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Biochemistry

Chemical synthesis of echistatin, a potent inhibitor of platelet aggregation from *Echis carinatus*: Synthesis and biological activity of selected analogs

(solid-phase synthesis/fibrinogen receptor antagonists)

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ABSTRACT **Echistatin, a polypeptide from the venom of the saw-scaled viper, *Echis carinatus*, containing 49 amino acids and 4 cystine bridges was synthesized by solid-phase methodology in 4% yield. In the final step, air oxidation of the octahydroderivative was found to be optimal at pH 8. The synthetic product was shown to be physically and biologically indistinguishable from native material. It inhibits fibrinogen-dependent platelet aggregation stimulated by ADP with IC_{50} = 3.3×10^{-8} M and also prevents aggregation initiated by thrombin, epinephrine, collagen, or platelet-activating factor. Reduction of purified synthetic echistatin to octahydroechistatin with dithiothreitol followed by air oxidation regenerated homogeneous echistatin in quantitative yield. This highly specific refolding strongly suggests that the linear sequence of octahydroechistatin contains all of the information that is required for the proper folding of the peptide. The sequence Arg^{24}-Gly-Asp of echistatin occurs also in adhesive glycoproteins that bind to the platelet fibrinogen receptor—a heterodimeric complex composed of glycoproteins IIb and IIIa. In an effort to evaluate the role of this putative binding site we have synthesized analogs of echistatin with substitution of Arg-24. Replacement with ornithine-24 (Orn-24) resulted in an analog having a platelet aggregation inhibitory activity with IC_{50} = 1.05×10^{-7} M. Substitution with Ala-24 gave IC_{50} = 6.1×10^{-7} M. The inhibitory activity of the corresponding short sequence analogs Arg-Gly-Asp-Phe ($IC_{50} = 6 \times 10^{-6}$ M), Orn-Gly-Asp-Phe ($IC_{50} = 1.3 \times 10^{-4}$ M), and Ala-Gly-Asp-Phe ($IC_{50} = 5.0 \times 10^{-4}$ M) was also determined. These results suggest that arginine plays a more important role in the binding of the tetrapeptide than in that of echistatin.**

Echistatin is a single-chain polypeptide that is isolated from the venom of the saw-scaled viper *Echis carinatus*. The complete amino acid sequence has been reported (1). The primary structure (Fig. 1) consists of 49 amino acid residues including 8 cysteines. Location of the four disulfide bridges in echistatin has not yet been determined. Echistatin contains the tripeptide unit Arg-Gly-Asp at residues 24–26, a sequence that appears in a variety of adhesive protein ligands and contributes to their interaction with specific membrane receptors (2). For example, the interaction of fibrinogen with its platelet receptor glycoprotein (GP)IIb/IIIa is likely mediated, at least in part, through one or both of the Arg-Gly-Asp sequences in fibrinogen. Trigramin, a platelet aggregation inhibitor, isolated from the venom of *Trimereserus gramineus* (3), also contains the Arg-Gly-Asp sequence within the isolated chymotryptic fragment Ala-Arg-Gly-Asp-Asp-Leu-Asp-Asp-Tyr-Cys. This fragment displays a 90% sequence homology with residues 23–32 of echistatin. Overall, echistatin shows a 55% identity with residues 28–72 of trigramin (4) and therefore it is likely that echistatin and trigramin are biologically related. Echistatin, however, is 4 times as potent an inhibitor of ADP-stimulated platelet aggregation and 3 times as potent in inhibiting ^{125}I-labeled fibrinogen binding to activated platelets, as is trigramin (1, 3). It would appear that, like trigramin, echistatin binds to the GPIIb/IIIa complex.

Inhibition of fibrinogen binding to GPIIb/IIIa complex has been shown in animal models to be an effective antithrombotic strategy (5–9). Murine monoclonal antibodies directed against GPIIb/IIIa block fibrinogen binding and inhibit platelet aggregation (5). In canine models, these antibodies promote thrombolysis (6) and prevent reocclusion (7) as well as preventing cyclic flow reductions in stenosed coronary arteries (8). Antibodies to GPIIb/IIIa will also prevent platelet deposition in extracorporeal arteriovenous shunts in a baboon model (9). Platelet aggregation appears to play a critical role in these models. The monoclonal antibodies are characterized by a prolonged effect on bleeding times and aggregation (9). It is unclear whether such a long duration of action represents a therapeutic advantage or whether shorter acting agents would be preferable. Since echistatin has a similar potency to monoclonal antibodies, it would be important to evaluate it in similar animal models of thrombosis.

