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Chemical synthesis and structural characterization of the RGD-protein decorsin: A potent inhibitor of platelet aggregation

PATRIZIA POLVERINO de LAURETO,[1] ELENA SCARAMELLA,[1] VINCENZO DE FILIPPIS,[1] ORIANO MARIN,[1] MARIA GABRIELLA DONI,[2] AND ANGELO FONTANA[1]

[1]CRIBI Biotechnology Centre, University of Padua, Via Trieste 75, 35121 Padua, Italy
[2]Institute of Human Physiology, University of Padua

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Abstract

Decorsin is a 39-residue RGD-protein crosslinked by three disulfide bridges isolated from the leech *Macrobdella decora* belonging to the family of GPIIb-IIIa antagonists and acting as a potent inhibitor of platelet aggregation. Here we report the solid-phase synthesis of decorsin using the Fmoc strategy. The crude polypeptide was purified by reverse-phase HPLC in its reduced form and allowed to refold in the presence of glutathione. The homogeneity of the synthetic oxidized decorsin was established by reverse-phase HPLC and capillary zone electrophoresis. The results of amino acid analysis after acid hydrolysis of the synthetic protein, NH_2-terminal sequencing and mass determination (4,377 Da) by electrospray mass spectrometry were in full agreement with this theory. The correct pairing of the three disulfide bridges in synthetic decorsin was determined by a combined approach of both peptide mapping using proteolytic enzymes and analysis of the disulfide chirality by CD spectroscopy in the near-UV region. Synthetic decorsin inhibited human platelet aggregation with an IC_{50} of ~0.1 μM, a figure quite similar to that determined utilizing decorsin from natural source. In particular, the synthetic protein was 2,000-fold more potent than a model RGD-peptide (e.g., Arg-Gly-Asp-Ser) in inhibiting platelet aggregation. Thermal denaturation experiments of synthetic decorsin, monitored by CD spectroscopy, revealed its high thermal stability (T_m ~ 74 °C). The features of the oxidative refolding process of reduced decorsin, as well as the thermal stability of the oxidized species, were compared with those previously determined for the NH_2-terminal core domain fragment 1–41 or 1–43 from hirudin. This fragment shows similarity in size, pairing of the three disulfides and three-dimensional structure with those of decorsin, even if very low sequence similarity. It is suggested that the less efficient oxidative folding and the enhanced thermal stability of decorsin in respect to those of hirudin core domain likely can be ascribed to the presence of the six Pro residues in the decorsin chain, whereas none is present in the hirudin domain. The results of this study indicate that decorsin can be obtained by solid-phase methodology in purity and quantities suitable for structural and functional studies and thus open the way to prepare by chemical methods novel decorsin derivatives containing unusual amino acids or even non-peptidic moieties.

Keywords: circular dichroism; decorsin; disulfide-rich peptides; mass spectrometry peptide mapping; platelet aggregation inhibitor; RGD-peptides; solid-phase peptide synthesis

Reprint requests to: Angelo Fontana, CRIBI Biotechnology Centre, University of Padua, Via Trieste 75, 35121 Padua, Italy; e-mail: fontana@civ.bio.unipd.it.

Abbreviations: [θ], mean residue ellipticity; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; CM-decorsin, reduced and S-carboxymethylated decorsin; CZE, capillary zone electrophoresis; DCC, dicyclohexylcarbodiimide; DCM, dichoromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DTNB, dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; EDT, ethanedithiol; EDTA, ethylenediaminetetracetic acid; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; Gdn·HCl, guanidine hydrochloride; GSH and GSSG, reduced and oxidized glutathione, respectively; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IC_{50}, concentration required to inhibit 50% platelet aggregation; NMP, N-methylpyrrolidone; PDI, protein disulfide isomerase; Pmc, 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl; PTH, phenylthiohydantoin; RGD, Arg-Gly-Asp; RP, reverse-phase; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; T_m, melting temperature; Tris, tris(hydroxymethyl)aminomethane; Trt, triphenylmethyl. Other abbreviations and symbols are those recommended by of the IUPAC-IUB Commission on Biochemical Nomenclature (*J Biol Chem* 247:977–983, 1972). All optically active amino acids are of the L-variety.

Enzymes: trypsin (EC 3.4.21.4); thermolysin (EC 3.4.24.4); thrombin (EC 3.4.21.5); protein disulfide isomerase (EC 5.3.4.1).

Blood-sucking leeches and snake venoms contain a variety of protein factors that potentially affect blood clotting, a complex process involving platelet aggregation. This phenomenon is mediated by the interaction of fibrinogen with the platelet membrane glycoprotein IIb-IIIa (GPIIb-IIIa), a member of the integrin family of cell adhesion and aggregation factors (Hynes, 1992; Lazarus & McDowell, 1993; Ginsberg et al., 1995). Inhibitors of the fibrinogen/GPIIb-IIIa interaction are presently investigated in a number of laboratories with the aim to possibly develop useful therapeutic agents for thrombotic diseases. In recent years, the development of suitable and reliable biological assays for platelet aggregation, as well as the availability of a variety of very efficient chromatographic methods for protein isolation, have led to the discovery of a series of very interesting, small, disulfide-rich proteins responsible for the hemostatic effect. Among the leech proteins, hirudin from *Hirudo medicinalis* (Markwardt, 1991; Stringer & Lindenfeld, 1992) or from *Hirudinaria manillensis* (Scacheri et al., 1993; Vindigni et al., 1994) is the most potent thrombin inhibitor so far known, thus inhibiting the key enzyme in the blood coagulation cascade. A number of other small proteins containing the Arg-Gly-Asp (RGD) adhesive recognition sequence found in many extracellular matrix proteins (fibrinogen, fibronectin, vitronectin, von Willbrand factor, and others; see Yamada, 1991, for a review) have been isolated from leeches or snake venom and characterized, among them decorsin, trigramin, kistrin, echistatin, mambin, ornatin, and hirustasin (Lazarus & McDowell, 1993). These small disulfide-rich proteins have been extensively studied in the past few years, and as a result of this, several of them have been produced and engineered by genetic methods. In particular, the RGD sequence is usually located in an exposed and flexible loop in proteins even showing different overall conformational folds. This has prompted several studies aimed to engineer several proteins and peptide scaffolds containing the RGD motif (Gurrath et al., 1992; Knapp et al., 1992; Scarborough et al., 1993; see Lazarus & McDowell, 1993, for an excellent review).

Decorsin is a 39-residue protein isolated from the leech *Macrobdella decora*, containing three intramolecular disulfide bonds (6–15, 17–27, and 22–38) and the RGD sequence (Fig. 1; Seymour et al., 1990). Decorsin inhibits the fibrinogen interaction with the platelet receptors and is related to disintegrins, a family of RGD-containing GPIIb-IIIa antagonists from snake venom (Lazarus & McDowell, 1993). The three-dimensional (3D) structure of decorsin in solution has been determined by NMR spectroscopy, and its structural motif was found analogous to that of other proteins with anticoagulant activity isolated from leeches, such as antistasin and ornatin (Krezel et al., 1994). Notably, the 3D structure of decorsin is very similar to that of the NH_2-terminal core domain 1–40 of hirudin (Folkers et al., 1989; Haruyama & Wüthrich, 1989; Rydel et al., 1990, 1991), even if the two polypeptide chains show little sequence similarity and different biological action.

In a previous study, we have described the solid-phase synthesis of the NH_2-terminal core domain 1–47 of hirudin (variant HM2) from *Hirudinaria manillensis*, also containing three intramolecular disulfide bridges (De Filippis et al., 1995). This synthesis allowed us to obtain in high yields a homogeneous and biologically active 47-residue polypeptide. Here, we describe the total chemical synthesis, folding, and purification and physicochemical and biological characterizations of decorsin. Synthetic decorsin inhibited the thrombin-induced platelet aggregation at a concentration similar to that observed with natural decorsin. Circular dichroism (CD) measurements were used to evaluate the features of the secondary and tertiary structure of the synthetic peptide, as well as its thermal stability. Differences in the process of oxidative folding and thermal stability between decorsin and the corresponding structurally analogous NH_2-terminal core fragment(s) of hirudin are discussed. Our results demonstrate that solid-phase chemical synthesis is an efficient methodology to produce a homogeneous, biologically active synthetic decorsin, constituting, therefore, a practical alternative to recombinant methods for producing this protein and its analogs.

