# NMR Study on a SRYD-containing Fibronectin-like Sequence (250–257) of Leishmania gp63: Contribution of Residual Water in the Dimethyl Sulfoxide Solution Structure

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The conformational characteristics of the $I^{250}$ASRYDQ$L^{257}$ synthetic octapeptide, which incorporates the SRYD adhesion site (252–255) of Leishmania gp63, have been investigated at pH 2 and 5, by means of 1D and 2D $^1$H NMR spectroscopy (temperature coefficient values, chemical shifts, vicinal coupling constants and NOE effects). It was found that elimination of residual water from the dimethyl sulfoxide (DMSO) solution at pH 2 provides exchange peaks in the ROESY and HOHAHA spectra similar to those obtained for the DMSO peptide solution at pH 5. This common structure is stabilized (i) by the formation of a type I β-turn involving the QNH→RCO interaction and (ii) by a possible interaction between the guanidinium and the D-β-carboxylate groups. After treatment with molecular sieves, the remaining residual water is redistributed between the peptide functional groups and participates in the rigidification of the new conformational state.

Recent studies on cell adhesion have revealed that the arginyl-glycylaspartate (RGD) sequence is the principal recognition segment in adhesive proteins that interact with a family of structurally related receptors (integrins).[1] Conformational studies on small linear inhibitor peptides containing the RGD sequence showed that they adopt a well defined conformation and that the receptor–inhibitor specificity may reside in the conformation of the RGD tripeptide.[2,3]

In a previous study it has been demonstrated that the major surface glycoprotein of Leishmania, gp63, a fibronectin-like molecule, plays a key role in parasite–macrophage interaction.[4] Binding of gp63 to macrophage receptors is inhibited by RGDS-containing synthetic peptides of fibronectin and by antibodies to these peptides, although gp63 lacks an RGDS sequence. A small peptide sequence SRYD (252–255) of gp63 has been identified as the minimum antibody-binding segment and the $I^{250}$ASRYDQ$L^{257}$ synthetic octapeptide, incorporating the SRYD tetrapeptide, efficiently inhibits parasite attachment to the macrophage receptors. It has been concluded that the SRYD sequence of gp63, which is conserved between different species, mimics antigenically and functionally the RGDS segment of fibronectin and represents the putative gp63 adhesion site.[5]

On the other hand, there is an on-going interest in the factors favouring the stabilization of secondary structures in peptides and proteins, for instance electrostatic ion-pair interactions or bound water molecules which may be found either within the interior or on the surface of the protein bridging hydrogen-bonding interactions.[6,7]

The significant role of residual water in biomolecules has been reported in a number of documents including the solution structure of interleukin 1β, bovine pancreatic trypsin inhibitor, and small peptide segments, which may act for the nucleation of α-helices.[8,9] Moreover, the native catalytically active conformation of enzymes is maintained by its bound water in organic solvents conferring upon the enzymes remarkable properties which are advantageous for many biotechnological applications.[10] In a previous study we have also demonstrated the relevance of residual water in the DMSO solution structure of small peptide hydrates. It was found that residual water remains tightly bound to the peptides, contributes to their rigidification and is not expelled by DMSO.[11]

This study describes the conformational properties of the $I^{250}$ASRYDQ$L^{257}$ synthetic octapeptide of Leishmania gp63, which is a relatively large peptide sequence with various possible secondary structures, in DMSO solution at pH 5 and 2, and also after elimination of residual water as determined by $^1$H NMR spectroscopy. DMSO was chosen as the solvent for our NMR experiments because it provides an amphiphilic environment and mimics physiological conditions.[12] Characterization of the local conformation of the SRYD moiety in relation to residual water could prove critical for an understanding of the receptor-mediated events of intracellular parasitism as well as the design of good vaccine candidates against leishmaniasis.

## Experimental

*Materials and Methods.*—The synthesis and the biological properties of the $I^{250}$ASRYDQ$L^{257}$ octapeptide have been reported previously.[5] The NMR samples were prepared by dissolving the solid material in $H_2O$ and adjusting the pH to the desired value with HCl or NaOH. The obtained aqueous solutions were lyophilized and then dried for 24 h at 40 °C. Weighed amounts of the peptide were dissolved in [$^2H_6$]DMSO at concentrations *ca.* $7 \times 10^{-3}$ mol dm$^{-3}$. At this concentration intermolecular associations were excluded, as was proved by dilution studies. For each experiment at pH 2, two DMSO peptide solutions were prepared and molecular sieves were added to one of these, while the second one was used without addition of molecular sieves.

