Significance of Bound Residual Water in the DMSO Solution Structure of Stable Peptide Hydrates

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Water molecules in peptide hydrates are widely known to be directly involved in the stabilization of the peptide structure in the solid state. In this work, the influence of tightly bound residual water molecules on the conformational properties of two hydrates of the L-Arg-L-Leu-Gly and Gly-L-Phe-L-Leu peptide lyophilized from water at acidic pH, has been investigated for the first time in DMSO solution by means of 1D and 2D ^{1}H NMR spectroscopy. Significant conformational changes have been observed in solution as the bound residual water is gradually expelled from the peptide hydrate. These observations point out the crucial role of bound water in fine-structural determinations of peptides in solution when evaluating NMR data.

Water molecules are often tightly bound to peptides and proteins in the solid state (crystalline or amorphous) and usually they cannot be removed even after various vigorous treatments.[1–5] The significant role of these water molecules bound to bioactive macromolecules has been noted in a variety of biological processes, *e.g.* oxygen transport by myoglobin, transport of certain polar solutes across membranes, and enzymatic catalysis.[6,7] Residual water molecules are also involved in stabilizing the structure of peptides or proteins through bridging hydrogen-bonding interactions.[8–10]

The exact amount of water bound to peptides in their crystalline state depends on the amino acid sequence of the peptide and on the mode of their crystallization, as has been successfully demonstrated for enkephalins. These small peptides are unique in the variety of crystallographic data available in the literature.[4,5] In fact, [Leu^5]-enkephalin has been obtained in four different crystalline forms with varying amounts of residual solvent molecules.

Evidence of water molecules bound to proteins in aqueous solution has come from a number of documents including the demonstration of a hydration layer through magnetic relaxation experiments,[11,12] and the measurement of bound water exchange with ^{18}O tracer techniques.[13] It is only very recently, however, that bound internal water has been directly observed in bovine pancreatic trypsin inhibitor in solution, by means of ROESY experiments.[14] The presence and localization of bound internal water in the solution structure of interleukin 1β have also been demonstrated using heteronuclear three-dimensional ^{1}H rotating-frame Overhauser ^{15}N–^{1}H multiple quantum coherence NMR spectroscopy (ROESY-HMQC).[15]

On the other hand, it has recently been shown that enzymes not only work vigorously in anhydrous organic media, but also, in this unnatural milieu, acquire remarkable properties such as enhanced stability, molecular memory, enantiomeric specificities and the ability to catalyse unusual reactions.[16] Obviously, these properties would be of great biotechnological interest because many enzymatic processes that are nearly impossible in water, are readily carried out in non-aqueous solvents.[17] It was found that as long as residual water is present, the enzyme should be active in organic solvents.[7]

Although it is generally admitted that water molecules in a peptide hydrate dissolved in an organic polar solvent are expelled to the solution, in fact there is no evidence reported in the literature for the actual comportment of these water molecules. Therefore, we have recently undertaken a systematic study on the influence of bound water on the conformational properties of two stable peptide hydrates, L-Arg-L-Leu-Gly and Gly-L-Phe-L-Leu, lyophilized from water at acidic pH, in DMSO solutions, by means of 1D and 2D ^{1}H NMR spectroscopy. L-Arg-L-Leu-Gly is the repeating fragment of sequential Arg-rich polypeptides,[18,19] which are capable of interacting with DNA,[20] while Gly-L-Phe-L-Leu is the [Leu^5]-enkephalin *C*-terminal tripeptide.[21]

Further information about the presence of residual water molecules in the solution structure of the L-Arg-L-Leu-Gly hydrate has been collected by means of HOHAHA experiments and ^{17}O NMR spectroscopy. The importance of bound water in conformational studies of peptides in DMSO solution, when evaluating NMR data, is also discussed.