Initial *in vivo* evaluation of the potential of echistatin necessitates the availability of larger quantities of material even for initial studies in animals than can be provided from natural sources. A number of peptides have been chemically synthesized in the size range of echistatin. Nonetheless, the degree of success is dependent on component amino acids. Particular difficulty is encountered with peptides having multiple cysteines. Synthetic challenges in the removal of their protecting groups and the achievement of proper folding remain. The goal of our studies was to develop a synthetic strategy for the total chemical synthesis of echistatin of sufficient generality to prepare and characterize quantities for *in vivo* evaluation and to evaluate structure–activity relationships through the synthesis of point substitution analogs. Of particular concern was the optimization of folding conditions in the formation of the four disulfide bridges.

MATERIALS AND METHODS

Materials. Butyloxycarbonyl (Boc)-*O*-benzylthreonine phenylacetamidomethyl (Pam) resin, Boc-protected amino acids, and all other reagents required for synthesis on the Applied Biosystems 430 A (ABI) peptide synthesizer were

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Abbreviations: GP, glycoprotein; Boc, butyloxycarbonyl; Orn, ornithine.

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Glu-Cys-Glu-Ser-Gly-Pro-Cys-Cys-Arg-Asn[10]-
Cys-Lys-Phe-Leu-Lys-Glu-Gly-Thr-Ile-Cys[20]-
Lys-Arg-Ala-Arg-Gly-Asp-Asp-Met-Asp-Asp[30]-
Tyr-Cys-Asn-Gly-Lys-Thr-Cys-Asp-Cys-Pro[40]-
Arg-Asn-Pro-His-Lys-Gly-Pro-Ala-Thr[49]

FIG. 1. The primary structure of echistatin.

obtained from the manufacturer. Side-chain protected aspartic acid, glutamic acid, and histidine were supplied by Bachem. The solvents dimethylformamide and methylene chloride (CH_2Cl_2) were obtained from Burdick and Jackson. Dithiothreitol was purchased from Bethesda Research Laboratories. Dithioerythritol was obtained from Chemical Dynamics (South Plainfield, NJ). *p*-Cresol and *p*-thiocresol were obtained from Aldrich.

Echistatin Synthesis. Starting with 0.50 mM (0.69 g) of Boc-*O*-benzylthreonine Pam resin (substitution at 0.72 mM of threonine per g of resin) the synthesis was carried out in a stepwise manner using the ABI automated peptide synthesizer (10). The amino acids were introduced using the manufacturer's prepacked cartridges (2 mM each). Side-chain protection was arginine (Tos), aspartic acid (OcHx), cysteine (Meb), glutamic acid (OcHx), histidine (Bom), lysine [Z(Cl)], serine (Bzl), threonine (Bzl), tyrosine[Z(Br)] [Tos, tosyl; cHx, cyclohexyl; Meb, 4-methylbenzyl; Z(Cl), 2-chlorobenzyloxycarbonyl; Bom, benzyloxymethyl; Bzl, benzyl; Z(Br), 2-bromobenzyloxycarbonyl]. Double coupling with symmetric anhydrides (preformed in CH_2Cl_2 followed by solvent exchange with dimethylformamide) were used for all Boc-protected amino acids except for arginine (Tos), asparagine, and histidine (Bom), where hydroxybenzotriazole esters in dimethylformamide were used in a double coupling protocol. To protect against undesired acid-catalyzed oxidations of cysteine and methionine (11) during trifluoroacetic acid deblocking, 0.1% (wt/vol) dithioerythritol was added as a scavenger. After the coupling of N-terminal glutamic acid, the Boc group was removed using trifluoroacetic acid and the peptide resin was dried. The final weight of N-terminal deblocked peptide resin was 4.15 g.

