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A peptide corresponding to the N-terminal 13 residues of T4 lysozyme forms an α-helix

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Solid-phase methods have been used to synthesize LYS(1–13), a peptide corresponding to the first 13 residues of T4 lysozyme. 2D ^{1}H NMR techniques were used to investigate its solution structure in the presence of SDS micelles. The identification of numerous medium-range NOESY crosspeaks and several slowly exchanging NH protons indicated the presence of an α-helical structure. This was confirmed by simulated annealing calculations performed using XPLOR.

Nuclear magnetic resonance; Protein folding; Peptide fragment; T4 lysozyme; Peptide conformation

1. INTRODUCTION

In 1973 Anfinsen [1] proposed that a protein's amino acid sequence may provide sufficient information for it to fold spontaneously. Since that time there has been considerable speculation as to how much of a protein is required for spontaneous folding. Most models of protein folding involve the initial formation of local regions of secondary structure that may be stabilized by packing against other elements of local folded structure. Although there is still debate as to how the ultimate 3D structure is actually achieved, models such as the 'hierarchical condensation' model [2], the framework model [3,4], and the 'molten-globule' approach [5], all require the initial formation of these elements of secondary structure. By implication, therefore, short peptides should, in some cases at least, fold autonomously into native-like structure. As a consequence, protein fragments [6] and small linear peptides [7] have been used to model regions of structure.

Although initial attempts to identify structure in small peptides were either unsuccessful or inconclusive [8], later studies have seen elements of secondary structure observed in peptides containing as few as 5 amino acids [9]. Stable α-helices have been observed for several peptides based on native sequences, including the C-peptide of ribonuclease A [10,11], and the Pα5 peptide corresponding to the α-helix of BPTI [12]. Using the RNAse A peptide as a model, Marqusee and Baldwin [13] designed, de novo, short peptides having as much as 80% helical content at 0°C. Clearly, short peptides can exhibit considerable secondary structure. It remains therefore to relate this structure to the early events in protein folding.

2D NMR techniques, combined with quench-flow detection of hydrogen–deuterium exchange of amide proteins, have been used recently to detect and characterize kinetic intermediates observed during protein folding. Proteins studied in this manner include RNase A [14], cytochrome *c* [15], barnase [16] and hen egg white lysozyme [17]. In each case it was possible to detect the presence of intermediates with some measure of native secondary structure.

The amide proton–deuterium exchange method of determining the structure of folding intermediates has been applied recently to T4 lysozyme [18]. The results showed that helix E (corresponding to residues 93–105) and helix A (residues 3–8) are most likely to be formed early during re-folding, and that these helices may form the framework for the re-folding of the rest of the protein. In view of this, and in light of evidence that α-helices in the native state reflect the intrinsic helical tendencies of the primary structure [19], we have synthesized and determined the solution conformation of a peptide, LYS(1–13), corresponding to the first 13 residues of T4 lysozyme. The aim of the study was to determine whether this fragment (which includes helix A) would adopt a helical conformation in the absence of

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; RNAse A, ribonuclease A; NMR, nuclear magnetic resonance; Fmoc, fluorenyl methyloxycarbonyl; SDS, sodium dodecyl sulphate; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; FID, free induction decay.

the remainder of the protein. Ideally, such an investigation would be carried out in aqueous solution. However, LYS(1–13) proved to be insoluble in water at the >0.5 mM concentrations required for such a study. A co-solvent system of SDS micelles in water was chosen for two reasons. Firstly, it was found that a mixture of 200 mM SDS in water was sufficient to produce a 2 mM solution of the peptide. Secondly, surfactant micelles have been known to stabilize helical structures in peptides where the primary sequence predicted an inherent propensity towards helix formation [20–22].

2. EXPERIMENTAL

2.1. *Peptide synthesis*

The peptide (MNIFEMLRIDEGL) was assembled by standard Fmoc solid-phase procedures [23] using an automated Milligen 9050 continuous flow peptide synthesizer. The termini of the peptide were not protected. Each amino acid was coupled as its N^{α}-Fmoc protected pentafluorophenyl ester and N^{α}-Fmoc deprotection was with 20% piperidine in dimethylformamide. At the end of the synthesis, the peptide resin was treated with 95% trifluoracetic acid/3% anisole/2% ethanethiol for 5 h at room temperature. Following its isolation, a portion of the crude deprotected peptide was purified by conventional reverse-phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 support using a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The purified product was characterized by RP-HPLC and, in addition, had an acceptable amino acid analysis following acid hydrolysis.

