The Three-Dimensional Structure of the RGD (Arg-Gly-Asp) Adhesion Sequence of Fibronectin; NMR Studies of the Peptides RGDS and GRGDSP

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The cell adhesion property of fibronectin and a number of proteins with similar properties resides in the RGD-sequence. The NMR spectrum of the active peptide GRGDSP at pH 3.5 shows that the peptide has the same stereostructure as that reported at pH 7.0. NMR measurements of the smallest active peptide RGDS at pH 3.5 and pH 5, including temperature gradients up to 70 °C, reveal similarities with GRGDSP. The NH-protons of aspartic acid and serine are only slowly exchanged indicating their participation in hydrogen bonds, which implies an exceptionally rigid stereostructure.

The multifunctional cell matrix protein fibronectin has been found to contain a 11.5 K domain comprising the cell adhesion function of the glycoprotein.[1] This function was later found to reside in the short Arg-Gly-Asp (RGD) sequence,[2] which has been demonstrated to occur in a number of proteins with suspected or actual adhesive properties. The receptor proteins have been given the name integrins,[3] and speculation has followed regarding the non-cross-reactivity between the different integrin–RGD-protein pairs. One idea is that the RGD sequence is a necessary but insufficient criterion for the reaction between the integrin and the RGD-protein, and that other sequences in the protein modify the reaction.[4] Another idea is that the RGD sequence adopts different three-dimensional structures in the various proteins, based on the neighbouring amino acids.[4] For the clarification of the nature of the interaction between the RGD-sequence and the integrins, the stereostructure of the RGD-group needs to be determined. Reed *et al.*[5,6] have reported the occurrence of nested β-bends in the peptides GRGDSP and GKGESP between the amino acids G and D(E) and between R(K) and S. This is a conclusion based on the complete assignment of all NMR signals at pH of the peptides, temperature dependence studies of the NH-proton signals, and ROESY experiments to confirm the closeness of certain protons.

In order to obtain further information on the nature of the folding of the RGD sequence we have synthesized a number of peptides related to fibronectin and studied their NMR spectra. In this paper we present ^{1}H and ^{13}C NMR spectra of the active peptides RGDS and GRGDSP at pH 3.5 (Fig. 1), and variable temperature studies of the NH signals of RGDS at pH 5.

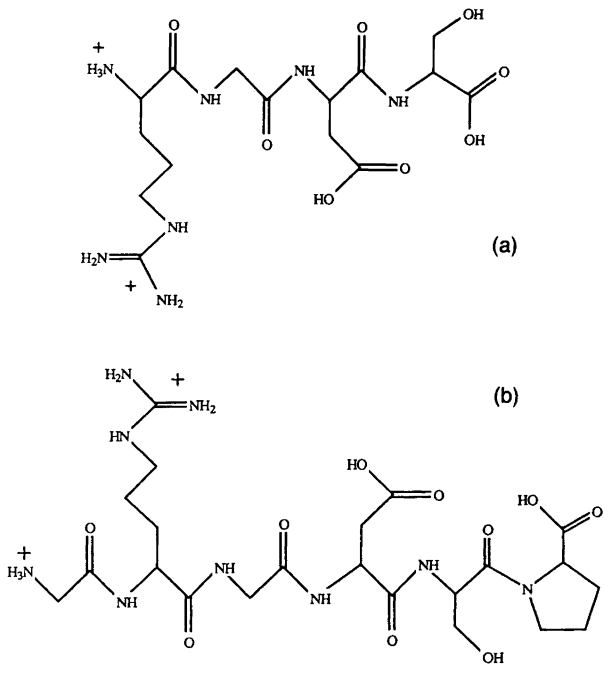

Fig. 1 (a) Tetrapeptide RGDS at pH 3.5; (b) Hexapeptide GRGDSP at pH 3.5.

Materials and methods

Throughout this paper the one letter code for amino acids is used.