HF Cleavage and Oxidation. The assembled peptide resin (2.0 g) was suspended in a mixture of 3 ml of 1:1 (vol/vol) *p*-thiocresol/*p*-cresol in an HF apparatus (Peninsula Laboratories; Type 1B). The system was evacuated with a mechanical vacuum pump and HF was condensed (30 ml) using liquid nitrogen cooling. After stirring at 0–5°C for 1.5 hr, the reaction mixture was evaporated *in vacuo* using a liquid nitrogen trap (20–30 min). The residue was triturated with ether, filtered, and washed three times with additional ether. The filtered residue was immediately transferred to 4 liters of a stirred solution of dilute acetic acid (0.4% in H_2O). After stirring for several minutes, the pH of the mixture was adjusted to 8.0 with ammonium hydroxide. After filtration to remove resin, the crude oxidation product was maintained without stirring at 5°C (18 hr) and subsequently at ambient temperature (19–20°C) for 3 days. Analytical HPLC was used to monitor the progress of the oxidation. A qualitative Ellman test (12) was used to monitor the disappearance of free sulfhydryl groups before proceeding with purification. This test was performed on a 1-ml sample, which was lyophilized to remove residual *p*-thiocresol.

Purification of Crude Oxidized Echistatin. The crude oxidized solution (4 liters) was acidified by addition of acetic acid (10 ml) and pumped directly onto a C_{18} silica (5 × 30 cm, 15 μm, 300 A) cartridge (Waters). The product was purified using preparative HPLC (Separations Technology, Wakefield, RI). A step gradient (100-ml increments) was generated from 1 liter each of successively increasing concentrations of mobile phase (solvent A, 0.1% trifluoroacetic acid/H_2O; solvent B, acetonitrile/0.1% trifluoroacetic acid). A flow rate of 70 ml/min was used to elute the product. Homogeneous (>95%) fractions as determined by reverse-phase HPLC (Vydac C_{18}, 218TP5415) were pooled and lyophilized to give 72 mg of product. The semipure product was contaminated with a less polar component as a shoulder to the product peak. The product was further purified by repassage on HPLC in the same manner as described above to yield echistatin (54 mg). Based on 0.25 mM starting resin, this weight represents a 4% overall yield. Homogeneity was demonstrated by analytical HPLC on C_{18} (Fig. 2), hydrophobic interaction chromatography using a propyl aspartamide support (The Nest Group, Southboro, MA) in 0.1 M K_3PO_4 (pH 3.0) and anion-exchange chromatography (Vydac 201TP104) in 0.05 M Na_3PO_4. Coinjection of synthetic product with native material, in the 0.1% trifluoroacetic acid system, gave a single peak (Fig. 2). Product was further characterized by amino acid analysis after hydrolysis with 6 M HCl (Table 1) and by automated Edman degradation (ABI 470A protein sequencer). A maximum of 1.9% preview was observed. Sequence analysis is a sensitive method for detection of deletions because it is cumulative at each step (13). The high yield of phenylthiohydantoin-derivatized amino acids from the first step also demonstrated that cyclization of the N-terminal glutamic acid to pyroglutamic acid had not occurred. FAB mass spectrometry in the presence of thioglycerol gave a major mass peak (M+H) of 5423 for the octahydro form and a secondary mass peak (M+H) of 5415 of the unreduced form. Molecular weight homogeneity was established by SDS/polyacrylamide gels (Fig. 3).

Reduction and Refolding of Synthetic Echistatin. Synthetic echistatin (0.50 mg) was dissolved in 1 ml of 0.07 M (pH 8.0) ammonium acetate (10 mM dithiothreitol) and the course of reduction was followed by analytical HPLC. After 1 hr, the starting material was converted quantitatively to a single reduced product (Fig. 2). Dialysis (24 hr) of the reduced product using a 12-mm diameter, M_r 1000 cutoff, cellulose tubing (Spectrum Medical Industries) against 4 liters of a 0.07 M ammonium acetate buffer (pH 8.0) produced only echistatin (Fig. 2). To demonstrate that the echistatin was in its fully reduced form prior to reoxidation, the dithiothreitol reduction of synthetic echistatin was repeated as described but in the presence of 6 M guanidine hydrochloride. Analytical HPLC confirmed that the reduced products had identical retention times. Isolation of reduced echistatin, by semipreparative HPLC, followed by quantitative Ellman analysis (12) showed the product to be in the octahydro form.