Experimental

Materials and Methods.—The synthesis and the conformational properties in aqueous solutions of the L-Arg-L-Leu-Gly and Gly-L-Phe-L-Leu tripeptides have been reported previously.[21,22] Samples were lyophilized from aqueous solutions acidified at pH 2 with 1 mol dm^{-3} hydrochloric acid. Each sample was dried for 24 h at 40 °C and then dissolved in [^{2}H$_6$]DMSO at concentrations of *ca.* 1.5 × 10^{-2} mol dm^{-3}. For each peptide studied two solutions were prepared, molecular sieves were added to one of these [^{2}H$_6$]DMSO peptide solutions, while the second one was used without addition of molecular sieves. All the solutions were kept at 22 °C.

1D and 2D ^{1}H NMR spectra were scanned on a Bruker AC 200 apparatus by using the standard COSY microprograms. Spectral width in F_1 and F_2 was 2500 Hz; 256 experiments in 1K data points in the F_2 dimension were performed; data points in t_1 were zero-filled to give a (512 × 512) data matrix and sine-bell apodization was used in both dimensions. The number of scans varied from 32–128.

Several ROESY experiments were carried out by changing the frequency offset of the carrier in order to be sure that the NOEs observed represented true proximity between protons and were not the result of Hartmann–Hahn transfer[23] coupled with incoherent magnetization transfer. The ROESY spectral parameters were 2K data points for both dimensions. A spin-locking mixing time of 350 ms was used with a radiofrequency field strength of 2.3 kHz. The MLEV 17 HOHAHA experiment[24] was scanned with a spin-locking mixing time of 100 ms and a radiofrequency field strength of 9.2 kHz. 1K data points were recorded for both dimensions.