The NMR spectra were recorded on a Bruker AC 200 spectrometer at 23 °C, by using the standard COSY, HOHAHA and ROESY microprograms. Spectral width in $F_1$ and $F_2$ was 3000 Hz; 512 experiments in 2K data points in the $F_2$ dimension were performed; sinebell squared and shifted (π/4) apodization was used in both dimensions. Several ROESY experiments were performed by changing the frequency offset of the carrier.[13] A spin-locking mixing time of 350 ms was used for ROESY experiments and of 100 ms for the MLEV 17 HOHAHA experiments.[14]

The NOESY spectra were recorded on a Bruker AM 400 spectrometer using two mixing times (150 and 350 ms). The spectral width in $F_1$ and $F_2$ was 5000 Hz; 512 experiments in

**Table 1** Proton magnetic resonance data of I[250]ASRYDQL[257] hydrate ($0.7 \times 10^{-2}$ mol $dm^{-3}$) in [$^2H_6$]DMSO, pH 2, at 296 K, referenced to tetramethylsilane[a]

| Residue | $^+NH_3$ | NH | $N^{\varepsilon}H$ | $N^{\eta}H$ | $C^{\alpha}H$ | $C^{\beta}H$ | $C^{\gamma}H$ | $C^{\delta}H$ | Others | $^3J_{NH-C^{\alpha}H}$ | Temperature coefficient ($10^{-3}$ ppm $K^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 8.22 | | | | 3.65 | 1.81 | 1.51<br>1.20 | 0.90 | | | |
| A | | 8.65 | | | 4.45 | 1.27 | | | | 7.3 | −2.5 |
| S | | 8.15 | | | 4.31 | 3.58 | | | OH 5.23 | 7.8 | −5.0 |
| R | | 8.08 | 7.68 | 7.15 | 4.23 | 1.65 | 1.43 | 3.05 | | 6.8 | −2.9 |
| Y | | 7.99 | | | 4.35 | 2.91<br>2.67 | | | Ar(*o*) 7.03<br>(*m*) 6.65<br>OH 9.22 | 7.8 | −3.4 |
| D | | 8.30 | | | 4.51 | 2.78<br>2.59 | | | | 7.7 | −7.1 |
| Q | | 7.77 | | | 4.27 | 1.91<br>1.75 | 2.11 | | 7.25<br>6.79 | 8.2 | −4.1 |
| L[b] | | 8.11 | | | 4.20 | 1.64 | 1.55 | 0.83 | | 7.9 | −5.1 |

[a] Solutions without molecular sieves. [b] $CO_2H$ at 12.42 ppm.

**Table 2** Proton magnetic resonance data of I[250]ASRYDQL[257] ($0.7 \times 10^{-2}$ mol $dm^{-3}$) in [$^2H_6$] DMSO, pH 2, at 295 K, reference to tetramethylsilance[a]

| Residue | NH | $N^{\varepsilon}H$ | $N^{\eta}H$ | $C^{\alpha}H$ | $C^{\beta}H$ | $C^{\gamma}H$ | $C^{\delta}H$ | Others | $^3J_{NH-C^{\alpha}\delta p_H}$ | Temperature coefficient ($10^{-3}$ ppm $K^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| I | | | | 3.51 | 1.76 | 1.47<br>1.12 | 0.86 | | | |
| A | 8.57 | | | 4.42 | 1.25 | | | | 6.9 | −6.3 |
| S | 8.21 | | | 4.28 | 3.58 | | | | 7.7 | −4.7 |
| R | 7.74 | 10.03 | 7.10 | 4.39 | 2.03<br>1.44 | | 3.19<br>2.94 | | 8.0 | −1.6 |
| Y | 8.63 | | | 4.25 | 3.00<br>2.72 | | | Ar(*o*) 7.02<br>(*m*) 6.65<br>OH 9.24 | 6.9 | −4.0 |
| D | 8.37 | | | 4.27 | 2.65<br>2.25 | | | | 8.0 | −1.3 |
| Q | 7.22 | | | 4.20 | 1.77<br>1.91 | 2.10 | | 7.26<br>6.79 | 8.8 | +0.0 |
| L | 7.22 | | | 4.12 | 1.62 | 1.52 | 0.83 | | 7.8 | −4.0 |

[a] Solutions 7 days after addition of molecular sieves.