Synthesis of Other Peptides. Preparation of [Orn[24]]- and [Ala[24]]echistatin (Orn, ornithine) followed the protocol described for echistatin. Homogeneity was established by analytical HPLC (system described in legend of Fig. 2) with retention times of 7.66 and 7.96 min, respectively. Amino acid composition was consistent with the structures (Table 1). Overall yields of both were comparable to that of echistatin. Synthesis of Arg-Gly-Asp-Phe, Orn-Gly-Asp-Phe, Ala-Gly-Asp-Phe, Arg-Gly-Asp-Asp, Orn-Gly-Asp-Asp, and Ala-Gly-Asp-Asp followed standard methodology (15). Structural identity and purity (>97%) of the tetrapeptides was confirmed by amino acid composition and proton NMR. Protection of ornithine was as the benzyloxycarbonyl derivative. To avoid cross-contamination of analogs, purification was achieved on dedicated reverse-phase C_{18} silica columns.

Platelet Aggregation Assay. Platelet aggregation was measured at 37°C in a Chronolog aggregometer. The reaction mixture contained washed human platelets (2×10^8 per ml), fibrinogen (100 μg/ml), Ca^{2+} (1 mM), and the platelet aggregation inhibitor fraction to be tested. The aggregation was initiated by adding 10 μM ADP 1 min after the other components had been added. The reaction was allowed to pro-

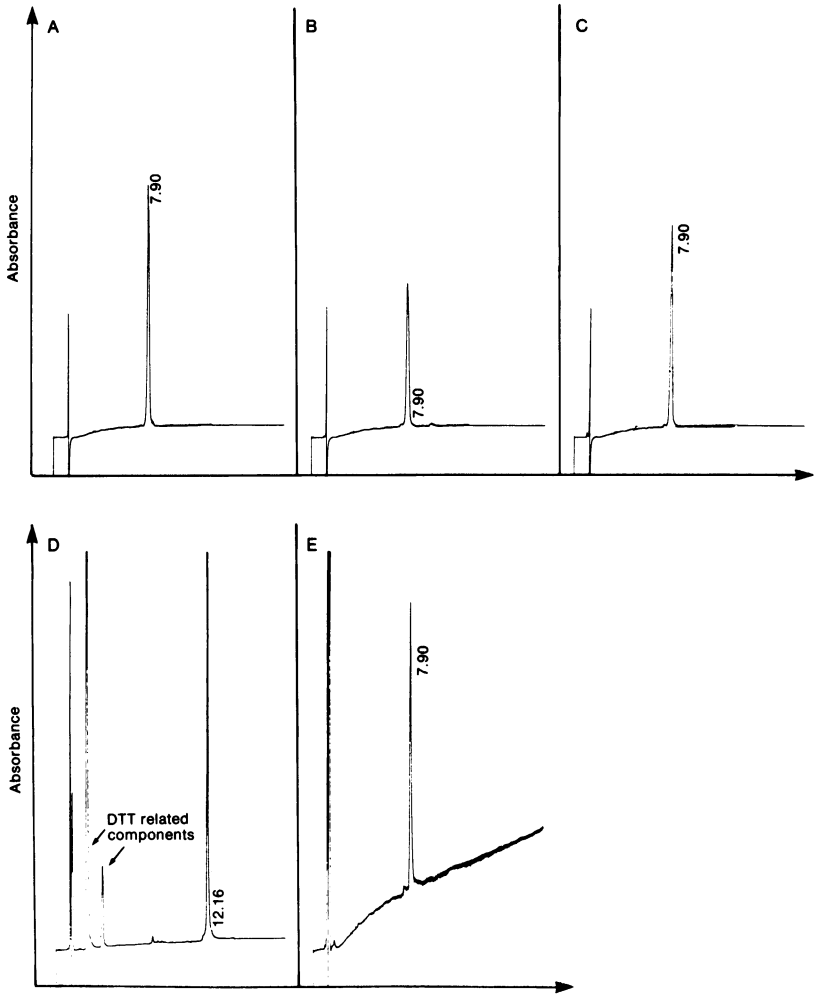

FIG. 2. Analytical HPLC. (*A*) Native echistatin. (*B*) Synthetic echistatin. (*C*) Coinjection of native + synthetic echistatin. (*D*) Dithiothreitol reduced echistatin. (*E*) Oxidized echistatin following dithiothreitol reduction. Column, Vydac C_{18} (4.6 × 150 mm). Solvent A, 0.1% trifluoroacetic acid/H_2O; solvent B, acetonitrile/0.1% trifluoroacetic acid. Gradient, 5–95% solvent B in 45 min. Flow rate, 1.5 ml/min. Absorbance (214 nm) plotted vs. time (min).

ceed for at least 2 min. The extent of inhibition of aggregation was expressed as the percentage of the rate of aggregation observed in the absence of inhibitor.