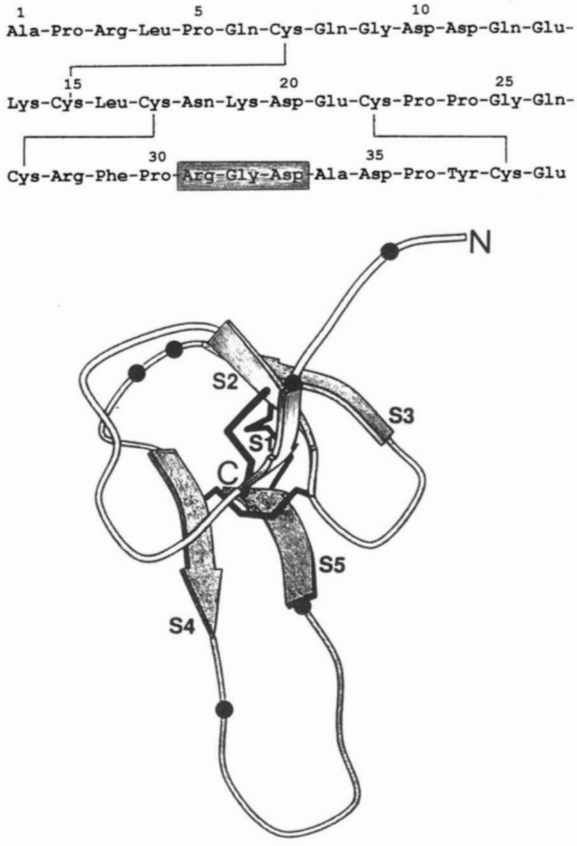

Fig. 1. (Top) Amino acid sequence of the 39-residue chain of decorsin from *Macrobdella decora* (Seymour et al., 1990). Disulfide bonds occur between Cys residues in position 7–15, 17–27, and 22–38 (Krezel et al., 1994). (Bottom) Schematic three-dimensional structure of decorsin showing the three disulfide bridges, the five β-strands (S1, from residue 5 to 7; S2, 15 to 17; S3, 20 to 22; S4, 26 to 29; S5, 36 to 39) as arrowed ribbons and the location of the six Pro residues (black dots) in position 2, 5, 23, 24, 30, and 36 of the chain. The ribbon drawing is adapted from Krezel et al. (1994).

Results

Synthesis and purification

The 39-residue polypeptide chain of decorsin was assembled in three days in a fully automated solid-phase synthesizer by the use of the Fmoc group for amino protection (Atherton & Sheppard, 1989; Fields & Noble, 1990) and the HBTU/HOBt activation procedure for the condensation reaction (Knorr et al., 1989). To improve the yields of coupling with difficult sequences, a double coupling cycle was utilized with hydrophobic or bulky amino acids

(Milton et al., 1990). Once the protected polypeptide chain had been assembled, it was deprotected and released from the resin using a mixture of TFA:ethanedithiol (EDT) (95:5, v/v) for two hours at 0 °C. To the cleavage mixture water was not added in order to possibly avoid the acid-mediated hydrolysis of the Asp35-Pro36 acid-labile peptide bond of decorsin (see Fig. 1; Piszkiewicz et al., 1970). After acidic cleavage of the peptide-resin, the crude reduced 39-residue peptide was obtained in ~75% yield.

The electrospray mass spectrometry (ES-MS) analysis of the crude peptide revealed, besides the expected species (experimental mass 4,384 ± 0.5 Da, calculated mass for the reduced form 4,384 Da), the presence of a significant amount of a byproduct of molecular mass of −18 Da (not shown). This side-product likely derived from the loss of one water molecule due to the formation of an aspartimide peptide moiety, often observed in the synthesis of Asp-containing peptides (Nicolas et al., 1989). To minimize this side reaction, the synthesis was conducted utilizing a shorter piperidine-treatment for removing the Fmoc protecting group, because the cyclization reaction takes place in alkali (Nicolas et al., 1989; Robson at al., 1990; Yang et al., 1994). The crude reduced peptide thus obtained was purified by micro-preparative RP-HPLC (Fig. 2A). A major peak of peptide material is seen in the chromatogram and the yield of reduced synthetic decorsin was estimated as ~45% from integration of the area under the chromatographic peaks in Figure 2A. The homogeneity of the purified reduced peptide was assessed by analytical HPLC (Fig. 2B) and capillary zone electrophoresis (CZE) (see below).

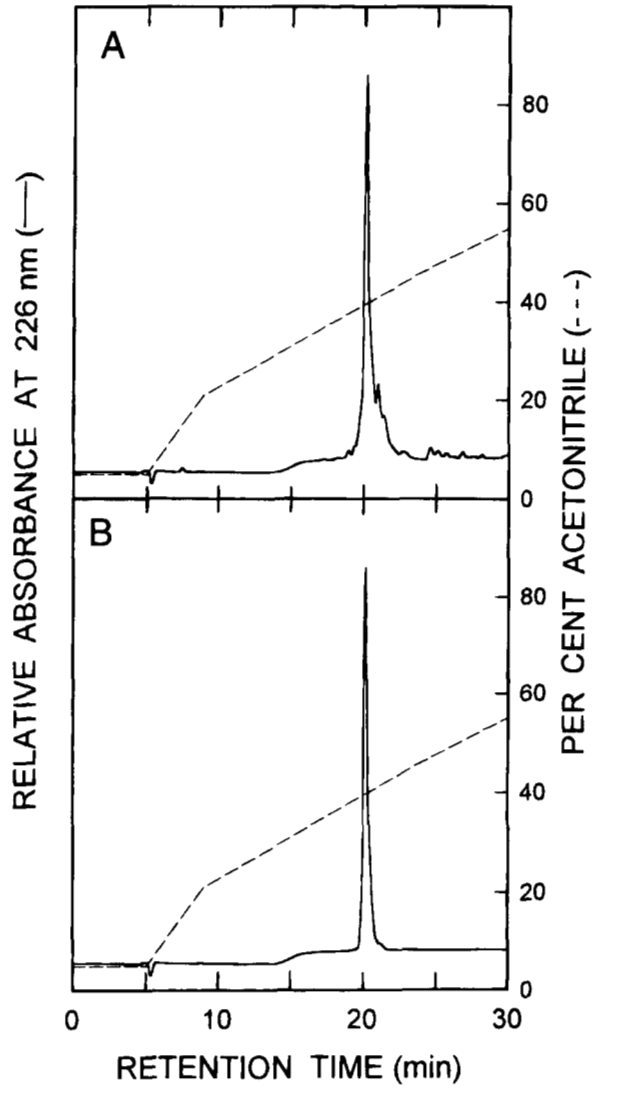

Fig. 2. RP-HPLC of synthetic decorsin: **A:** Crude protein after deprotection from the resin. **B:** Purified protein after micro-preparative HPLC. The samples were dissolved in 0.1% TFA and loaded onto a Vydac C18 column (4.6 × 150 mm). Elution was carried out at a flow rate of 0.6 mL/min with a gradient of acetonitrile in 0.1% TFA. The effluent from the column was monitored by absorbance measurements at 226 nm.