2K data points in the $F_2$ dimension were performed; data points $t_1$ were zero-filled to give a (1K × 1K) data matrix and sinebell squared and shifted ($\pi/4$) apodization was used in both dimensions.

## Results and Discussion

$^1H$ *Resonance Assignments.*—The combined use of COSY, HOHAHA and ROESY experiments allowed the complete assignment of all proton resonances of the octapeptide, which is in agreement with previously reported $^1H$ NMR studies.[11,15] All the proton chemical shift data, for the peptide at pH 2 without molecular sieves and after addition of molecular sieves are listed in Tables 1 and 2, respectively. Table 3 summarizes the proton assignments of the octapeptide at pH 5.

*Conformational Changes of the* I[250]ASRYDQL[257] *Octapeptide in* [$^2H_6$]DMSO *Solution.*—The conformational perturbations of the peptide at pH 5 in comparison with the peptide hydrate at pH 2 can be appreciated by plotting the difference between the backbone chemical shifts.[16] It appeared that almost all the resonances were more or less affected [Fig. 1(*a*)]. Thus, the chemical shift differences of the $C^{\alpha}H$ and NH protons of leucine probably reflect the deprotonation of the *C*-terminal group upon raising of the pH, while the $IC^{\alpha}H$ shift denotes changes in the equilibrium between the ammonium and the

**Table 3** Proton magnetic resonance data of $I^{250}$ASRYDQL$^{257}$ ($0.7 \times 10^{-2}$ mol $dm^{-3}$) in [$^2H_6$]DMSO, pH 5, at 296 K referenced to tetramethylsilane

| Residue | NH | $N^{\varepsilon}H$ | $N^{\eta}H$ | $C^{\alpha}H$ | $C^{\beta}H$ | $C^{\gamma}H$ | $C^{\delta}H$ | Others | $^3J_{NH\text{-}C^{\alpha}H}$ | Temperature coefficient ($10^{-3}$ ppm $K^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| I | | | | 3.15 | 1.69 | 1.40<br>1.08 | 0.85<br>0.82 | | | |
| A | 8.48 | | | 4.38 | 1.22 | | | | 8.1 | −6.3 |
| S | 8.22 | | | 4.28 | 3.60<br>3.55 | | | | 7.7 | −4.3 |
| R | 7.73 | 10.18 | 7.05 | 4.38 | 2.05<br>1.46 | 1.45<br>1.56 | 3.20<br>2.96 | | 8.0 | −1.7 |
| Y | 8.63 | | | 4.26 | 2.99<br>2.74 | | | Ar(*o*) 7.03<br>(*m*) 6.65<br>OH 9.22 | 6.9 | −3.8 |
| D | 8.37 | | | 4.25 | 2.66<br>2.24 | | | | 7.8 | +0.5 |
| Q | 7.23 | | | 4.17 | 1.80<br>1.95 | 2.10 | | 6.74<br>7.30 | 8.4 | +0.2 |
| L | 7.66 | | | 4.02 | 1.63 | 1.48 | 0.87 | | 7.7 | −2.4 |