NMR Spectroscopy. Proton NMR spectra were recorded with a Varian VXR-400S spectrometer at 400 MHz and a sample temperature of 25°C. ^2H_2O (Merck Isotopes) was used as the solvent in all cases. Chemical shifts are referenced to internal sodium 3-trimethylsilylpropionate-2,2,3,3-d_4. The HO^2H resonance from residual water in the samples was suppressed by presaturation. p^2H values refer to direct readings with a pH meter and are uncorrected for the deuterium isotope effect.

Table 1. Amino acid analysis of synthetic echistatin and analogs

	Echistatin	[Orn^{24}]Echistatin	[Ala^{24}]Echistatin
Lys	5.00 (5)	4.88 (5)	5.09 (5)
His	1.00 (1)	1.03 (1)	1.00 (1)
Arg	3.87 (4)	2.83 (3)	2.94 (3)
Asp	8.13 (8)	8.14 (8)	8.37 (8)
Thr*	2.99 (3)	2.96 (3)	3.14 (3)
Ser*	1.04 (1)	1.00 (1)	1.03 (1)
Glu	3.07 (3)	3.05 (3)	3.00 (3)
Pro	4.08 (4)	4.38 (4)	4.29 (4)
Gly	5.03 (5)	5.00 (5)	5.15 (5)
Ala	2.10 (2)	2.04 (2)	3.07 (3)
Cys†	7.67 (8)	8.03 (8)	7.38 (8)
Met‡	0.84 (1)	0.85 (1)	0.84 (1)
Leu	0.98 (1)	0.97 (1)	0.95 (1)
Phe	0.95 (1)	0.94 (1)	0.95 (1)
Tyr	1.00 (1)	1.01 (1)	1.00 (1)
Ile§	0.99 (1)	0.92 (1)	0.99 (1)
Orn		0.98 (1)	

Amino acid analysis after hydrolysis with 6 M HCl at 100°C for 70 hr (theoretical number in parentheses). Orn, ornithine.
*Corrected for decomposition during hydrolysis.
†Determined as cysteic acid following performic acid oxidation.
‡Uncorrected.
§Ile + allo-Ile.

RESULTS AND DISCUSSION

The primary objective of our studies was the chemical synthesis of the 49-amino acid-containing echistatin. Assembly of the primary structure was achieved by the Merrifield solid-phase method (10, 16). Appropriate selection of side-

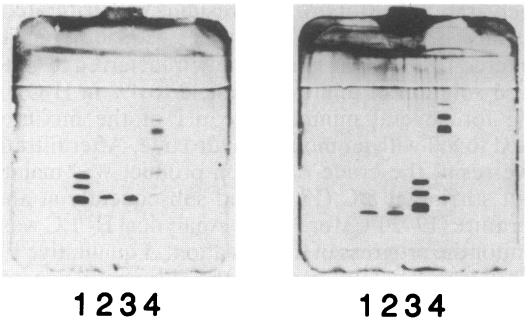

FIG. 3. SDS/PAGE of native and synthetic echistatin on 20% polyacrylamide PhastGels with SDS buffer strips. (*Left*) Nonreduced: BDH polypeptide standards (16,949, 14,404, 8159, 6214 kDa) (lane 1), synthetic echistatin (lane 2), native echistatin (lane 3), Pharmacia low molecular mass standards (94,000, 67,000, 43,000, 30,000, 20,100, 14,400 kDa) (lane 4). (*Right*) Reduced with 15 mM dithiothreitol: synthetic echistatin (lane 1), native echistatin (lane 2), BDH polypeptide standards (lane 3), Pharmacia low molecular mass standards (lane 4). Gels were silver stained (14).