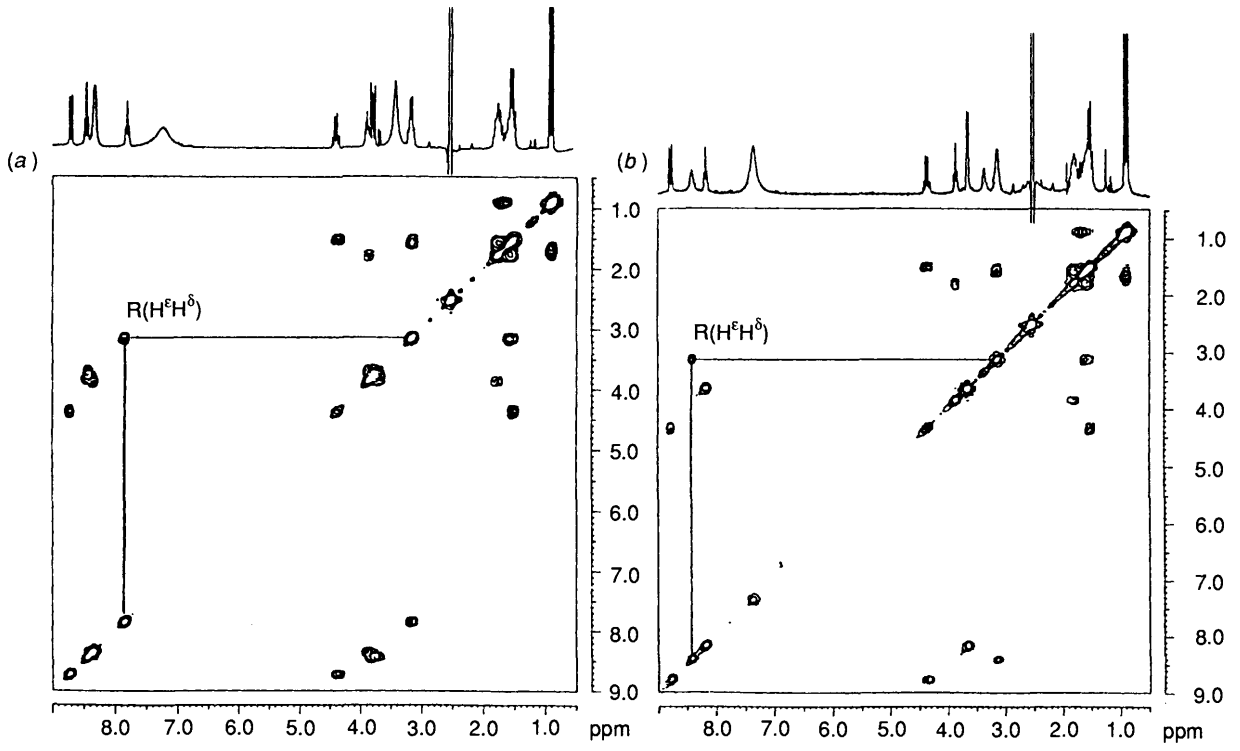

Fig. 1 Homonuclear correlated 2D spectrum (COSY) of the L-Arg-L-Leu-Gly hydrate in [$^2H_6$]DMSO without molecular sieves (*a*), after addition of molecular sieves (*b*)

Table 1 Proton magnetic resonance data of the L-Arg-L-Leu-Gly and Gly-L-Phe-L-Leu tripeptides (1.5×10^{-2} mol dm^{-3}) in [$^2H_6$]DMSO at 295 K from tetramethylsilane

Peptide	Residue	NH_3^+	NH[c]	N$^{\varepsilon}$H	N$^{\eta}$H$_2$	C$^{\alpha}$H	C$^{\beta}$H	C$^{\gamma}$H	C$^{\delta}$H	C$^{\varepsilon}$H
L-Arg-L-Leu-Gly[a]	Arg	8.28		7.85	7.23	3.90	1.84	1.62	3.20	
	Leu		8.75 (−2.17)			4.38	1.55	1.80	0.98	
	Gly		8.44 (−5.42)			3.71				
						3.81				
L-Arg-L-Leu-Gly[b]	Arg			8.40	7.39	3.90	1.84	1.62	3.16	
	Leu		8.75 (−2.56)			4.35	1.55	1.80	0.98	
	Gly		8.18 (−4.44)			3.66				
Gly-L-Phe-L-Leu[a]	Gly	8.15				3.55				
						3.36				
	Phe		8.68 (−3.67)			4.65	3.06		7.23	7.30
							2.73			
	Leu		8.50 (−5.83)			4.21	1.54	1.62	0.85	
									0.91	
Gly-L-Phe-L-Leu[b]	Gly	8.83				3.49				
						3.31				
	Phe		8.62 (−5.46)			4.64	3.06		7.23	7.30
							2.73			
	Leu		8.41 (−5.07)			4.21	1.54	1.62	0.85	
									0.91	

[a] Solutions without molecular sieves. [b] 72 h after addition of molecular sieves. [c] The temperature coefficients (10^{-3} ppm K^{-1}) are specified in parentheses for the NH proton signals.

Results and Discussion

Signal Assignment of the L-*Arg*-L-*Leu-Gly Hydrate in* [$^2H_6$]*DMSO.*—All the proton resonances (NH, C$^{\alpha}$H and side chain aliphatic protons) were assigned on the basis of COSY connectivities (Fig. 1, Table 1). The N$^{\eta}$H protons of the arginine guanidinium group showed a broad resonance centred at 7.23 ppm which corresponds to four protons. This assignment is in agreement with previously reported ^{1}H NMR studies on arginine-containing peptides.[25,26] The presence of the $-NH_3^+$ resonance at 8.28 ppm corresponding to three protons [Fig. 2(*a*), Table 1] and that of the $-CO_2H$ at 12.48 ppm confirmed the ionization state of the tripeptide at pH 2. The NOE cross-peaks were observed in the ROESY spectrum of the [$^2H_6$]-DMSO solution of the L-Arg-L-Leu-Gly hydrate (Fig. 3). The presence of intense NOE cross-peaks, including $d_{\alpha N}$ (*i*, *i* + 1) and $d_{N\alpha}$ (*i*, *i*) NOE connectivities for the DMSO solution without molecular sieves [Fig. 3(*a*)], indicated a constrained structure of the tripeptide in the presence of the peptide-bound residual water molecules not expelled by the DMSO molecules. The ABX system for the C$^{\alpha}$H$_2$ of Gly, which is the carboxy terminal residue, can be related to some mobility restrictions of this amino acid and the two $^3J_{NH\text{-}C^{\alpha}H}$ coupling constant values

(5.7 and 5.9 Hz) are consistent with the rotational angle value[27] of $\varphi = +70°$.