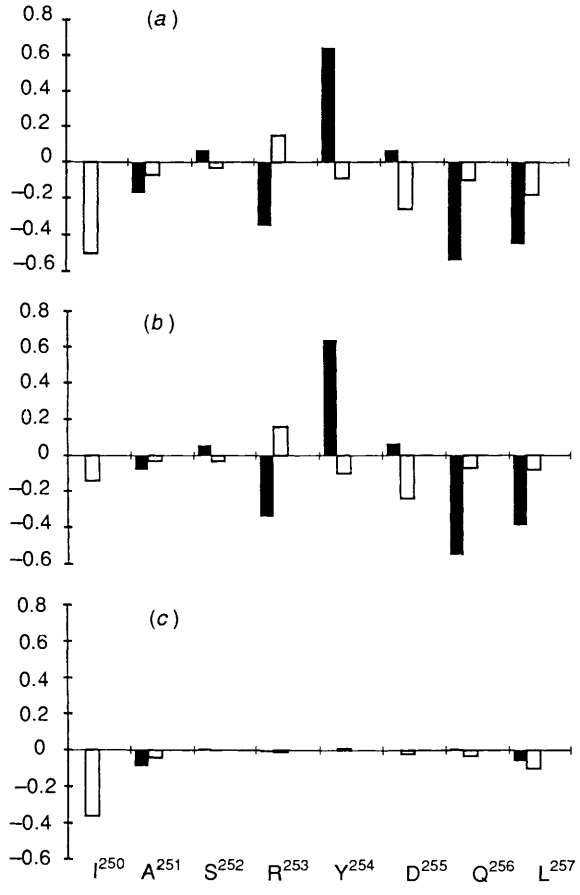


**Fig. 1** Perturbations of the backbone proton resonances for (*a*) the IASRYDQL octapeptide at pH 5 with reference to the octapeptide hydrate at pH 2; (*b*) the octapeptide after removal of residual water at pH 2 with reference to the octapeptide hydrate at pH 2; (*c*) the octapeptide at pH 5 with reference to the octapeptide after removal of residual water at pH 2; the open and filled boxes refer to the $C^{\alpha}H$ and NH resonances, respectively

amino state of the *N*-terminal group.[17] Additionally, the observed differences in the remaining signals (YNH, QNH, RNH and $DC^{\alpha}H$) suggest considerable conformational modifications of the peptide backbone.

The temperature coefficient values (below $-3 \times 10^{-3}$ ppm $K^{-1}$) of almost all the NHs of the octapeptide hydrate indicate that they are exposed to the solvent (Table 1). In particular, the small absolute $\Delta\delta/\Delta T$ value of ANH ($-2.5 \times 10^{-3}$ ppm $K^{-1}$) can be attributed either to the presence of the adjacent charged ammonium group or to solvent-shielding. Moreover, the temperature coefficient values of RNH ($-2.9 \times 10^{-3}$ ppm $K^{-1}$) denote that this amide proton is not entirely exposed to the solvent and is probably involved in an intramolecular interaction stabilizing the peptide structure.

The ROESY spectrum of the peptide hydrate at pH 2 [Fig. 2(*a*)] showed few and weak ROE connectivities between successive amide protons compared with that at pH 5 [Fig. 2(*b*)]. For instance the YNH/DNH cross peak, which in combination with a low absolute temperature coefficient value for the QNH could be a diagnostic for the presence of a type Iβ-turn in the sequence -R-Y-D-Q-, is absent at pH 2 [Fig. 2(*a*)], in contrast with pH 5 [Fig. 2(*b*), Table 3]. Additionally, the weak DNH/QNH connectivity [Fig. 2(*a*)] lends support to a flexible conformation at pH 2.

Addition of molecular sieves to the DMSO peptide solution at pH 2 resulted in the progressive elimination of the water signals (resonance peak at 3.3 ppm) and a variety of spectral modifications were observed (Fig. 3, Tables 1 and 2). The backbone chemical shift differences between the peptide after removing a substantial amount of trace water and the peptide hydrate at pH 2 are shown in Fig. 1(*b*), and the NH and $C^{\alpha}H$ chemical shift changes of the central sequence (RYDQ) indicate significant conformational perturbation after eliminating most of the water.

Emphasis should also be placed on the low absolute temperature coefficient values of the RNH, DNH and QNH amide protons obtained after addition of molecular sieves to the DMSO peptide solution (Table 2). These indicate that these NH groups are not entirely exposed to the solvent and they are probably involved in intramolecular interactions. The previously cited variations of the temperature coefficient values (Tables 1 and 2) suggest that the environment of almost all the