The peptides were synthesized manually using Merrifield's solid phase technique with t-butyloxycarbonyl (Boc)-protected amino acids.[7] The first Boc-amino acid was covalently linked to a Biorad® chloromethylated poly-

styrene resin (1.25 mequiv. g^{-1} substitution) according to the KF method[8] in DMF. Boc-serine and Boc-aspartic acid were further protected as *O*-benzyl deerivatives, and Boc-arginine was N^G-tosylated. Coupling of the Boc-amino acids to the *N*-terminal of the growing peptide was performed as symmetric anhydrides using diisopropyl carbodiimide (DIPCDI) as an activator with CH_2Cl_2 as the coupling solvent. Boc-glycine and Boc-arginine were coupled as active esters in the presence of 1-hydroxybenzotriazole (HOBT) with CH_2Cl_2/DMF 10:1 as the coupling solvent. The coupling reaction was monitored for completion with a ninhydrin test.[9] Boc-Arg-Tos was coupled three times for completion.

The complete peptides were deprotected and cleaved from the resin with Low-High HF[10] and purified on a Sephadex® G15-column in 0.1 M ammonium bicarbonate solution. Further purification was achieved by reversed-phase liquid chromatography. We used a Pharmacia FPLC Gradient Programmer equipped with a Waters 10 μ C18-column in a Z-module and a water–acetonitrile–0.05 % TFA gradient. Detection was UV at 214 nm.

The purity was checked by FAB–MS (fast atom bombardment mass spectrometry). This was carried out on a JEOL double-focusing mass spectrometer with an FAB ion source, and a JMA DA6000 data system. An aliquot of the sample was dissolved in 0.1 % TFA (Aq) and evaporated on the FAB-target, then 0.7 μl 70 % aqueous formic acid and a glycerol matrix were added, and the sample was bombarded with 10 keV Xenon atoms. Spectra of positive ions were recorded in the range *m/z* 40–1200 with a resolution of 3000.

Samples for NMR spectroscopy were prepared at 30–60 mM peptide concentration, with pH 3.5(0.5) in either 99.9 % ^2H_2O or 90 % H_2O/10 % ^2H_2O. (For ^2H_2O solutions, the pH refers to the pH-meter reading without correction for isotope effect.) The NMR measurements were recorded on a JEOL GSX 270 and a JEOL GSX 400 spectrometer. Assignments were made using two-dimensional COSY,[11] two-dimensional double quantum filter COSY,[12] C–H correlated spectroscopy[13] and phase-sensitive COSY[14,15] at 37 °C. Chemical shifts are given in parts per million (ppm) relative to trimethylsilylpropionic acid (TSP =0.00) for ^1H spectra and dioxane (δ_C=67.4) for ^{13}C. Prior to the NMR measurements in ^2H_2O the samples were lyophilized three times in ^2H_2O.

Results

Synthesis and FAB–MS. Synthesis and purification of RGDS and GRGDSP, was monitored by FAB–MS (*m/z* 490 and 588 respectively). Although glycine and arginine were coupled in the presence of HOBT, double incorporation of arginine in RGDS was observed in the FAB–MS as an additional minor peak at *m/z* 590. In GRGDSP double incorporation of arginine and glycine gave rise to small additional peaks at *m/z* 743 (doubly incorporated arginine peptide) and *m/z* 644 (doubly incorporated glycine peptide).

In order to locate NMR peaks from persistent impurities due to the double incorporation of arginine, we synthesized a pentapeptide RRGDS, and a heptapeptide GRRGDSP. A small amount of RGDS was purified by HPLC of the Boc derivative which was more than 98 % pure by NMR spectroscopy.

Table 1. ^1H NMR shifts[a] of non-exchangeable protons in (a) the hexapeptide GRGDSP and (b) the tetrapeptide RGDS.