Conformational Changes of the Peptide in [$^2H_6$]*DMSO Solution in the Presence of Molecular Sieves.*—The spectral modifications, observed after the addition of molecular sieves, a widely used dehydrating agent, are analysed as follows. Most of the water traces (signal presented at 3.4 ppm in Fig. 2) contained and/or absorbed by the solvent ([$^2H_6$]DMSO) were eliminated within 12 h without any alteration of the original NMR spectrum [Fig. 2(*a*)]. However, when the amount of water went on decreasing after the water:peptide ratio became less than one, considerable changes occurred [see Figs. 2(*b*), (*c*) and Table 1]. (*i*) The $-NH_3^+$ signal at 8.28 ppm vanished and was detected as a broad distortion of the baseline probably due to solvation and fast exchange [Fig. 2(*b*)]; (*ii*) the Arg $N^\varepsilon H$ signal shifted to low fields, whereas the GlyNH signal shifted to high fields; (*iii*) the broad signal of Arg $N^\eta H_2$ shifted downfield and became sharp; (*iv*) the Gly $C^\alpha H_2$ protons became magnetically equivalent [Fig. 2(*c*)] whereas the ROE cross peaks between the $ArgNH_3^+$ and Arg $C^\alpha H$ protons disappeared [Fig. 3(*b*)].

When molecular sieves were taken out and the DMSO solution was subjected to rehydration, the water signal increased without any further modification of the peptide spectrum [Fig. 2(*d*)]. The initial peptide spectrum was recovered only when DMSO solution was diluted with water, repeatedly lyophilized after adjustment to pH 2, and taken up again in [$^2H_6$]DMSO.

These results indicate that residual water (*i*) is strongly bound to the peptide, (*ii*) contributes to the rigidification of the peptide conformation and (*iii*) is not expelled by DMSO. Moreover, the elimination of residual water by means of molecular sieves has an irreversible effect, as water cannot recover its original

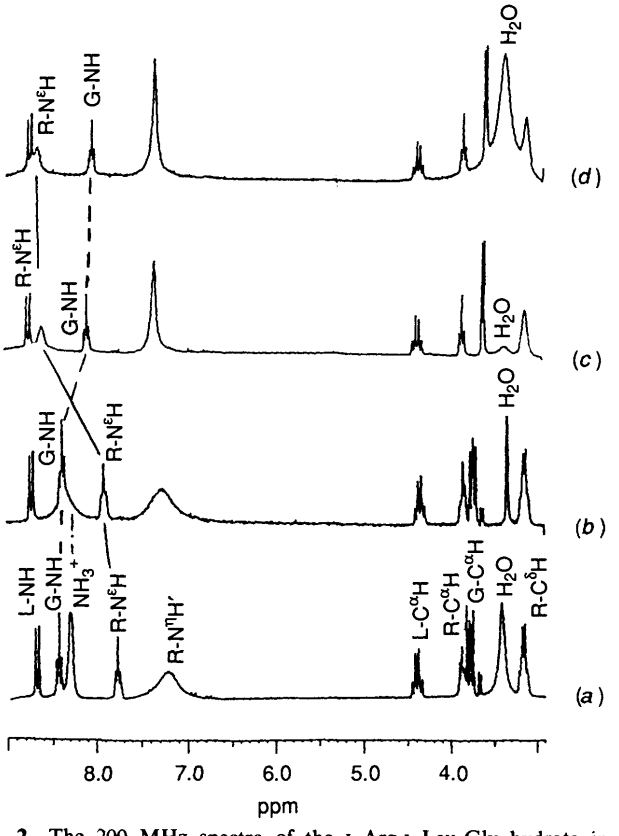

Fig. 2 The 200 MHz spectra of the L-Arg-L-Leu-Gly hydrate in [$^2H_6$]DMSO without molecular sieves (*a*), 24 h after addition of molecular sieves (*b*), 72 h after addition of molecular sieves (*c*), subjected to rehydration (*d*)

