecht, 3521 GG Utrecht, The Netherlands

Received December 5, 1986; Revised Manuscript Received March 19, 1987

ABSTRACT: The adenovirus DNA-binding protein (AdDBP) is a multifunctional protein required for viral DNA replication and control of transcription. We have studied the binding of AdDBP to single-stranded M13 DNA and to the homopolynucleotides poly(rA), poly(dA), and poly(dT) by means of circular dichroism (CD) and optical density (OD) measurements. The binding to all these polynucleotides was strong and nearly stoichiometric. Titration experiments showed that the size of the binding site is 9–11 nucleotides long for M13 DNA, poly(dA), and poly(rA). A higher value (15.0 ± 0.8) was found for poly(dT). Pronounced changes in the circular dichroism and optical density spectra were observed upon binding of AdDBP. In general, both the positive peak around 260–270 nm and the negative peak around 240–250 nm in the CD spectra decreased in intensity, and a shift of the crossover point to longer wavelengths was found. The OD spectra observed upon binding of AdDBP are remarkably similar to those obtained with prokaryotic helix-destabilizing proteins like bacteriophage T4 gene 32 protein and fd gene 5 protein. The data can best be interpreted by assuming that the AdDBP–polynucleotide complex has a regular, rigid, and extended configuration that satifies two criteria: (1) a considerable tilt of the bases in combination with (2) a small rotation per base and/or a shift of the bases closer to the helix axis.

The adenovirus DNA-binding protein (AdDBP)[1] is the major product of region E2 and is synthesized in high amounts early in infection of permissive cells (van der Vliet & Levine, 1973). Analysis of the phenotype of various temperature-sensitive and host-range mutants has indicated that the protein is multifunctional. It is essential for viral DNA replication (van der Vliet et al., 1975; Friefeld et al., 1983) and is involved in the control of early and late transcription (Carter & Blanton, 1978;

† This work was supported by the Netherlands Foundation for Biophysics and the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Foundation for the Advancement of Pure Research (ZWO).

‡ Physics Laboratory of the Free University.

§ State University of Utrecht.

[1] Abbreviations: AdDBP, adenovirus DNA-binding protein; CD, circular dichroism; GP32, T4 gene 32 protein; GP5, gene 5 protein; OD, optical density; dx, measure for the distance to the helix axis for a base as mentioned in Scheerhagen et al. (1986a); rotpb, rotation per base as mentioned in Scheerhagen et al. (1986a); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ss, single stranded.

0006-2960/87/0426-4646$01.50/0