Amino acid	α-H	β-H	γ-H	δ-H
(a) GRGDSP				
Gly	3.89	–	–	–
Arg	4.35(7.8)	β1 1.88(–) β2 1.76(–) (–)	1.67(–)	3.22(8.7)
Gly	α1 3.97 α2 3.92 (αα 12.8)	–	–	–
Asp	4.71(7.6, 5.6)	β1 2.83(5.6) β2 2.75(7.6) (ββ 16.8)	–	–
Ser	4.77(6.6, 5.3)	β1 3.91(5.3) β2 3.81(6.6) (ββ 10.5)	–	–
Pro	4.30(8, 5)	β1 2.25(8) β2 1.93(5)	2.01(–)	3.72(–)
(b) RGDS				
Arg	4.10(6.5)	β1 2.01(βγ 5) β2 1.95(βγ 5)	1.71(–)	3.24(6.7)
Gly	α1 4.10 α2 4.00 (αα 16.6)	–	–	–
Asp	4.75(7.9, 5.1)	β1 2.89(5.1) β2 2.78(7.9) (ββ 16.6)	–	–
Ser	4.34(5.2, 4.1)	β1 3.90(5.2) β2 3.87(4.1) (ββ 17.2)	–	–

[a]The values in parentheses are spin–spin coupling constants in Hz. (–) = not detectable.

NMR spectroscopy. Assignments of ^1H NMR peaks are collected in Table 1 (non-exchangeable protons), and Table 2 (exchangeable protons).

One can easily ascertain that the shifts of the non-exchangeable protons in GRGDSP at pH 3.5 differ insignificantly from those obtained at pH 7.[5] The values for the non-exchangeable protons in RGDS, show a high degree of similarity to those of GRGDSP, except those located near the new terminals. Noteworthy is the great separation of the values for the β-protons in serine and aspartic acid,

Table 2. NMR shifts of exchangeable NH-protons.[a]

Peptide	Gly	Arg[b]	Arg[b]	Gly	Asp	Ser	Pro
RGDS	–	–	X	8.75 (5.5)	8.47 (7.5)	7.96 (7.4)	–
RRGDS	–	X	8.80 (5–6)	8.64 (5–6)	8.26 (7.2)	7.96 (7.3)	–
GRGDSP	X	–	8.62 (6)	8.56 (5–6)	8.20 (7.5)	8.14 (7.0)	–
GRRGDSP	X	8.59 (6.1)	8.52 (6.2)	8.43 (5–6)	8.27 (7.3)	8.14 (7.0)	–

[a]The values in parentheses are spin–spin coupling constants in Hz. X marks the *N*-terminal amines where shift values could not be detected. [b]The shift value for the guanidine protons are for η-NH resp. ε-NH; RGDS 7.19 resp. 6.65, RRGDS 7.23 resp. 6.66 for both arginines, GRGDSP 7.15 resp. 6.65, GRRGDSP 7.16 resp. 6.63 for both arginines.

indicating a high degree of ordered structure in those regions. This can be seen in both peptides.

The shifts and coupling constants of the exchangeable protons (Table 2) in GRGDSP also coincide well with the results of Reed *et al.*[5] However, the low pH value enables us to observe the arginine amide proton, which is clearly separated from the glycine amide signal. From GRGDSP to RGDS a downfield shift of about 0.2–0.3 ppm is observed for the amide signals of glycine and aspartic acid.

The penta- and hepta-peptide amide proton values are included in Table 2 in order to enable the exclusion of small impurity signals due to the double incorporation of arginine. Examining the amide protons at pH 3.5 over a temperature range of 25–70 °C at five degree intervals [Figs. 2(a) and 2(b)], reveals that the amide proton of glycine in RGDS, and of both glycine and arginine in GRGDSP are exchanged. The aspartic acid and serine signals of both peptides remain intense, which indicates participation of these protons in hydrogen bonds. However, in a similar experiment at pH 5 [Fig. 2(c)] the amide proton of aspartic acid in RGDS is exchanged when the temperature reaches 70 °C. The ^{13}C NMR values in Table 3 show the expected