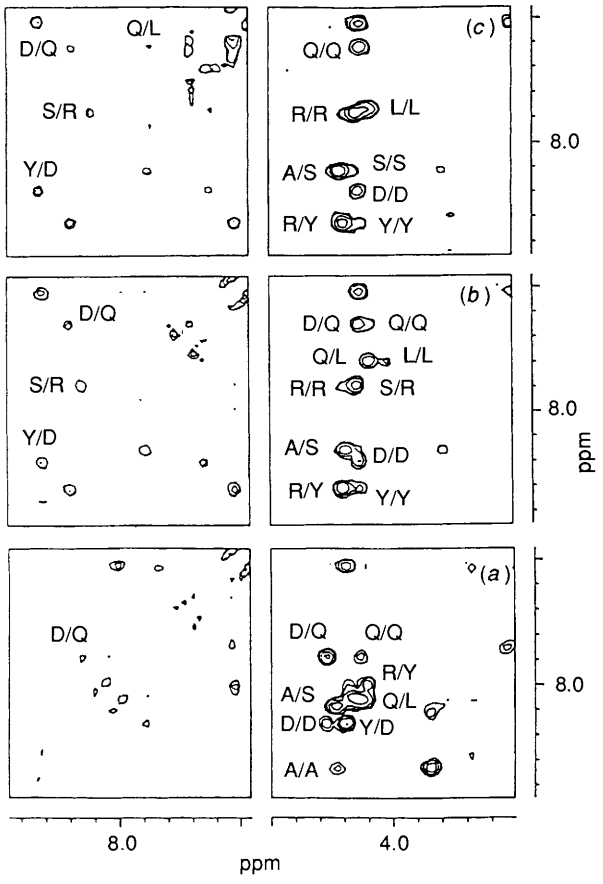


Fig. 2 The NH/NH and $C^{\alpha}H$/NH portions of the 200 MHz ROESY spectra of IASRYDQL octapeptide in [$^2H_6$]DMSO without molecular sieves at pH 2 (*a*), at pH 5 (*b*), and seven days after addition of molecular sieves at pH 2 (*c*)

NH protons was affected. Interestingly, low absolute $\Delta\delta/\Delta T$ values were also measured for the same amide protons of the octapeptide solution at pH 5, in which traces of water remained bound to the peptide even after vigorous treatment with molecular sieves (Table 3).

Comparison of the ROE connectivities of the initial peptide solution at pH 2 and after treatment with molecular sieves, showed that the residual water contributes to the stabilization of the peptide conformation [Figs. 2(*a*) and (*c*)]. In addition, the density of the ROE connectivities between $NH_{(i)}/NH_{(i+i)}$ protons after addition of molecular sieves, is comparable to that of the ROE cross peaks between consecutive amide protons of the peptide at pH 5 [Figs. 2(*b*) and (*c*)].

Taking into account the similarities of the temperature coefficient values (Tables 2 and 3) (a unique exception is the temperature coefficient value of LNH, which probably reflects the different ionization states of the *C*-terminal carboxylic group at the two pH values) and the $NH_{(i)}/NH_{(i+1)}$ connectivities of the peptide after removal of water, as well as at pH 5 [Figs. 2(*b*) and (*c*)], we can assume that the octapeptide adopts similar conformations in both cases. Additional confirmation for this assumption is obtained from the backbone chemical-shift differences of the DMSO octapeptide solution [Fig. 1(*c*)]. It appears that the unique resonance differences are those of the $C^{\alpha}H$ protons corresponding to the *N*- and *C*-terminal residues (I and L, respectively), probably due to changes of charge. In contrast, the very small perturbation of the proton chemical shifts of residues S, R, Y, D and Q indicates that the magnetic environment of the peptide backbone is preserved. Furthermore, comparison of the coupling constant values of the peptide after elimination of water and at pH 5 highlights the conformational similarities of the molecule in both cases (Tables 2 and 3).

Rather intense ROE connectivities were observed between the successive amide protons YNH/DNH and DNH/QNH at pH 5 and 2 after treatment with molecular sieves. The absence of a strong $YC^{\alpha}H$/DNH connectivity was confirmed by recording a NOESY spectrum at 400 MHz, of which selected rows are shown in Fig. 4. These findings, as well as the low absolute temperature coefficient value of QNH (Tables 2 and 3) argue in favour of a type Iβ-turn [18] at the -R-Y-D-Q *C*-terminal part of the octapeptide involving the QNH→RCO interaction. Intense ROE connectivities were also found between successive $C^{\alpha}H_i/NH_{i+1}$ and $NH_i/NH_{i+1}$ protons ($AC^{\alpha}H$/SNH and SNH/RNH, respectively), as well as between $SC^{\alpha}H$/RNH [Fig. 2(*b*) and (*c*)]. These qualitative ROE data and the low absolute temperature coefficient values of RNH (Tables 2 and 3) amide proton denote a non-random structure at the *N*-terminal part of the octapeptide. The considerably downfield shift [11,15] of the $RN^{\varepsilon}H$ signal (*ca.* 10.1 ppm) and the unusually large chemical shift difference of the magnetically non-equivalent $DC^{\beta}H_2$ protons ($\Delta\delta$ = 0.4) may possibly indicate an interaction between the quanidinium and the D-β-carboxylate groups at pH 5 and 2 after removal of trace water.