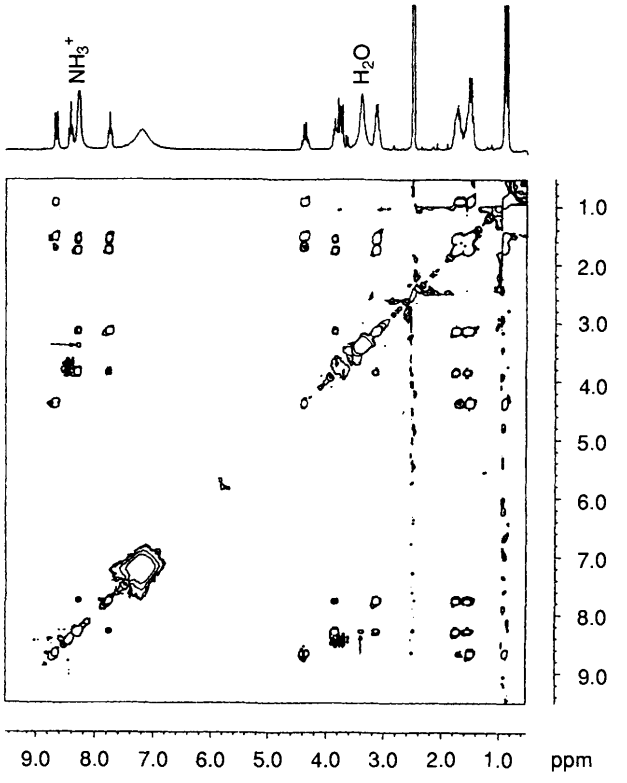

Fig. 4 HOHAHA spectrum of the L-Arg-L-Leu-Gly hydrate in [$^2H_6$]DMSO without molecular sieves. The arrow shows the chemical exchange cross-peak between $ArgNH_3^+$ and water.

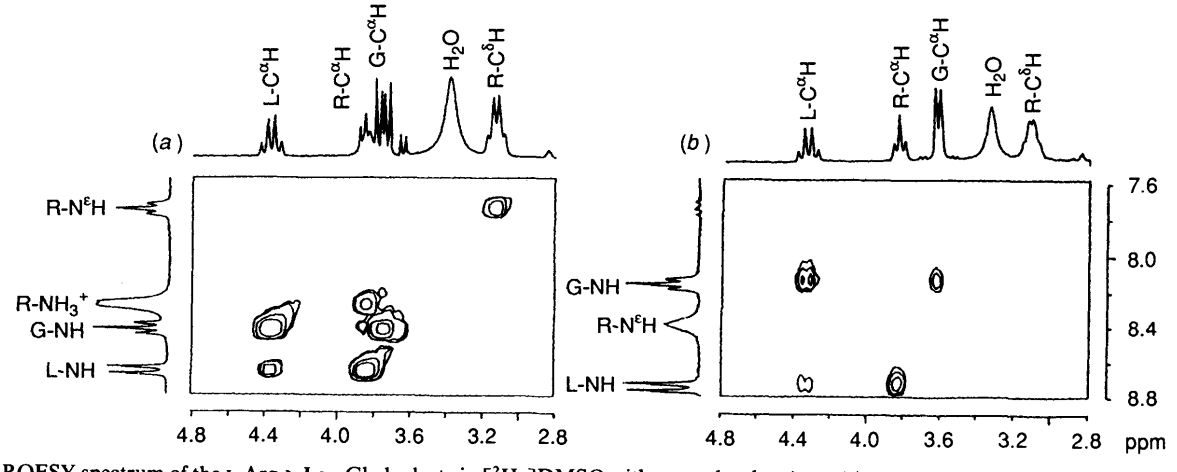

Fig. 3 ROESY spectrum of the L-Arg-L-Leu-Gly hydrate in [$^2H_6$]DMSO without molecular sieves (*a*), after addition of molecular sieves (*b*)

position after being reintroduced into the solution, and results in a new conformation of the peptide.