Disulfide formation

Small-scale experiments of oxidative folding were conducted utilizing a sample of the reduced 39-residue peptide purified to homogeneity (see above; Fig. 2B). Initial trials were conducted following essentially the same experimental conditions (air oxidation in 0.1 M $NaHCO_3$, pH 8.3, in the presence of 0.1 mM β-mercaptoethanol) previously shown to determine highly efficient (92–95%) refolding of fragment 1–47 of hirudin HM2 (De Filippis et al., 1995). Briefly, RP-HPLC analysis of aliquots taken from the re-folding mixture revealed that the process was very slow and gave poor yields of the refolded species. The oxidized, correctly folded decorsin was expected to elute earlier from the RP-HPLC C18 column than the reduced peptide or misfolded species, because a proper disulfide crosslinking would lead to the burial of hydrophobic side chains of the peptide and thus to a peptide species with a lower affinity for the hydrophobic matrix of the C18 column (Woo et al., 1989; De Filippis et al., 1995; Pierret et al., 1995). A series of experiments were then performed by varying the experimental conditions to find out the optimal folding conditions. The redox system constituted by reduced (GSH) and oxidized (GSSG) glutathione (1–2 mM) was next investigated (Saxena & Wetlaufer, 1970; Creighton, 1984a). Different GSH/GSSG ratios were employed, because it is known that both the kinetics and yields of disulfide bond formation are affected by varying the oxidation potential of the re-folding mixture (Saxena & Wetlaufer, 1970; Pierret et al., 1995). Because even the use of the GSH/GSSG system did not provide quantitative refolding, the beneficial role of adding a catalytic amount of protein disulfide isomerase (PDI) (Creighton et al., 1980; Freedman, 1984) was investigated.

The best conditions of oxidative folding of the reduced synthetic peptide so far developed involve the use of 2 mM GSH, 1.5 mM GSSG, and PDI at an enzyme:peptide ratio of 1:500 (by weight) and a peptide concentration of 0.2 mg/mL in 0.1 M Tris-HCl, pH 8.5, containing 1 mM EDTA. Some RP-HPLC chromatograms of aliquots taken at intervals from the optimized refolding reaction mixture are shown in Figure 3. At the initial stages of the reaction, a major peak is seen in the chromatogram preceding that of the fully reduced species (R) and following that of the final oxidized species (N). The protein material of this peak (named III) was isolated and analyzed (see also below). It was found that species III was devoid of –SH groups, but contained mis-folded species, because proteolytic digestion of component III and analysis by NH_2-terminal sequencing and mass spectrometry of the resulting peptides provided evidence of a non-native disulfide Cys7–Cys38, besides the native one Cys7–Cys14 (data not shown). The protein species III was found to accumulate in the re-folding mixture if GSH was not in excess over GSSG, whereas in the presence

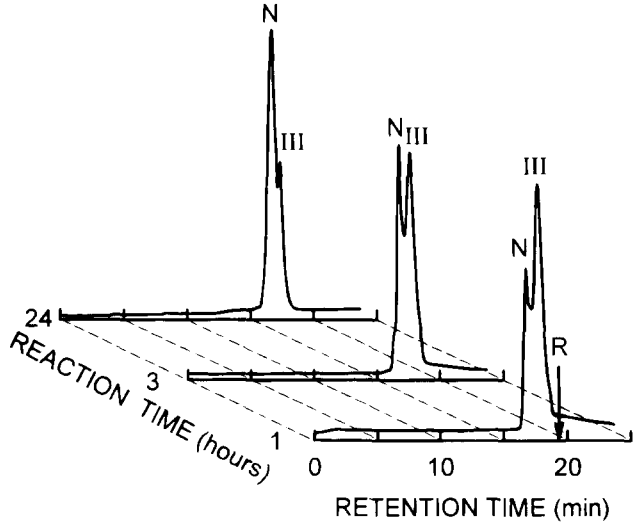

Fig. 3. RP-HPLC analysis of the time-dependent oxidative folding of reduced decorsin. Fully reduced, HPLC-purified decorsin was allowed to fold at room temperature in 0.1 M Tris-HCl, pH 8.5, containing 0.2 M NaCl, 1 mM EDTA, 2 mM GSH, and 1.5 mM GSSG. The refolding mixture contained also protein disulfide isomerase (PDI) (see Materials and methods). Aliquots of the reaction mixture (10 μL) were analyzed at intervals using a Vydac C18 column (4.6 × 150 mm) eluted with a gradient of acetonitrile in 0.1% aqueous TFA from 5 to 21% in 4 min and from 21 to 46% in 20 min. The protein concentration in the effluent from the column was determined by absorption measurements at 226 nm. The arrow indicates the retention time of fully reduced decorsin (R).

of the reducing agent was converted to a species (N) earlier eluted from the C18 column. Nevertheless, the chromatographic data of Figure 3 show that even after 24 h reaction under optimized conditions the N species is not unique and accounts for ~70% of the protein material eluted from the column. The oxidative folding was then conducted on the crude, reduced peptide as obtained after the acid cleavage of the peptide-resin utilizing a procedure similar to that employed with the purified species (see above). It was estimated that the overall yield of the final oxidized species was ~10% (including peptide chain assembly, resin cleavage, and oxidative folding).

Analytical characterization

The purity and identity of the oxidized synthetic decorsin were established by utilizing a variety of analytical methods. The purified oxidized (N) and reduced (R) species of synthetic decorsin appear to be highly homogeneous by RP-HPLC analysis (Fig. 4A). The electrophoretic mobility on capillary zone electrophoresis (CZE) of the N-species is higher than that of the R-species (see Fig. 4B), as expected from the fact that the charge to radius ratio is smaller in the oxidized, folded species than in the reduced, unfolded one (Kleparnik & Bocek, 1991). The CZE-analysis of the reduced species shows that the main peak is preceded by small amounts of other components, which are assumed to arise from the partial oxidation of the sample of the reduced species. Analysis by ES-MS (not shown) provided clear-cut evidence for the identity and purity of the synthetic product, because the experimental molecular mass of the oxidized folded protein (4,377 ± 0.5 Da) is exactly that calculated from the amino acid composition of decorsin with three disulfide bridges (4,377.8 Da). Quantitative amino acid analysis of the synthetic product after acid hydrolysis was in agreement with theory (not shown).

The correctness of the amino acid sequence of synthetic decorsin was established by NH_2-terminal sequence analysis by the Edman degradation (15 cycles; data not shown) and by fingerprint-

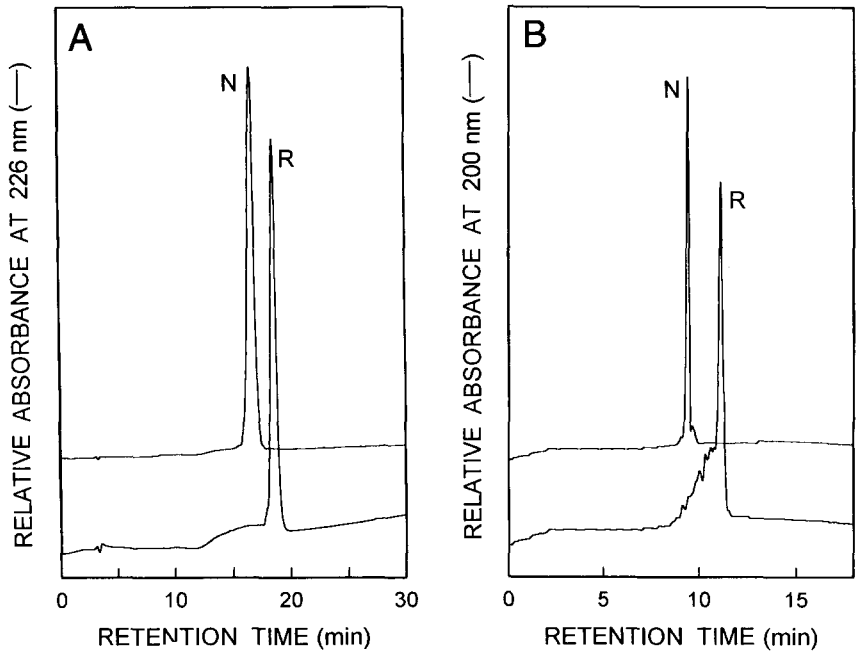

Fig. 4. Comparison between fully reduced (R) and folded/native (N) decorsin by RP-HPLC (**A**) and capillary zone electrophoresis (CZE) (**B**) (see Materials and methods for experimental details).