We conclude that the peptide structure at pH 5 and 2 after removal of the trace water is stabilized (*i*) by the formation of a type I β-turn involving the QNH→RCO interaction and (*ii*) by a possible interaction between the guanidinium and the D-β-carboxylate groups.

*Location of Residual Water in the* $I^{250}$ASRYDQL$^{257}$ *Octapeptide Structure.*—Cross-peaks between $IN^{+}H_3$, SOH, YOH and LCOOH protons and those of water molecules were observed in a HOHAHA experiment of the octapeptide in DMSO solution at pH 2 without molecular sieves. Although cross peaks due to chemical exchange can be obtained in a HOHAHA spectrum for easily exchangeable protons, only the exchange cross-peaks between $IN^{+}H_3$, SOH, YOH, LCOOH and residual water were noted, whereas exchange cross-peaks of the above mentioned functional groups between each other were absent. This fact provides evidence for the occurrence of residual water in the vicinity of the above-mentioned residues and that water participates in the exchange process.[8]

Further information on the position of the peptide-bound water was obtained from the exchange cross-peaks found in a ROESY spectrum (cross-peaks of the same sign as the diagonal peaks) similar to those detected in the HOHAHA experiment. Additionally, ROE connectivities [19] between the aromatic $YC^{3,5}H$ and $YC^{2,6}H$ protons and $H_2O$ molecules (cross-peaks of opposite sign to the diagonal peaks) were detected in the ROESY spectrum (Fig. 5). The intense ROE cross-peak between $YC^{3,5}H$ and water could possibly arise from a two-step interaction whereby magnetization is transferred *via* exchange from water to YOH followed by through-space NOE transfer to $YC^{3,5}H$. In addition, the less intense ROE connectivity between $YC^{2,6}H$ and water could occur *via* a similar mechanism followed by an additional HOHAHA transfer step between $YC^{3,5}H$ and $YC^{2,6}H$. A similar mechanism for the magnetization transfer was reported for the indole ring protons of W, where the ROESY cross peaks arising from this mechanism have the same sign as the cross-peaks resulting from a direct ROE transfer.[13,20] The weak ROE connectivity identified between $SC^{\alpha}H$ and residual water should also derive from a similar magnetization-transfer pathway.

The above-mentioned findings provide evidence for the presence of residual water in close proximity to the $IN^{+}H_3$ (proton donor) and the SOH, YOH and LCOOH groups (operating either as proton donors or proton acceptors).