Evidence for the existence of the peptide hydrate in DMSO solution also came from ^{17}O NMR spectroscopy. A sample of the L-Arg-L-Leu-Gly tripeptide was lyophilized from $H_2{}^{17}O$ (10% ^{17}O enrichment), acidified at pH 2 and dissolved in [$^2H_6$]DMSO. The $H_2{}^{17}O$ molecules showed a chemical shift at 6.5 ppm with reference to H_2O in DMSO, which clearly demonstrates its interaction with the peptide.

Additional information on the position of the peptide-bound water has been obtained from HOHAHA experiments on the L-Arg-L-Leu-Gly tripeptide in DMSO solution without molecular sieves (Fig. 4). It is known that cross-peaks due to chemical exchange can be observed in a HOHAHA spectrum for fast exchangeable protons. Thus, the ammonium, carboxylic and guanidinium groups of the L-Arg-L-Leu-Gly tripeptide can provide cross-peaks between themselves and with the residual water, as far as water participates in the chemical exchange. Besides all the expected connectivities in the HOHAHA spectrum, only the cross-peak due to the chemical exchange between $ArgNH_3^+$ and water was observed. This finding provides evidence for the presence of residual water in close proximity to the *N*-terminal charged group in the peptide.

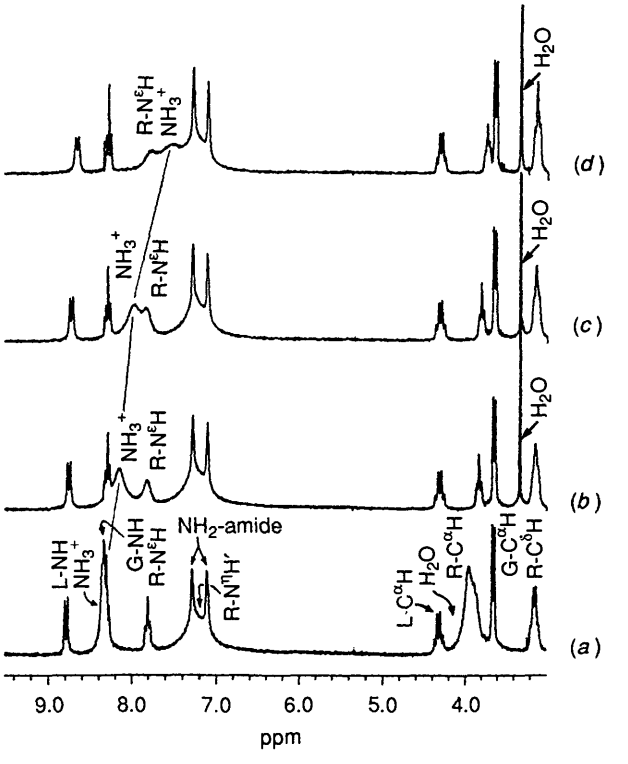

Fig. 5 Spectra of the L-Arg-L-Leu-GlyNH_2 tripeptide **b** in [$^2H_6$]DMSO without molecular sieves (*a*), 24 h after addition of molecular sieves (*b*), 72 h after addition of molecular sieves (*c*), 12 days after addition of molecular sieves (*d*)