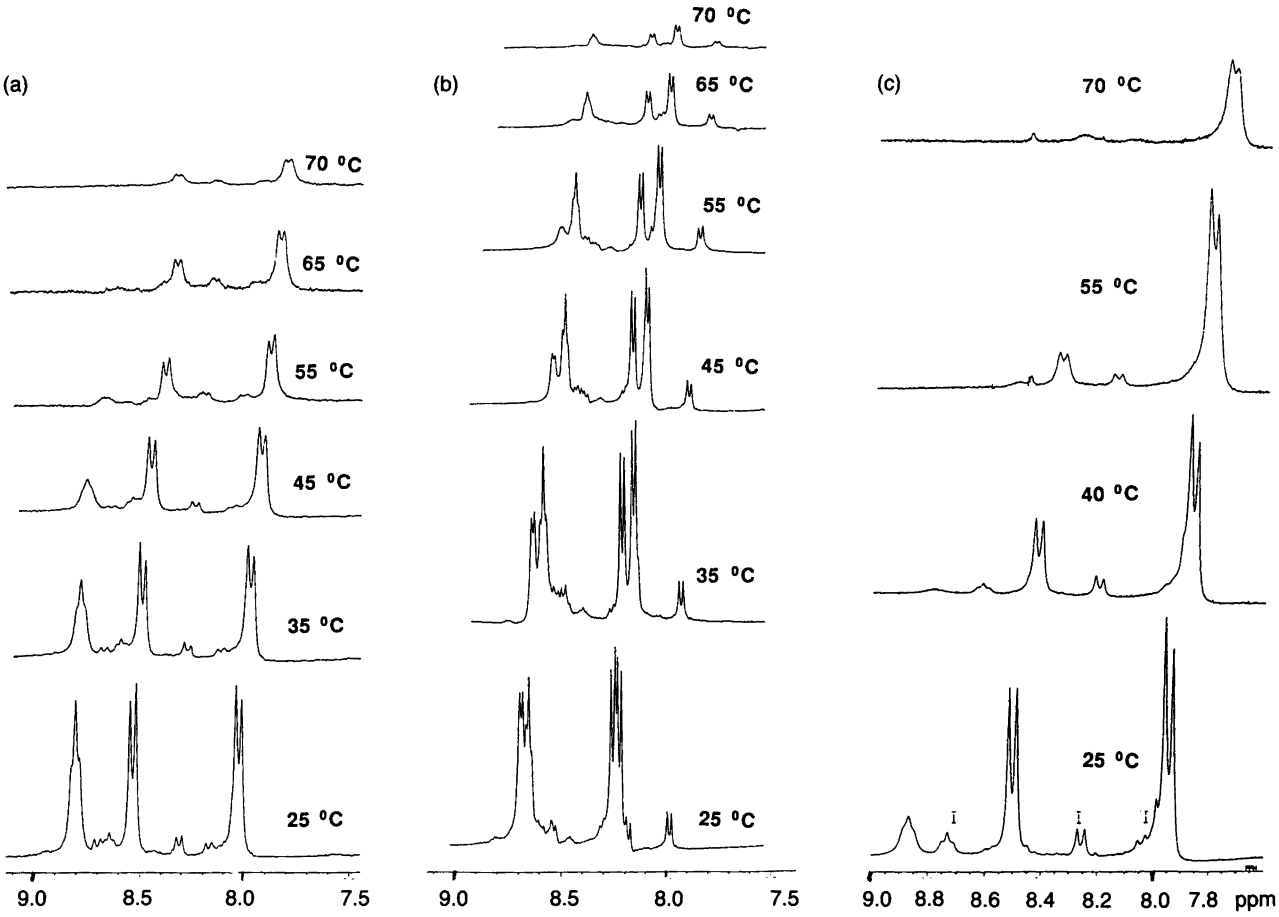

Fig. 2. Variable temperature spectra at pH 3.5 in ^1H_2O for (a) the tetrapeptide RGDS and (b) hexapeptide GRGDSP at T = 25, 35, 45, 55, 65 and 70 °C. (c) Variable temperature spectra at pH 5 in ^1H_2O for the tetrapeptide RGDS at T = 25, 40, 55 and 70 °C. The small peaks in (a) and (c) are due to the pentapeptide RRGDS, and in (b) are due to the heptapeptide GRRGDSP and a *cis* conformation.[5]