ing analysis. A fully reduced and S-carboxymethylated protein (CM-decorsin) was digested with trypsin and the resulting peptide mixture was separated by RP-HPLC (see Fig. S1, Electronic Appendix). The fragments thus isolated were identified by ES-MS (see Table S1, Electronic Appendix) and NH_2-terminal sequencing (data not shown). Fragments 4–14, 15–28, 15–39, 29–31, and 32–39 were thus identified (see Fig. S1 and Table S1, Electronic Appendix), in agreement with the location along the chain of decorsin of the basic residues (Lys and Arg) susceptible to tryptic hydrolysis (see Fig. 1). The NH_2-terminal peptide Ala-Pro-Arg was not isolated, most likely because this small hydrophilic peptide elutes with the solvent front from the RP-HPLC column (Fig. S1). Peptide fragments originating from the tryptic hydrolysis of the Lys29–Asp30 peptide bond were not found. This can be explained by the fact that acidic residues (Glu, Asp) on either side of the basic ones (Lys, Arg) reduce significantly the rate of tryptic hydrolysis (Walsh, 1970).

The peptide mapping technique was also applied to synthetic oxidized decorsin to establish the correct disulfide pairing along the polypeptide chain (see Fig. 1). The traditional strategy for determining disulfide topology in proteins involves proteolytic cleavage of the polypeptide chain to produce disulfide-bridged peptides, which can be isolated to homogeneity and analyzed. In the case of oxidized decorsin, it was necessary to isolate disulfide-linked peptides individually encompassing one of the three disulfide bridges of the protein (7–15, 17–27, 22–38) (see Fig. 1; Krezel et al., 1994). The highly crosslinked protein species and the absence of suitable sites of proteolytic cleavage along the chain made this strategy only partially successful. The actual data of these experiments are reported in the Electronic Appendix. Briefly, tryptic hydrolysis of oxidized decorsin was shown to occur only at the level of the Arg residues in positions 3, 28, and 32, and not at the level of the two Lys residues in positions 14 and 19. Three nicked/truncated decorsin species were thus isolated from the digest: 4–28/32–39, 4–31/32–39, and 4–28/29–39 (see Fig. S2 and Table S2, Electronic Appendix). These species all contain the three disulfide bridges of the intact protein (see Fig. 1).

To possibly produce fragment species with a single disulfide bridge, the tryptic fragment 4–28/32–39 was additionally fragmented with thermolysin, a protease of broad substrate specificity (Heinrikson, 1977) and thus expected to produce extensive protein fragmentation. From the thermolytic digest of fragment 4–28/32–39 two additional fragment species (5–15 and 16–28/32–39) were isolated as a result of a selective cleavage at the amino side of Leu16 (see Fig. 1). The isolation of the thermolytic fragment 5–15 provided evidence of the correct disulfide pairing between Cys5 and Cys15. On the other hand, fragment 16–28/32–39 contains two disulfide bridges deriving from the pairing by oxidation of the Cys residues in positions 17, 22, 27, and 38. These four –SH groups can form three disulfide topologies, including the native one 17–27 and 22–38. Thus, the results of the peptide mapping technique are in agreement with a correct disulfide pairing in the synthetic oxidized decorsin, but do not provide the complete analysis of the disulfide topology. However, it should be noted that all the proteolytic fragments isolated from purified, oxidized decorsin are in agreement with a correct pairing of the disulfides, whereas this was not the case when the peptide mapping procedure was applied to the mis-folded species III (see above). Moreover, spectroscopic data obtained by near-UV CD provided evidence that the chirality of the disulfide bridges of oxidized decorsin is that expected for the native species determined by NMR (Krezel et al., 1994) (see Discussion).

Spectroscopic characterization

The conformational analysis of synthetic decorsin was conducted by CD measurements in the far- and near-UV region (Fig. 5). The far-UV CD spectrum of synthetic refolded decorsin shows two negative bands centered at 195 and 237 nm and two broad positive bands at 204 and 218 nm, while CM-decorsin displays a single strong negative band at 200 nm, characteristic of polypeptides in a random conformation (Brahms & Brahms, 1980; Johnson, 1990). It has been shown by NMR spectroscopy that the solution structure of decorsin contains a substantial amount of β-like secondary struc-

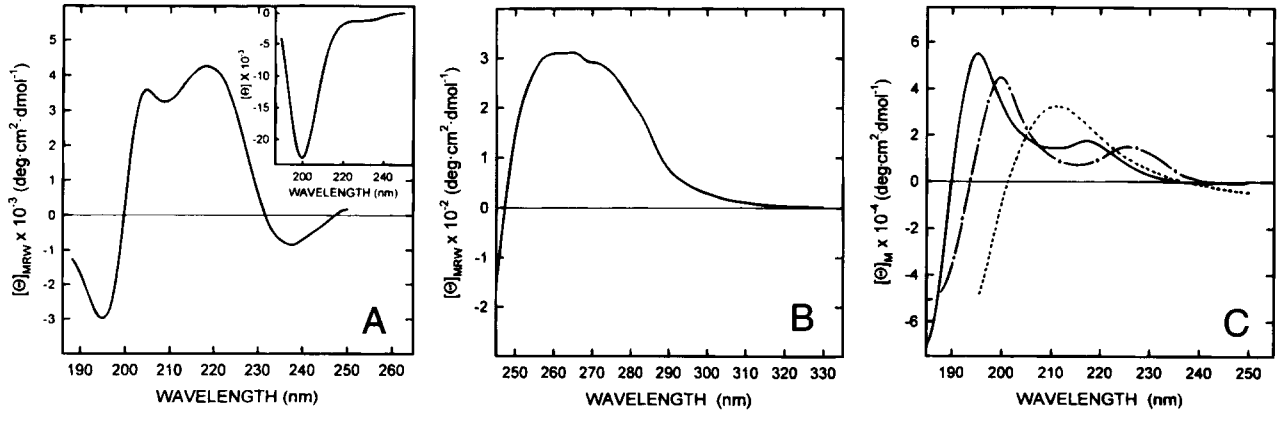

Fig. 5. Spectroscopic characterization of synthetic decorsin. **A:** Far-UV CD spectrum of refolded oxidized decorsin. Inset: Far-UV CD spectrum of CM-decorsin. **B:** Near-UV CD spectrum of refolded oxidized decorsin. **C:** Far-UV CD spectra of the model compounds Ac-Tyr-NH_2 (—), Ac-Phe-NH_2 (—·—), and cystine (····). The molar concentration of cystine was three times that of the other two model compounds because decorsin contains three disulfides (see Fig. 1). All CD spectra were taken at 25 °C in 10 mM sodium phosphate buffer, pH 7.0, using a 1- or 10-mm pathlength quartz cuvette in the far- and near-UV region, respectively. The ellipticity values were expressed as mean residue ellipticity, $[\theta]_{MRW}$, or molar ellipticity, $[\theta]_M$.

ture (44%) (Krezel et al., 1994) and that its overall fold is very similar to that of the NH_2-terminal domain of hirudin HV1 (residues 1–40) (Folkers et al., 1989; Haruyama & Wüthrich, 1989; Szyperski et al., 1992). Nevertheless, the far-UV CD spectrum of decorsin does not show a negative band at 215–220 nm and a positive one at 195–200 nm typical of a β-protein (Brahms & Brahms, 1980; Johnson, 1990) and is different from that reported for intact hirudin HV1 (Otto & Seckler, 1991) or for the NH_2-terminal domain 1-47 of hirudin HM2 (De Filippis et al., 1995). Because the CD signal due to the peptide chromophore in the far-UV region can be masked by the contributions of aromatic residues and disulfide bonds (Manning & Woody, 1989; Vuilleumier et al., 1993), we recorded the far-UV CD spectra of model compounds acetyl-tyrosinamide (Ac-Tyr-NH_2), acetyl-phenylalanyl-amide (Ac-Phe-NH_2) and cystine (Fig. 5C). The spectra of both Ac-Tyr-NH_2 and Ac-Phe-NH_2 show two positive bands at 220–230 nm and 195–200 nm, while the spectrum of cystine is characterized by a broad positive band centered at 210 nm and a strong negative one at wavelengths shorter than 200 nm. Therefore, it can be proposed that the unusual features of the far-UV CD spectrum of decorsin are mainly determined by the positive contributions of the aromatic moieties and of the three disulfide bonds of the protein, therefore overwhelming the weak negative CD signal at 215–220 nm of the peptide bonds embedded in a β-structure. The contributions of aromatic and disulfide bonds become negligible in the far-UV CD spectrum of CM-decorsin, because the strong CD signal of the peptide bond in a random conformation greatly dominates (Fig. 5A, inset).