chain protection was made in an effort to minimize the formation of by-products during synthesis and HF deprotection. A double-coupling protocol was used for peptide resin assembly and coupling efficiency was monitored by ninhydrin analysis of residual free amine (17). Coupling yields were greater than 98.5% in all but three cycles. The additional couplings of Arg-9 and Arg-22 were performed four times and Ile-19 three times. After these repeated couplings, the ninhydrin analysis was improved to a coupling completion level >97%. The final 49-peptide resin, which was isolated in a 93% overall yield, was cleaved from the resin using the high HF procedure (18) in the presence of *p*-cresol and *p*-thiocresol as scavengers. Use of anisole as a scavenger or the application of the low–high HF procedure (18) proved to be less satisfactory because of the generation of complex and difficult to purify mixtures. After HF treatment, the crude reduced product was subjected to air oxidation in ammonium acetate buffer. Probe oxidations at pH 6.5, 7.0, 7.5, and 8.0 over a period of 4 days indicated the optimum pH to be 8.0. Completeness of reaction was established by Ellman analysis (12) and analytical HPLC. The crude product was purified by preparative HPLC by loading the oxidation reaction mixture directly onto a C_{18} silica reverse-phase column. This technique allowed efficient concentration of 4 liters of solution containing crude echistatin onto the column support followed by a gradient elution for the isolation of the purified product. After a single pass, the product peak was >95% pure. A second gradient elution of the product produced material that was homogeneous by analytical HPLC in the same solvent system in a 4% overall yield. Application of this concerted purification technique enabled us to isolate echistatin with a high level of purity.

The product was characterized for structure and purity. Amino acid analysis after 70 hr of acid hydrolysis and performic acid oxidation showed ratios within ±5% of expected values (Table 1). Sequence analysis of echistatin carried out for 47 cycles gave the expected results. Coinjection of synthetic and native material on analytical reverse-phase HPLC produced a single symmetrical peak (Fig. 2). Proton NMR of synthetic echistatin (p^2H = 3.15 for 1.2 mg of the trifluoroacetic acid salt dissolved in 0.6 ml of ^2H_2O) showed a single methionine methyl group at 2.07 ppm, well resolved phenylalanine, tyrosine, and histidine aromatic resonances, and 8 slowly exchanging amide proton resonances (still present in spectrum after 8 days at 25°C in ^2H_2O). Warming the ^2H_2O solution to 65°C for 45–60 min resulted in complete exchange of the amide protons by solvent deuterons. Within the limits of detection (10%) proton NMR in ^2H_2O established that the methionine had not oxidized to methionine oxide, which is expected to resonate at ≈2.75 ppm. The NMR also indicated that alkylation of side-chain functionalities had not occurred. Amino acid composition, following 70 hr of acid hydrolysis, of CNBr-treated echistatin was consistent with the expected conversion of methionine to homoserine. This observation further established the absence of methionine sulfoxide. Analysis of the product by SDS/polyacrylamide electrophoresis under nonreducing and reducing conditions showed a single peptide band that was indistinguishable from native echistatin (Fig. 3). Further evidence of correct molecular weight was seen in a mass of 5423 (M+H) of the octahydro form in the presence of thioglycerol.

In examining the analytical chromatograms during the oxidation of crude echistatin, we were impressed by the apparent efficiency of the folding. In an effort to determine the yield of the oxidation, we reduced pure synthetic echistatin to octahydroechistatin with dithiothreitol and regenerated homogeneous echistatin in nearly a quantitative yield. The high level of conversion was established by HPLC analysis of the product before and after reduction and reoxidation. Isolation by HPLC of reduced echistatin followed by quantitative Ellman analysis (12) confirmed the product to be in the fully reduced octahydro form. This highly specific refolding shows that the linear sequence of octahydroechistatin contains all of the information that is required for proper folding of the peptide.