Table 3. ^{13}C NMR shifts for (a) GRGDSP and (b) RGDS.

Amino acid	α-C	β-C	γ-C	δ-C
(a) GRGDSP				
Gly	41.36	–	–	–
Arg	54.84	28.83	25.09	41.48
Gly	43.58	–	–	–
Asp	51.58	37.65	–	–
Ser	54.64	61.54	–	–
Pro	62.10	29.98	25.38	48.58
(b) RGDS				
Arg	53.68	28.69	24.27	41.20
Gly	43.31	–	–	–
Asp	51.54	37.65	–	–
Ser	57.51	62.58	–	–

variations due to pH and chain length. Furthermore, the aspartic acid β-carbon and the serine α-carbon signals in RGDS are small and broadened.

Discussion

Measuring the NMR spectra of proteins or peptides at pH 3.5 induces an unnatural condition with charges other than those of the same molecules at around pH 7. However, NH signals are easier to locate and are usually sharper at low pH due to the slower hydrogen exchange.[17] Reed *et al.*[5] found in GRGDSP at pH 7 that the NH signals in H_2O of serine and aspartic acid remained intact when the sample was heated to 56 °C. This is indicative of hydrogen bonds engaging these hydrogen atoms. Molecular modelling provided a highly probable structure involving two hydrogen bonds G→D4 and R2→S5 consistent with two β-bends, the former in an equilibrium between type I and III, the second of type III.[5] We find at pH 3.5 that the peptide GRGDSP shows a similar retention of NH-signals in H_2O even at 70 °C, indicating conservation of the nested β-bend structure at low pH. This peptide can induce and inhibit the cell attachment action of cells characteristic for fibronectin. Even the smaller peptide RGDS has this property, although under more restricted conditions. In the synthesis of RGDS the first three *C*-terminal amino acids are easily incorporated, but arginine poses problems, probably because of a secondary structure making the amino group inaccessible. This leads to unusually long coupling times in the presence of HOBT.

The assignment of NMR signals at pH 3.5 for the tetrapeptide RGDS and the hexapeptide GRGDSP accords well with the data obtained at pH 7 by Reed,[5] which indicated the same conformation at both pH values. The proton exchange between the NH groups and water is slower at low pH and a temperature of 70 °C had to be used to induce the complete extinction of the glycine NH signal. However, the serine and aspartic acid amide hydrogen signals were still strong. Assuming a β-bend R1→S4, the engagement of the aspartic acid amide hydrogen deserves an explanation. Raising the pH to 5 would create an NH exchange situation at 25 °C similar to that obtained at pH 7 at 0 °C.[6] The NH signals of D and S are then still strong but the aspartic acid NH signal is extinguished at 70 °C indicating a weaker hydrogen bond engaging the aspartic acid NH than that engaging the serine NH. Model building of the molecule inserting a hydrogen bond between serine and arginine gives the NH of aspartic acid only few alternaitves for hydrogen bonding, the major being to the aspartic acid carboxy group, the other to the carbonyl group of arginine to form a combined β- and γ-bend. The pattern of hydrogen bonding may thus be the key to the known different receptor activities.[4,16]

We have thus found that the short peptide RGDS in aqueous solution exists in a mainly flexible conformation although with a considerable contribution of hydrogen-bonded structures indicative of a β-bend. On-going studies on the conformation of longer active peptides will show whether this has any bearing upon the specificity of these adhesion proteins.

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