The near-UV CD spectrum of refolded decorsin (Fig. 5B) shows a broad positive band in the 250–290 nm region, due to the absorption of the three disulfide bonds of decorsin (Strickland, 1974; Kahn, 1979). The positive CD signal in the 250–290 nm region, as well as the molar ellipticity value, provide evidence that the three disulfides of the refolded synthetic decorsin are in a right-handed conformation, as expected from the NMR solution structure of natural decorsin (Krezel et al., 1994) (see Discussion). The appearance of a weak Tyr fine structure at 275–280 nm, superimposed to the disulfide absorption, indicates that the single Tyr37 is located in a relatively rigid and asymmetric environment in the decorsin molecule (Strickland, 1974). The contribution of the single Phe29, expected as low-intensity bands in the 250–270 nm region (Strickland, 1974), is not detected, likely masked by the strong CD signal of the three disulfides.

Thermal stability

Figure 6 shows the thermal denaturation profile of synthetic decorsin as determined by measuring the decrease of the CD signal at 265 nm as a function of temperature between 15 and 90 °C. This decrease arises mainly from the changes in conformation of the disulfide bridges of the protein (Kahn, 1979; see also De Filippis et al., 1995). The heat-induced denaturation profile of decorsin is characterized by a broad conformational transition in the temperature range 60–90 °C, but even at 90 °C the CD signal does not reach a constant value, as typically seen in the post-transition region of a protein denaturation process. Differentiation of the melting curve shows a peak at ~74 °C (Fig. 6, inset), which we take as the melting temperature (T_m) of the synthetic decorsin. The thermal denaturation of decorsin is fully reversible, as judged from the recovery of the ellipticity value at 265 nm upon cooling.

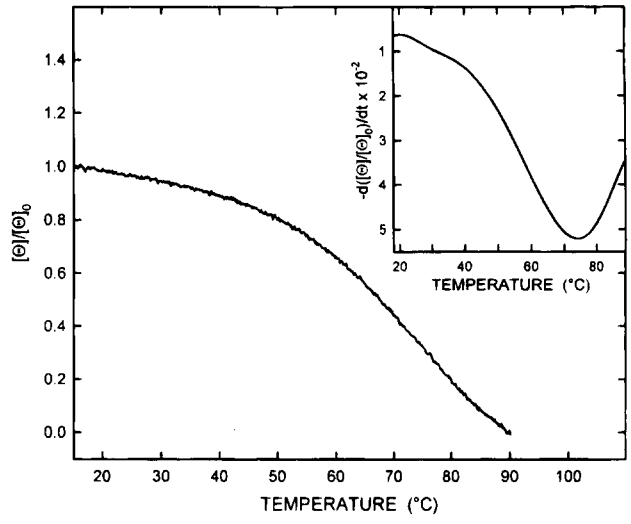

Fig. 6. Thermal unfolding of synthetic decorsin followed by monitoring the CD signal at 265 nm. Measurements were conducted in 10 mM sodium phosphate buffer, pH 7.0. Data are given as the $[\theta]/[\theta]_0$ ratio, where $[\theta]_0$ is the mean residue ellipticity value measured at 15 °C. Inset: Second derivative profile of the melting curve.

Biological activity

Synthetic decorsin inhibits the thrombin-activated fibrinogen-dependent aggregation of human platelets in a dose-dependent manner with an IC_{50} ~0.1 μM (Fig. 7). This figure for IC_{50} is quite similar to that previously reported for natural decorsin (com-

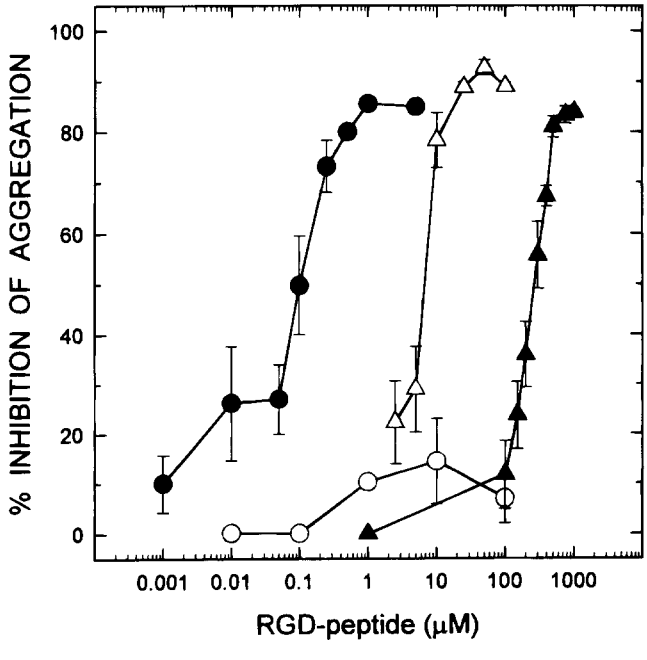

Fig. 7. Comparison of the effect of synthetic oxidized decorsin (●), CM-decorsin (○), and synthetic peptides Arg-Gly-Asp-Trp (Δ) and Arg-Gly-Asp-Ser (▲) on human platelet aggregation (see Materials and methods for experimental details).

plete inhibition with 1 μM decorsin and IC_{50} ~500 nM; Seymour et al., 1990). On the other hand, the fully unfolded CM-decorsin (see Fig. 5A, inset) shows a much lower inhibitory potency when assayed under the same experimental conditions as those employed for the folded, disulfide-crosslinked protein. For comparative purposes, platelet aggregation assays were conducted also utilizing two small RGD-peptides, which are much less potent inhibitors than decorsin. In particular, the synthetic decorsin was 2,000-fold more potent inhibitor than Arg-Gly-Asp-Ser (see Fig. 7). The figures for IC_{50} of the peptides herewith examined are in line with those previously determined by other authors utilizing RGD peptides (Gurrath et al., 1992; Scarborough et al., 1993). For example, the IC_{50} for Arg-Gly-Asp-Phe is reported to be 6.1 μM (Garski et al., 1989), that for Arg-Gly-Asp-Asp 203 μM (Garski et al., 1989), and that for Gly-Arg-Asp-Val 75 μM (Seymour et al., 1990).