2.2 *NMR spectroscopy*

As LYS(1–13) was insoluble in aqueous solution, samples for ^{1}H NMR spectroscopy were prepared in either 99.99% D_2O or 90% H_2O/10% D_2O with 200 mM SDS_{d25} at pH 5.4, and contained 2 mM peptide. The following spectra were recorded at 500 MHz on a Bruker AMX 500 spectrometer at 293 K: NOESY [24] with mixing times of 300 and 150 ms, TOCSY and clean-TOCSY with no trim pulses [25–28] and a mixing time of 65 ms, and DQFCOSY [29]. All spectra were recorded using the time-proportional phase incrementation (TPPI) method [30]. Typically 400 t_1 increments (600 for DQFCOSY) were acquired for 4,000 data points with 16 transients (96 for NOESY) and a spectral width of 6,024 Hz. Water suppression for the NOESY sequence was achieved using a semi-selective jump-return sequence [31] together with very mild pre-saturation. Continuous low-powered pre-saturation during the recycle delay was used to suppress the water signal for clean-TOCSY and DQFCOSY experiments.

All spectra were processed on a Silicon Graphics 4D/30 workstation using the FELIX program (Hare Research, Inc). A polynomial function was applied to each FID prior to Fourier transformation. The data were also multiplied by a 60°-shifted sine-bell window function in each dimension before transformation to produce matrices consisting of 2,000×2,000 real data points. Baseline correction on processed spectra was applied as required. Peak volume calculations on the 150 ms NOESY spectra were performed within FELIX. The volumes were classified as strong, medium, weak or very weak, corresponding to interproton distance restraints of 1.8–2.7, 1.8–3.5, 1.8–5.0, and 1.8–6.0 Å, respectively [32,33]. Appropriate pseudoatom corrections were applied to non-stereospecifically assigned methylene and methyl protons [34] and, in addition, 1.5 Å was added to the upper limits of distances involving methyl protons.

2.3. *Structure calculations*

The set of 3D structures was generated using XPLOR [35,36]. An ab initio-simulated annealing protocol [32], starting from a template structure with randomized coordinates, was applied to generate a set of 25 structures. These were subjected to a restrained Powell energy minimization [37] using a refined force-field incorporating an explicit hydrogen-bonded potential [35]. Statistics on the refined structures were also performed within XPLOR and structure superpositions were accomplished using INSIGHT (Biosym).

3. RESULTS AND DISCUSSION

3.1. *^{1}H NMR assignments*

The spin systems of the individual amino acid residues of LYS(1–13) were identified by means of through-bond connectivities found from DQFCOSY and clean-TOCSY experiments in H_20 and D_20. Incorporation of LYS(1–13) into the SDS micelle increases the effective molecular weight of the peptide many-fold, and this explains why the clean-TOCSY experiment, which is tailored for larger molecular weight compounds [28], produced less phase distortion and fewer artifacts than the conventional TOCSY. The long mixing time (300 ms) NOESY experiment was useful in confirming spin systems identified in the clean-TOCSY experiments. The procedure outlined by Wüthrich [38] was employed to make a complete sequential assignment from the 150 ms NOESY spectra in H_20 and D_20. Although the considerable overlap observed for the NH and CαH resonances of residues 5–9 made their assignments difficult, ambiguities of assignment were overcome by analysis of the CαH-CβH region of the NOESY spectrum and the identification of several medium-range connectivities involving these residues. Fig. 1 shows part of the NH region of the NOESY spectrum and the corresponding connectivities, while the complete ^{1}H NMR assignment of LYS(1–13) is provided in Table I.

3.2. *Secondary structure analysis*

As both the crystal and ^{1}H NMR structures of intact T4 lysozyme indicate a well-defined α-helical region spanning residues 3–10 [39–41], initial efforts were concentrated on detecting signs of the presence of α-helical structures in LYS(1–13). Firstly, a series of weak NH–CαH, together with stronger NH–NH, inter-residue connectivities were seen between residues 3–11. In addition, several CαH–NH_{i+3} and CαH-$C\beta H_{i+3}$ connectivities, characteristic of α-helical structures, were observed in the same region (Figure 2).