In order to get further insight on the functional role of the residual water in the solution structure of the L-Arg-L-Leu-Gly tripeptide, NMR experiments have been performed on the following model compounds: **a** Ac-L-Arg-L-Leu-Gly-NH_2, **b** L-Arg-L-Leu-GlyNH_2, **c** L-Nva-L-Leu-Gly and **d** Ac-L-Arg-L-Leu-Gly. After addition of molecular sieves to compound **a**, in which the *N*-terminal ammonium group and the *C*-terminal carboxylic group were converted into acetyl and amide groups, respectively, no change appeared in the NMR spectrum, except for a decrease of the water signal and a shift to higher fields (from 4.1 ppm to 3.3 ppm). The $ArgNH_3^+$ signal of the amide tripeptide **b** shifted upfield (from 8.35 ppm to <7.5 ppm) and broadened when the amount of water decreased (Fig. 5). However, in contrast with the L-Arg-L-Leu-Gly tripeptide (Fig. 2), the ArgN$^{\varepsilon}$H, ArgN$^{\eta}H_4$ and GlyC$^{\alpha}H_2$ signals of compounds **a** and **b** remained unchanged when water was progressively eliminated. We can assume that the residual water is not directly bound to the guanidinium group, but strongly affects the *N*-terminal ammonium group. This conclusion was also confirmed by the NMR spectrum of compound **c**, in which the charged guanidinium group was substituted by an aliphatic group. After treatment by molecular sieves, no change was observed, apart from a downfield shift of the $NvaNH_3^+$ signal, as was also observed for the $ArgNH_3^+$ group in the L-Arg-L-Leu-Gly tripeptide (Fig. 2). Taking into account the presented NMR data (HOHAHA experiment, ABX system for the C$^{\alpha}H_2$ of Gly and NMR experiments of model compounds) we can propose that water links the amino and carboxy ends of the molecule, as shown in Fig. 6(*a*).

On the other hand, the ArgN$^{\varepsilon}$H signal of the *N*-acetylated tripeptide (compound **d**) with free carboxylic group shifted to low fields (Fig. 7), as was also observed for the ArgN$^{\varepsilon}$H signal of L-Arg-Leu-Gly when water was progressively eliminated. Taking into consideration this observation and the fact that the ArgN$^{\varepsilon}$H signal of the tripeptide amides (compounds **a** and **b**) remained unchanged when water was taken out, we can assume that an interaction occurred between the ArgN$^{\varepsilon}$H group and the carboxylic group of the L-Arg-L-Leu-Gly tripeptide in DMSO, when bound water was eliminated, as shown in Fig. 6(*b*). Strong downfield shift of the ArgN$^{\varepsilon}$H signal has been also reported[25,26] when this group participated in a hydrogen-bonding interaction.

From the above we conclude that such conformational changes as observed for the L-Arg-L-Leu-Gly hydrate, when bound water is removed, are not particular to the Arg-containing tripeptides, but are rather due to the ability of the tripeptide sequence to form stable hydrates. Similar effects as

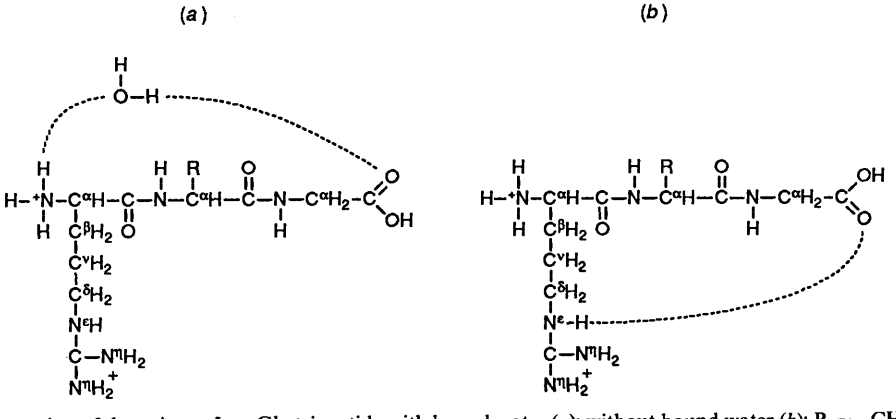

Fig. 6 Proposed conformation of the L-Arg-L-Leu-Gly tripeptide with bound water (*a*); without bound water (*b*); R = $-CH_2CH(CH_3)_2$