Discussion

Recombinant DNA technology is nowadays the method of choice for the biological synthesis of proteins or large peptides, whereas the solid-phase methodology remains the most popular strategy for the synthesis of peptides (Atherton & Sheppard, 1989). However, the difficulty of obtaining homogeneous peptides by solid-phase synthesis is expected to grow rapidly as the size of the target peptide increases (Kent, 1988). Nevertheless, the results of this study show that the combined use of Fmoc-chemistry for the N_α-protection (Fields & Noble, 1990) and of uronium salts (HBTU) for the activation of the carboxyl group (Knorr et al., 1989) can provide a successful synthesis of the 39-residue polypeptide chain of decorsin. In addition, the synthesis of this protein containing three disulfide bonds was expected to be difficult, both for an efficient and reversible protection of –SH groups during the synthesis, as well as for the correct pairing of disulfide bonds during the oxidative re-folding process of the reduced, synthetic polypeptide, because the six cysteine residues can form 15 different disulfide-crosslinked species (Creighton, 1984a; Benham & Jafri, 1993). On the other hand, the oxidative re-folding process of a reduced synthetic polypeptide to the native disulfide-crosslinked species leads to the formation of a globular entity with most of its hydrophobic groups buried in the protein interior and thus showing a lower affinity than the open chain polypeptide or mi-sfolded species for the hydrophobic matrix of the C18 column in RP-HPLC (Kent, 1988; Woo et al., 1989). Therefore, as a consequence of oxidative folding, the folded molecule is eluted earlier by the organic solvent (acetonitrile) than other polypeptide species, thereby significantly facilitating the purification of the correctly folded and biologically active end product (see Results).

Oxidative refolding

A number of experiments with varied experimental conditions were conducted to optimize the oxidative refolding of the synthetic, reduced decorsin to the final, native protein with the correct pairing of the disulfide bonds (Creighton, 1984a). The best results were obtained by including in the refolding buffer a GSH/GSSG redox mixture (Saxena & Wetlaufer, 1970). The beneficial role of PDI (Creighton et al., 1980; Freedman, 1984) in achieving higher yields of correctly folded species has been also demonstrated. However, the yields of correctly folded decorsin from the purified reduced species were at most ~70% after 24 h reaction and, moreover, other fully oxidised, non-native species were present in the refolding mixture (see Results). These findings contrast with the highly efficient and clean reoxidation of hirudin HV1 and its NH_2-terminal fragment 1–43 (Chatrenet & Chang, 1993; Chang, 1994, 1995; see also Thannhauser et al., 1997) or fragment 1–47 (De Filippis et al., 1995) or 1–41 (unpublished) of hirudin HM2. Similar fast, quantitative, and correct refolding has been described for other small disulfide-crosslinked proteins, such as charybdotoxin (37-residue chain, three disulfides) (Vita et al., 1993, 1995; Pierret et al., 1995).

The relatively difficult refolding of reduced decorsin was not anticipated, considering the identity of the overall fold and, in particular, of the number and spacing of Cys residues in both decorsin and hirudin's fragment 1–40(41) (Krezel et al., 1994). A possible explanation can be as follows. Decorsin has six Pro residues along its polypeptide chain (see Fig. 1), each of which likely can exist in a *cis* or *trans* peptide bond configuration in the unfolded reduced decorsin (Brandts et al., 1975). Because all peptide bonds in the native folded decorsin are *trans* (Krezel et al., 1994), it might be expected that *cis* ↔ *trans* transitions at the level of Xaa-Pro peptide bonds are required for a correct folding. Because this isomerization is relatively slow (Brandts et al., 1975), it may be well that rater stable, incorrectly folded molecules with non-native disulfide bonds are formed and trapped during the folding process, as indeed experimentally verified (see Results). Of interest, hirudin HV1 (Chatrenet & Chang, 1993; Chang, 1994, 1995) or hirudin HM2 (Scacheri et al., 1993; Vindigni et al., 1994) contain three Pro residues in their polypeptide chain, but all located in the C-terminal disordered tail and fully outside the disulfide-crosslinked NH_2-terminal core. In particular, fragment 1–41 of hirudin HM2 (Vindigni et al., 1994, and unpubl. obs.) or charybdotoxin (Vita et al., 1993, 1995; Pierret et al., 1995) do not contain any Pro and oxidatively re-fold very efficiently. Our proposal of a participation of Pro isomerization in the oxidative refolding process of reduced decorsin remains to be experimentally verified by demonstrating a catalytic effect of proline isomerase (Lang & Schmid, 1988; Fischer & Schmid, 1990; Fischer, 1994). Moreover, in future studies it will be possible to study the refolding process of synthetic decorsin analogs with Pro-Xaa exchange(s).

Conformational analysis

The conformational characterization of synthetic refolded decorsin has been conducted by CD spectroscopy. Although the far-UV CD spectrum is strongly influenced by aromatic and disulfide chromophores and thus is not diagnostic of the actual secondary structure of decorsin (see Results, Fig. 5A and C), the near-UV CD spectrum is dominated by the CD signal of the protein disulfides and provides useful informations on the overall fold of the synthetic protein. The wavelength at which the maximum absorption (λ_{max}) of the disulfide chromophore occurs is strongly conformation dependent, being shifted from 255 to 330 nm for X_3 angle (C_β–S–S–C_β) values of 90° and 30°, respectively (Neubert & Carmack, 1974). Moreover, the sign of the disulfide CD band can be related to the chirality of the disulfide bridges, because a positive or negative dichroic signal is observed if they are in a right-handed or a left-handed chirality, respectively (see Kahn, 1979, for a review). On this basis, the positive ellipticity in the near-UV region of re-folded synthetic decorsin provides evidence that the three disulfides are in a right-handed conformation, in full agreement with the disulfide chirality observed in the NMR solution structure

of natural decorsin (X_3 angle values comprised between +79° and +95°; PDB code 1DEC; Krezel et al., 1994). It is of interest to recall the characteristics of the near-UV CD spectrum of charybdotoxin (37-residue chain, three disulfides). The near-UV CD spectrum of charybdotoxin shows a broad negative disulfide absorption in the 250–300 nm region, with a figure for $[\theta]$ of about −14,000 deg·cm^2·dmol^{-1} at 265 nm (Vita et al., 1993; Pierret et al., 1995), in agreement with the left-handed chirality of the three disulfides (X_3 angle values ranging from −84° to −91°) observed in the NMR structure of charybdotoxin (PDB code 2CRD; Bontems et al., 1992). Moreover, it is of interest to observe that the overall shape of the near-UV CD spectrum and molar ellipticity value at 265 nm of synthetic refolded decorsin ($[\theta]_M$ = 12,120 deg·cm^2·dmol^{-1}) are closely similar to those of hirudin HM2 NH_2-terminal domain 1–47 ($[\theta]_M$ = 12,440 deg·cm^2·dmol^{-1}) (De Filippis et al., 1995). Indeed, the analysis of either X-ray and NMR structure of the full-length hirudin (Folkers et al., 1989; Haruyama & Wüthrich, 1989; Rydel et al., 1991) and of its NH_2-terminal domain 1–51 (Szyperski et al., 1992) reveals that the three disulfide bonds are in a right-handed conformation, with X_3 dihedral angles ranging from +80° and +97°. Therefore, the close similarity of the near-UV CD spectrum of synthetic decorsin with that of hirudin NH_2-terminal fragment 1–47 (De Filippis et al., 1995) can be taken as an evidence that both molecules, in spite of their poor sequence similarity, share the same disulfide bond topology and thus a common fold, in agreement with previous NMR data (Krezel et al., 1994).

Protein stability

It has been postulated that, in the absence of the stabilizing role of disulfide crosslinks or ligand binding, a stable folded polypeptide (domain) should be constituted by at least ~50 amino acid residues (Privalov & Gill, 1988). For rather small polypeptides, therefore, such as the 39-residue chain of decorsin, it is mandatory to stabilize their folded conformation via disulfide crosslinking. Indeed, the non-crosslinked chain of CM-decorsin is fully unfolded, easily digested by proteases, and essentially lacks biological activity (see Results). In analogy to several small, disulfide-crosslinked proteins (toxins, protease inhibitors) that show high thermostability, decorsin is characterized by a quite unusual thermal stability (T_m ~ 74 °C), as determined by monitoring its thermal unfolding by CD spectroscopy (see Fig. 6). This T_m is quite higher than that of hirudin HV1 (T_m = 65 °C; Otto & Seckler, 1991) and HM2 (T_m = 61.5 °C) or hirudin HM2 fragments 1–47 (T_m = 62.5 °C; De Filippis et al., 1995) and 1–41 (T_m = 52 °C) (Vindigni et al., 1994, and unpubl. obs.). Thus, decorsin and the core domain 1–41 of hirudin, despite having the same size, strict similarity of three-dimensional structure (Krezel et al., 1994) and both crosslinked by three disulfides with the same spacing of cysteine residues, show a ΔT_m of ~22 °C.