When molecular sieves were added to the DMSO peptide

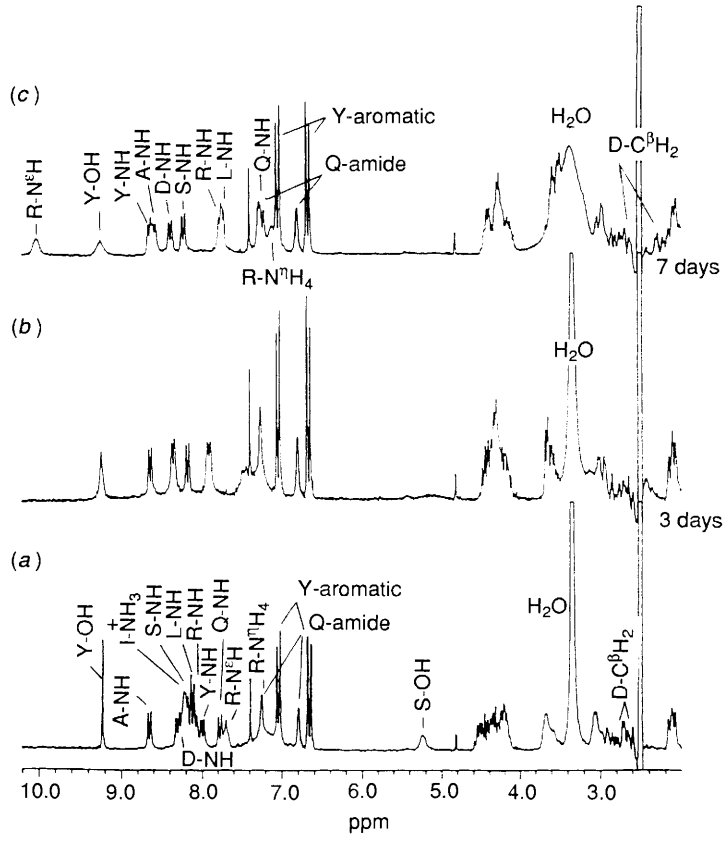


**Fig. 3** The 200 MHz $^1$H NMR spectra of IASRYDQL octapeptide in [$^2H_6$]DMSO without molecular sieves (*a*), and three days (*b*) and seven days (*c*) after addition of molecular sieves

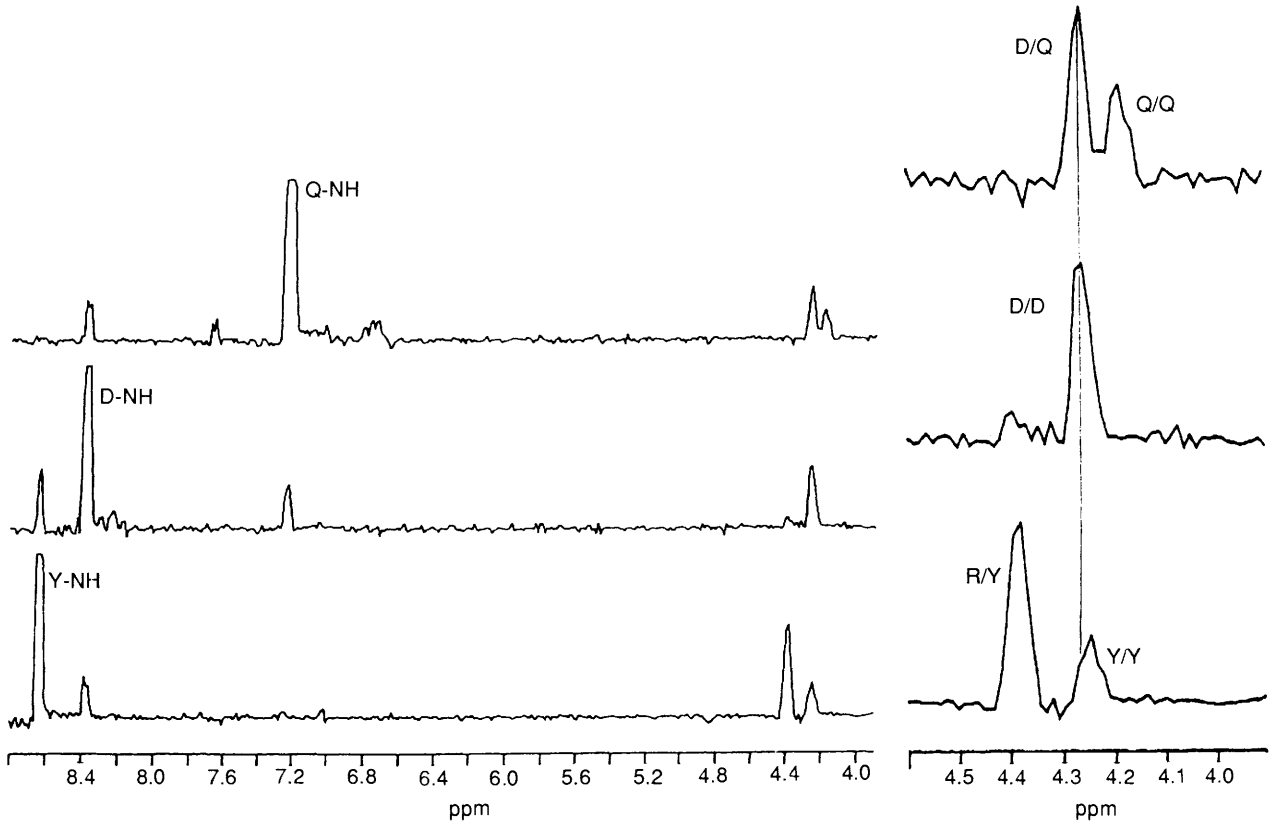


**Fig. 4** Selected rows from the 400 MHz NOESY spectrum of IASRYDQL in [$^2H_6$]DMSO at pH 5 and their expansion in the C$^{\alpha}$H region