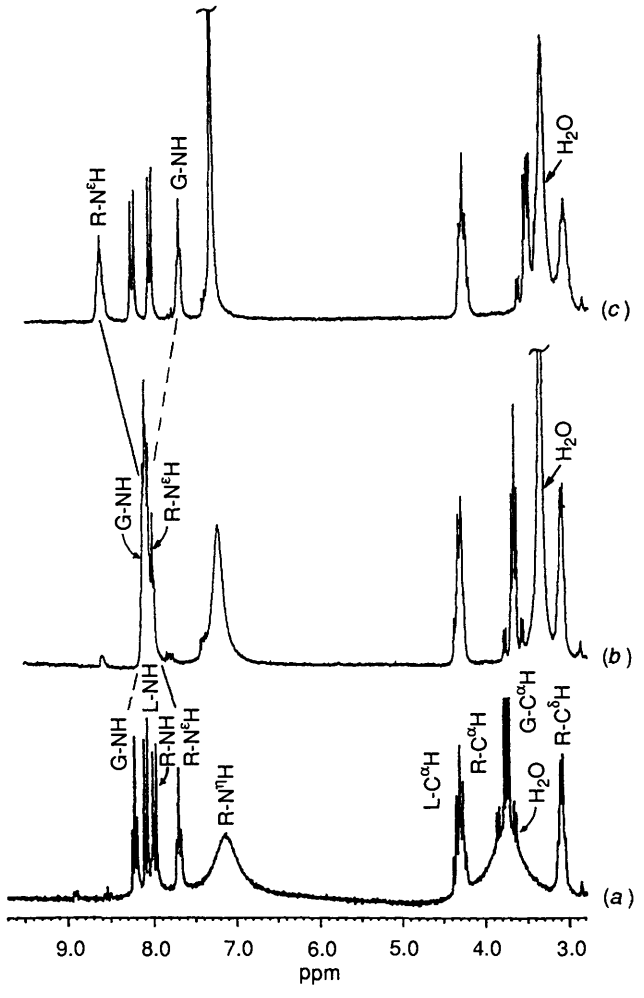

Fig. 7 Spectra of the Ac-L-Arg-L-Leu-Gly tripeptide **d** in [$^2H_6$]DMSO without molecular sieves (*a*), 10 days after addition of molecular sieves (*b*), 20 days after addition of molecular sieves (*c*)

those described previously, although less intense, were also observed in the DMSO solution of the Gly-L-Phe-L-Leu hydrate when treated with molecular sieves (Table 1).

Changes were also observed on the temperature coefficients of the NH groups of L-Arg-L-Leu-Gly and Gly-L-Phe-L-Leu hydrates before and after elimination of residual water with molecular sieves (Table 1). This fact indicates that the exposure of the amide protons to the solvent is affected by the removal of water. Intermolecular interactions were excluded in our studies, since no significant concentration dependence was observed for the NH chemical shifts in the range 3×10^{-3}–4.0×10^{-2} mol dm^{-3}.

Conclusions

The NMR results presented here point out the possible persistence of a peptide hydrate in DMSO solution when water molecules are gradually eliminated. Residual water is strongly bound to the L-Arg-L-Leu-Gly hydrate, and this contributes to the rigidity of the molecule and is not expelled by DMSO. Elimination of residual water by means of molecular sieves has an irreversible effect on the peptide and a new conformation results. It is concluded that the role of the residual water molecules in DMSO solution has to be investigated when evaluating NMR data, *e.g.* changes in the chemical shifts, the temperature coefficients and the NOE connectivities, for peptide conformational studies.

Acknowledgements

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