It is tempting to speculate on the molecular reasons for the enhanced stability of decorsin in respect to hirudin and its fragments 1–47 and 1–41. In studying the molecular mechanism determining the enhanced stability of proteins and enzymes isolated from thermophilic bacteria in respect to their mesophilic counterparts (Jaenicke, 1991; Fontana, 1991a, 1991b), Suzuki and coworkers observed that, for homologous proteins, there is a strong correlation between the increase in the number of Pro residues and the enhancement of protein thermostability (Watanabe et al., 1994). The protein stabilizing role of Pro appears to be due to the steric constraints of the pyrrolidine ring, which promote a conformationally more rigid main chain (Matthews et al., 1987). It has been estimated that the folded form of a protein is stabilized by 0.5–1.0 kcal/mol when a Pro residue is introduced into a structurally non-disruptive position (e.g., not at a level of a helix; Nicholson et al., 1992; Gray et al., 1996). On this basis, it is relevant to observe that in the 39-residue chain of decorsin there are up to six Pro-residues (Seymour et al., 1990) (see Fig. 1), with a relative Pro abundance of 15.3%, much higher than the 5.1% frequency of Pro residues in natural proteins (McCaldon & Argos, 1988). Moreover, all Pro residues in decorsin are in *trans* conformation with ϕ and ψ angles of −64° ± 10 and 148° ± 20, respectively, and thus in an energetically favorable geometry (Ramachandran & Sasisekharan, 1968; Brandts et al., 1975; Creighton, 1984b). Moreover, all Pro residues are mostly located in loops or β-turns joining adjacent secondary structure elements (see Fig. 1; Krezel et al., 1994). At variance, hirudin HV1 (Dodt et al., 1985) or hirudin HM2 (Scacheri et al., 1993) have three Pro residues in their sequence (4.5% relative abundance), hirudin HM2 core fragment 1–47 has two Pro residues, whereas fragment 1–41 lacks Pro (Scacheri et al., 1993; Vindigni et al., 1994). These considerations prompt us to propose that the major determinant of the enhanced thermal stability of decorsin in respect to hirudin and its fragments resides in the stabilizing role of Pro residues. In addition, the cyclic nature of Pro severely impairs rotation about its N-C_α bond and restricts the number of possible local conformations of the protein backbone, thus confering to the protein more rigidity, which correlates with protein heat stability (Vihinen, 1987; Fontana, 1991a, 1991b; Varley & Pain, 1991). Indeed, in contrast to the mobile finger-like structure (residues 32–35) of hirudin (Folkers et al., 1989; Haruyama & Wüthrich, 1989; Szyperski et al., 1992), the recognition site (RGD-loop) of decorsin is well defined and quite rigid in the NMR structure of the protein, because Pro30 and Pro36 flank the RGD sequence (see Fig. 1; Krezel et al., 1994).

Conclusions

The successful chemical synthesis of decorsin here described opens the way to prepare analogs of this important protein to elucidate its structure–function relationships and to investigate the functional and pharmacological aspects of RGD-containing integrin antagonists. The possibility to produce decorsin analogs containing non-coded amino acids or non-peptidic moieties is a specific advantage of the chemical procedure here described. The protein motif of small disulfide-rich polypeptides has been already exploited as a versatile scaffold for engineering stable mini-proteins with desired and novel structural and functional properties (Knapp et al., 1992; Szardenings et al., 1995). For example, the α/β motif of scorpion toxins, crosslinked by three disulfide bridges, has been utilized for producing new protein entities with metal binding properties (Vita et al., 1995) or receptor binding activity (Drakopoulou et al., 1996; Zinn-Justin et al., 1996). The all β-fold of decorsin and hirudin (Krezel et al., 1994), characterized by a conserved spacing of the six cysteine residues and a high sequence permissiveness, can be also used for protein engineering purposes. The chemical approach described here and previously (De Filippis et al., 1995) appears to compete with biological expression systems in both rapidity and efficiency and to provide a practical route for the production of small decorsin- or hirudin-like proteins with novel activities.

A significant aspect of this study is that decorsin shows a slower and incomplete oxidative refolding process and an enhanced ther-

mostability in respect to those of NH_2-terminal core domain of hirudin, despite their similarity in three-dimensional structure and the identity of pairing of the six cysteine residues (Krezel et al., 1994). These differences have been ascribed to the presence of the six Pro residues in decorsin, while these residues are absent in the hirudin domain. This proposal is in line with the fact that the *cis-trans* Pro isomerization (Brandts et al., 1975) is known to be a late and relatively slow folding event hampering fast protein folding (Fischer & Schmid, 1990) and that an enhanced content of Pro residues has been previously proposed as a mechanism of protein stabilization in thermophilic enzymes and verified by protein engineering experiments (Matthews et al., 1987; Watanabe et al., 1994).

Materials and methods

Materials

Fmoc-L-amino acids were purchased from Novabiochem (Laufelfingen, Switzerland). The solvents and reagents used for peptide synthesis and those for peptide/protein sequencing were obtained from Applied Biosystems (Foster City, California), while those for amino acid analysis were from Millipore-Waters (Milford, Massachusetts). Trypsin (TPCK-treated) and protein disulfide isomerase (PDI) were purchased from Boehringer (Mannheim, Germany), while thermolysin from *Bacillus thermoproteolyticus* was from Sigma (St. Louis, Missouri). The RGD-containing peptides Arg-Gly-Asp-Trp and Arg-Gly-Asp-Ser were synthesized in our laboratory by solid-phase methods and their purity and identity established by HPLC and amino acid analysis. Other chemicals were of analytical reagent grade and were obtained from Fluka (Buchs, Switzerland).

Solid-phase peptide synthesis

Decorsin was synthesized by the solid-phase method/Fmoc strategy (Fields & Noble, 1990) on 4-hydroxymethyl-phenoxymethyl-copolystyrene-1% divinylbenzene-resin (0.96 mmol/g) using an automated peptide synthesizer (Applied Biosystems, model 431A). Synthesis was performed on a 0.05 mmol scale by adapting the procedure suggested by the manufacturer. Fmoc-protected amino acids were used with the following side-chain protection: *tert*-butyl ether (tBu) for Tyr; *tert*-butyl ester (OtBu) for Glu and Asp; trityl (Trt) for Cys, Asn, and Gln; *tert*-butyloxycarbonyl (Boc) for the ϵ-amino group of Lys and 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl (Pmc) for the guanido group of Arg. Deprotection of the Fmoc group was obtained by a 10-min treatment with 20% piperidine in N-methylpyrrolidone (NMP). The chain elongation was performed using 10-fold excess (0.5 mmol) of Fmoc-amino acid, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) (1:1:1) in the presence of a 20-fold excess of diisopropylethylamine (DIEA). The deprotection of the Fmoc NH_2-protecting group was monitored by absorbance measurements at 301 nm after every coupling step to determine the extent of cleavage. The capping with acetic anidride was routinely carried out after introduction of hydrophobic (Phe, Tyr, Leu) and Arg residues, as suggested by Milton et al. (1990). After completion of the last cycle, the resin was washed with NMP, dichloromethane (DCM)/methanol (1:1, v/v), DCM and dried in vacuo. The synthetic peptide was cleaved from the resin and deprotected by treating the peptide-resin with a 95:5 (v/v) mixture of TFA:1,2-ethanedithiol (EDT) for 2 h at 0 °C. The resin was filtered and cold diethyl ether was added to the solution to precipitate the crude, reduced peptide. The pellet was recovered by centrifugation at 3000 rpm and than resuspended in diethyl ether and centrifuged for three times. The crude, reduced peptide was stored at −20 °C under nitrogen.