solution additional exchange cross-peaks were observed in a HOHAHA experiment using the same previously defined spectral conditions. In particular, besides the YOH/$H_2O$ exchange cross-peak, RN$^{\eta}$H$_4$/$H_2O$ and ANH/$H_2O$ connectivities were also observed, while N$^{+}$H$_3$/$H_2O$, COOH/$H_2O$ and SOH/$H_2O$ exchange cross-peaks were not detected. It is also

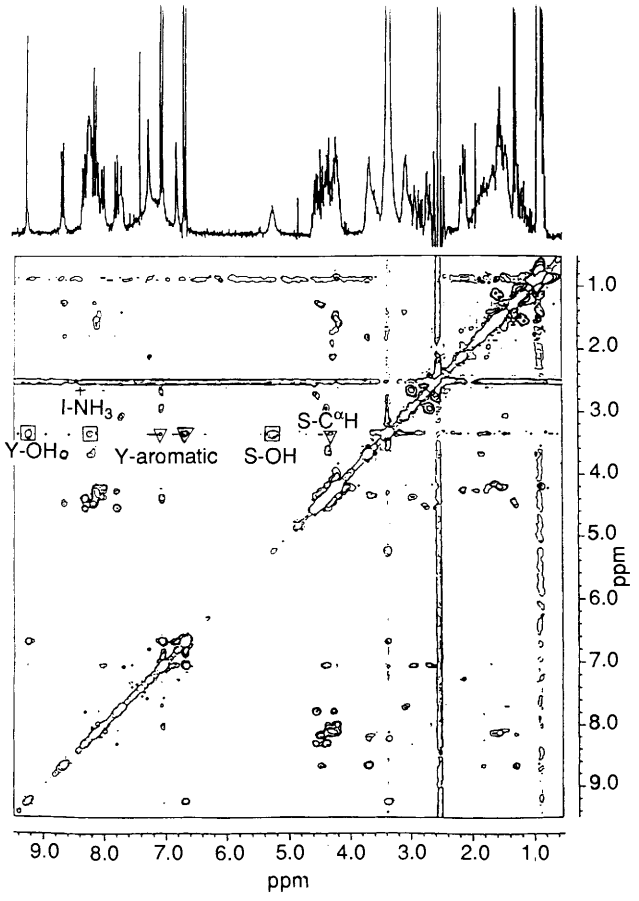


**Fig. 5** The 200 MHz ROESY spectrum of IASRYDQL in [$^2H_6$]-DMSO without molecular sieves at pH 2; interactions with water giving cross-peaks of the same sign (□) and of opposite sign (▽) to the diagonal peaks

noteworthy that the ROESY cross-peaks between $YC^{3,5}H/H_2O$ and $YC^{2,6}/H_2O$ were not noted after the elimination of the bulk water, probably due to variations in the exchange rate between the hydroxy groups and the residual water. Evidence for this change in the chemical exchange rate also comes from the alteration of the SOH and YOH resonance peak widths.

## Conclusions

The most prominent results obtained from our NMR study is that elimination of trace water from a DMSO solution of the $I^{250}$ASRYDQL$^{257}$ synthetic octapeptide (250–257) of Leishmania gp63 at pH 2, induces conformational changes in the molecule and the rearrangement of the remaining residual water contributes to its rigidification, resulting in a new structure similar to that at pH 5. This structure is stabilized (*i*) by the formation of a type I β-turn involving the QNH→RCO interaction and (*ii*) by a possible interaction between the guanidinium and the D-β-carboxylate groups.

The present study also contributes to the understanding of the catalytically active conformation of enzymes in organic solvents[10] and shows the importance of the initially used solvent in the conformational properties of the final peptide solution.[21]

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