The sequence Arg-Gly-Asp is also present in the platelet aggregation inhibitor trigramin (3, 4). In addition, adhesive proteins including fibronectin, von Willebrand factor, and human fibrinogen contain the Arg-Gly-Asp sequence. In human fibrinogen, it occurs twice within the Aα chain at positions 95–97 and 572–574 (19). This tripeptide sequence

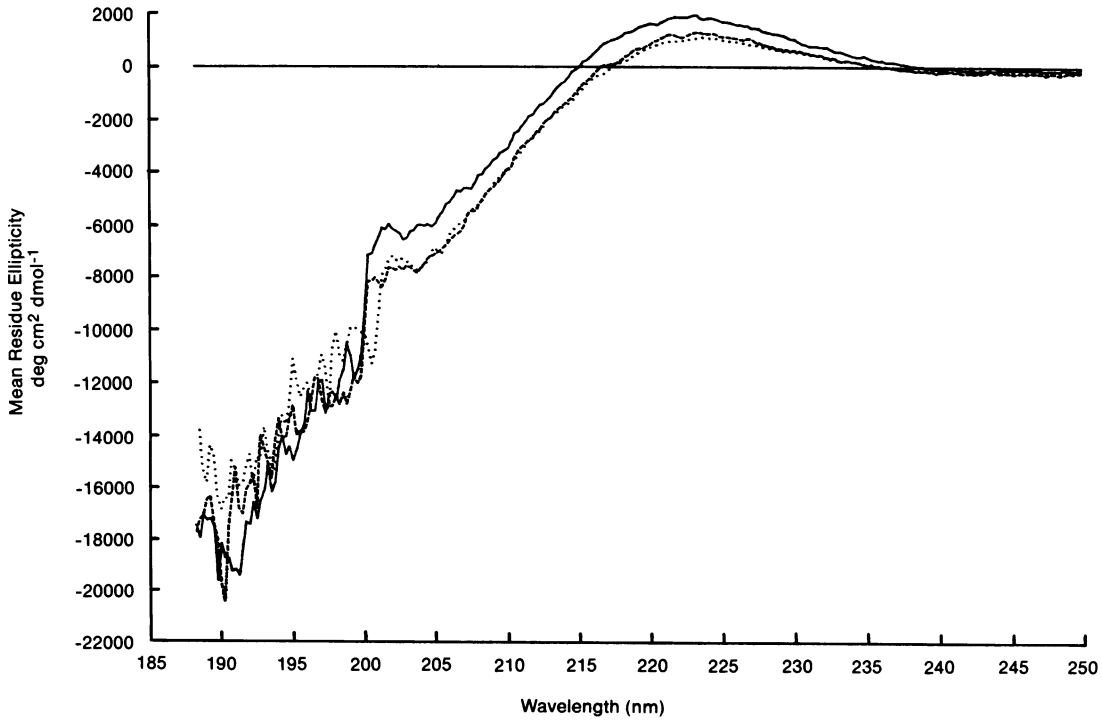

FIG. 4. Circular dichroism spectra of echistatin (——), [Ala^{24}] (····), and [Orn^{24}] (----) echistatin.

appears to be involved in the interaction of these adhesive proteins with the platelet fibrinogen receptor (20–22). In an attempt to evaluate the role of arginine in this putative binding site we have prepared [Orn^{24}]echistatin and [Ala^{24}]echistatin by the methods described. As in the case of echistatin, the arrangement of the disulfide bridges in these analogs is currently unknown. Circular dichroism spectra for echistatin, [Orn^{24}]echistatin, and [Ala^{24}]echistatin (Fig. 4) are consistent with a disulfide pairing that is identical for all three structures. These spectra are also consistent with a similar backbone conformation in all three peptides. Echistatin inhibits the ADP-induced fibrinogen-dependent aggregation of human gel-filtered platelets in a dose-dependent manner with an IC_{50} of 3.3×10^{-8} M (Fig. 5). Substitution of Arg-24 by ornithine gave an IC_{50} of 1.0×10^{-7} M, while replacement with the uncharged and shortened residue alanine gave an IC_{50} of 6.1×10^{-7} M (Fig. 5). These results confirm a role for arginine in the Arg-Gly-Asp sequence of echistatin since replacement of Arg-24 ($pK'_3 = 13.2$) with ornithine ($pK'_3 = 10.76$) reduced the level of inhibitory activity by a factor of 3, while substitution with alanine reduced the activity by a factor of ≈20 (Table 2). Whether the loss of activity comes from deletion of the charge or removal of the hydrophobic polymethylene chain is not yet clear. The guanidino group *per se* is not a key contributing factor. The significant remaining activity of the alanine analog suggests the presence of one or more additional binding sites in echistatin. The sequence Arg-Gly-Asp-Phe from Aα fibrinogen 95–98 is one of the most potent tetrapeptide inhibitors of aggregation of washed or gel filtered human platelets reported to date with an IC_{50} of 4–10 μM (23). Inhibitory activity of analogs in the tetrapeptide series wherein arginine was replaced with ornithine or alanine as in the corresponding echistatin analogs is presented in Table 2. A comparison with the Arg-Gly-Asp-Asp sequence (echistatin 24–27) is also presented (Table 2). These results show that the guanidino group of arginine plays a more important role in the binding of the tetrapeptide than in that of echistatin.