HPLC purification and oxidative folding

Analytical and micro-preparative HPLC of the crude, reduced peptide was performed on a Waters (Milford, California) model 510 instrument equipped with a tunable absorbance detector (model 484), using a Vydac C18 column (150 × 4.6 mm) purchased from The Separations Group (Hesperia, California). The peptide material was eluted with a linear gradient of 0.1% TFA in water versus 0.1% TFA/acetonitrile from 5 to 21% in 4 min and from 21 to 46% in 20 min. Peptide containing fractions were pooled and lyophilized.

Purified, synthetic decorsin was dissolved (~0.2 mg/mL) in the oxidative refolding buffer (0.1 M Tris-HCl, pH 8.5, containing 1 mM EDTA, 0.2 M NaCl, 2 mM GSH and 1.5 mM GSSG) (Saxena & Wetlaufer, 1970; Creighton, 1984a) and allowed to refold at room temperature (Chatrenet & Chang, 1993; Chang, 1994, 1995). Some experiments were conducted also in the presence of protein disulfide isomerase (PDI) (Creighton et al., 1980; Freedman, 1984) at an enzyme/protein ratio of 1:500 (by weight). The refolding was monitored by removing at intervals aliquots (10 μL) from the refolding mixture and loading them on a RP-HPLC column eluted with a gradient of acetonitrile in 0.1% TFA. The rate of oxidation of cysteine thiol groups was determined using the Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) (Habeeb, 1972).

Analytical characterization

For quantitative amino acid analysis, the peptide/protein samples were hydrolyzed in gas phase with 6 N HCl containing 0.1% phenol for 1 h at 150 °C utilizing the Pico-Tag workstation (Waters). The mixture of free amino acids was reacted with phenylisothiocyanate and the resulting phenylthiocarbamoyl (PTC)-derivatives analyzed by RP-HPLC (Heinrikson & Meredith, 1984) utilizing a Pico-Tag C18 column (3.9 × 150 mm; Waters) and a Perkin-Elmer liquid chromatograph (model 410-Bio) connected to an automatic injection system (Perkin-Elmer, model ISS-101).

Peptide/protein NH_2-terminal sequence analysis was performed by Edman degradation using an Applied Biosystems peptide/protein sequencer model 477A connected on line to a PTH analyzer model 120A, following the standard procedure recommended by the manufacturer.

Capillary zone electrophoresis (CZE) was performed on a Bio-Rad instrument (Richmond, California) model HPE-100 using a fused silica capillary (35 cm × 50 μm). Samples were dissolved in 0.1 M sodium phosphate buffer (pH 2.5) and loaded at an electric field of 10 kV for 7 s. The separation was conducted at room temperature and the effluent was monitored by absorbance measurements at 200 nm.

Electrospray mass spectrometry (ES-MS) was carried out on a PE-SCIEX (Thornhill, Ontario, Canada) single quadrupole (API-1) mass spectrometer equipped with a nebulization-assisted electrospray ionization source. The ion-spray was 5 kV, the orifice plate voltage of 90 V, and the mass range from 700 to 1600 m/z was

scanned with a step size of 0.1. The spectra were acquired and deconvoluted by the MacSpec software given by the manufacturer.

Reduction and S-carboxymethylation of cysteine residues

Purified, oxidized decorsin (500 μg) was reduced under nitrogen at 37 °C for 2 h in 0.5 M Tris-HCl buffer (pH 8.0) containing 2 mM EDTA, 6 M Gdn·HCl and dithiothreitol (DTT; 1.1 mg). The S-carboxymethylation reaction of cysteine residues was carried out by adding iodoacetic acid (1.5 mg) to the reaction mixture. After 30 min reaction, removal of excess of DTT and reaction byproducts was carried out by RP-HPLC on a Vydac C18 column using the same acetonitrile/water gradient employed for the purification of the synthetic crude polypeptide (see above).

Peptide mapping

Fingerprinting analysis of reduced S-carboxymethylated decorsin (CM-decorsin) or of oxidatively folded decorsin was conducted using trypsin as proteolytic enzyme. Proteolysis was conducted in 0.1 M NH_4HCO_3 buffer, pH 7.8, at 37 °C. Proteolysis of a tryptic fragment of oxidized decorsin (see Results) with thermolysin was conducted in 50 mM Tris-HCl, pH 7.0, containing 5 mM $CaCl_2$, at 37 °C. The digestion mixtures were analyzed by RP-HPLC on a Vydac C18 column (150 × 4.6 mm) eluted with a gradient of 0.1% TFA in acetonitrile.

Spectroscopic characterization

Protein concentration was determined by absorption measurements at 280 nm on a double-beam Lambda-2 spectrophotometer from Perkin-Elmer. The solutions were clarified by filtration through a 0.45 μm filter (Whatman). Extinction coefficients at 280 nm for reduced and oxidized forms of decorsin were calculated according to Gill & von Hippel (1989) and taken as 0.29 and 0.46 $mg^{-1} \cdot cm^{-1}$, respectively.

Circular dichroism (CD) spectra were recorded with a Jasco J-710 (Tokyo, Japan) spectropolarimeter equipped with a thermostatted cell holder and a Neslab (Newington, New Hampshire) model RTE-110 water circulating bath. The instrument was calibrated with *d*-(+)10-camphorsulfonic acid (Toumadje et al., 1992). Far- and near-UV CD spectra were recorded at 25 °C at a protein concentration of 30 and 160 μM and using 0.1- and 1-cm pathlength quartz cells in the far- and near-UV region, respectively. The mean residue ellipticity $[\theta]$ $(deg \cdot cm^2 \cdot dmol^{-1})$ was calculated from the formula $[\theta]_{MRW} = (\theta_{obs}/10)(MRW/lc)$, where θ_{obs} is the observed ellipticity at a given wavelength, MRW is the mean residue weight of decorsin taken as 112.5 Da, l is the cuvette pathlength in cm, and c is the protein concentration in g/mL. Alternatively, the CD data were expressed as molar ellipticity, $[\theta]_M$.

The thermal unfolding of synthetic decorsin was monitored by recording the decrease of the CD signal at 265 nm as a function of the sample temperature (heating rate 50 °C/h). Both CD signal and temperature data were recorded simultaneously by a computer program provided by Jasco. The melting temperature (T_m) was determined from the derivative curve of the CD signal at 265 nm versus temperature. The reversibility of the thermal unfolding process was determined by measuring the recovery of the CD signal upon cooling to 20 °C.

Platelet aggregation assay

The biological activity of decorsin was tested by assaying the inhibition of platelet aggregation. Fresh blood was drawn from a healthy volunteer and immediately mixed with one sixth volume of the anticoagulant solution (85 mM sodium citrate, 70 mM citric acid, 110 mM dextrose). Platelet-rich plasma was prepared according to Radomski & Moncada (1983) and was incubated with lysine acetylsalicylate (1 mM, 20 min) to prevent generation of prostaglandin endoperoxides and thromboxane. The cells were pelleted by centrifugation at 900 rpm for 10 min at room temperature and resuspended in 20 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 10 mM glucose. The platelet count was adjusted to 2×10^8 cells/mL and the suspension was kept at room temperature for 2–3 h. Prior to measurements, the external calcium concentration was adjusted to 0.5 mM and the cells were equilibrated at 37 °C for 3 min. Platelet aggregation induced by thrombin (0.05 U/mL) was evaluated with an Elvi Logos aggregometer at 1,000 rpm stirring velocity in the presence of known concentrations of decorsin or RGD-peptides.

Supplementary material in electronic appendix

Two figures showing RP-HPLC separations of proteolytic digests of synthetic decorsin, as well as two tables reporting the analyses by mass spectrometry and NH_2-terminal sequencing of proteolytic fragments of decorsin, can be found in the Electronic Appendix.

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