In preliminary *in vivo* studies, synthetic echistatin has been shown to be a very effective antithrombotic agent. Further experiments will document whether potent fibrinogen receptor antagonists such as echistatin are to be of therapeutic value.

In conclusion, this work demonstrates the total chemical synthesis of echistatin, a 49-amino acid peptide sequence having four disulfide bridges. The product possesses chemical and biological properties indistinguishable from those of natural echistatin. The described synthetic scheme was shown to be applicable to the syntheses of point substitution analogs of echistatin. *In vitro* testing of these analogs has provided additional support to the importance of the Arg-Gly-Asp sequence in adhesive interactions at the platelet surface.

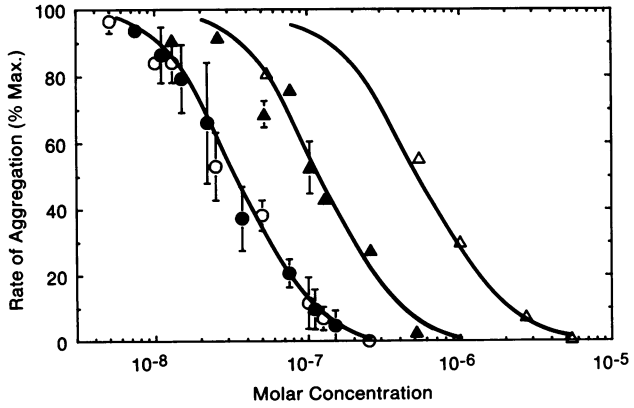

FIG. 5. Human gel-filtered platelets (2×10^8 cells per ml) were incubated at 37°C with 0.1 mg of human fibrinogen per ml, 1 mM $CaCl_2$, and synthetic echistatin (○), native echistatin (●), [Orn^{24}]echistatin (▲), or [Ala^{24}]echistatin (△). Aggregation was initiated by the addition of 10 μM ADP and initial rates were measured and reported as the percent of control aggregation in the absence of any echistatin. Data are the mean ± SEM of determinations made in platelets from two to four individual donors performed in duplicate.

Table 2. Effect of echistatin and analogs on inhibition of human platelet aggregation

Peptide	IC_{50}, μM (95% confidence limits)
Native echistatin	0.032 (0.026–0.039)
Synthetic echistatin	0.033 (0.026–0.041)
[Orn^{24}]Echistatin	0.105 (0.089–0.129)
[Ala^{24}]Echistatin	0.606 (0.282–0.919)
Arg-Gly-Asp-Phe	6.1 (4.6–7.6)
Orn-Gly-Asp-Phe	134 (80–205)
Ala-Gly-Asp-Phe	500
Arg-Gly-Asp-Asp	203
Orn-Gly-Asp-Asp	2000
Ala-Gly-Asp-Asp	2000

IC_{50} was determined by using platelets from two to four donors. Determinations were performed in duplicate for each donor.

The authors wish to dedicate this manuscript to the memory of Dr. Max Tishler, our long time friend and mentor. We would like to acknowledge Dr. Paul S. Anderson for his support and encouragement, Dr. Mark Polokoff and Mr. Gerard Bensen for supplies of native echistatin, Mr. Carl F. Homnick for hydrophobic interaction chromatography and anion-exchange chromatography, Mr. John Rodkey for sequence analyses, Dr. H. Ramjit for mass spectroscopy, Mrs. Lori Wassel for amino acid analyses, Mr. Charles Chang for platelet aggregation studies, and Mrs. Vera W. Finley for typing the manuscript.

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