Other criteria for the determination of α-helical structures include the observation of slowly exchanging NH protons, and low values (4–5 Hz) for the $^3J_{NH-C\alpha H}$ coupling constants [38]. In proteins and larger peptides, slowly exchanging NH protons may be present after days and, in some cases, even months. However, for small peptides the detection of slowly exchanging NH protons is not common since NH protons are more exposed to the solvent. In the case of LYS(1–13) it was decided that a relatively short period would suffice for the classification of a 'slowly exchanging' NH proton. In order to detect such protons, a TOCSY spectrum of a freshly prepared solution of LYS(1–13) in D_2O/

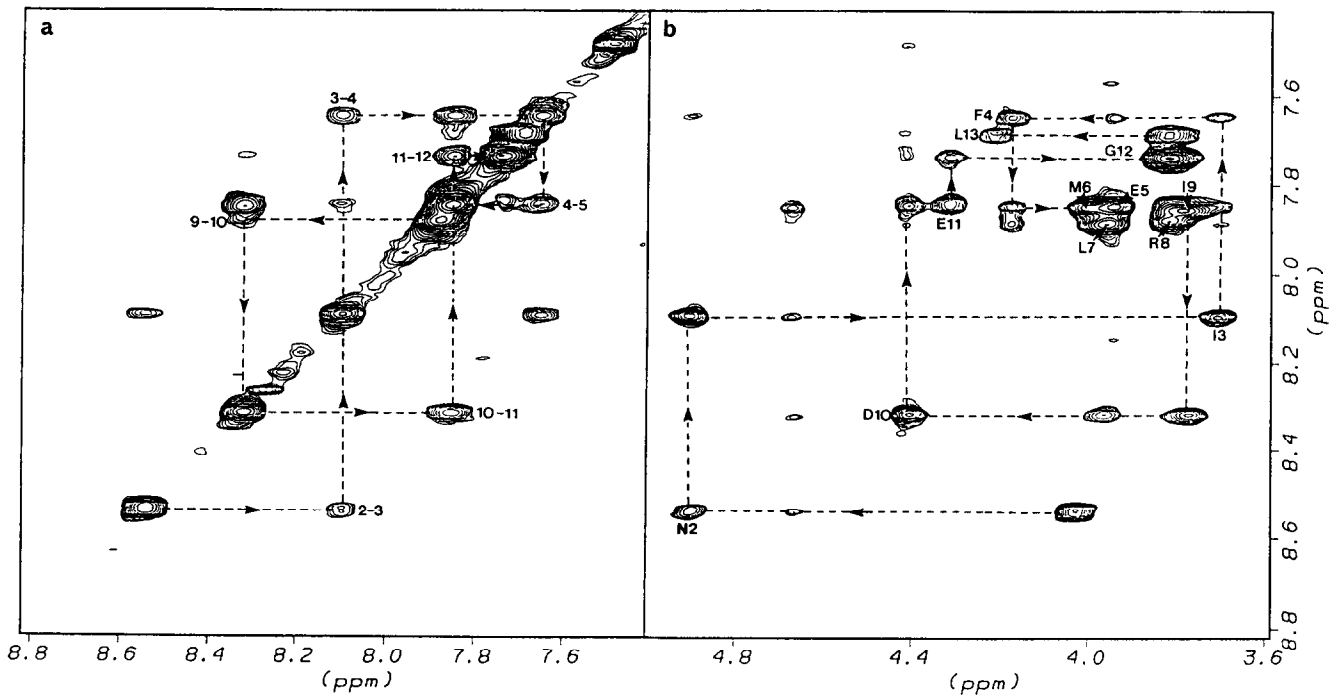

Fig. 1. (a) NH/NH and (b) NH/CαH region of the 150 ms NOESY spectrum of SDS_{d25}-bound LYS(1–13) in 90% H_2O, pH 5.4, 293 K showing sequential assignments.

micelles was run, commencing 0.5 h after dissolution and running for a period of 4 h. Several NH protons along the stretch 3–11 were detected in the TOCSY spectrum, indicating slow exchange with solvent D_2O. These are indicated in Fig. 2.

The broadness of the lines brought about by the increased viscosity due to to the SDS micelles caused the signals to be weak in the NH–CαH region of the DQFCOSY spectrum. As a consequence, coupling constants were unobtainable at reasonable accuracy, even

Table I

^1H NMR chemical shifts and resonance assignments for LYS(1–13) in aqueous SDS_{d25}, pH 5.4, at 293 K

Residue	Chemical shift (ppm)			
	NH	CαH	CβH	Other
M1		4.07	2.13	CγH 2.50 SCH_3 1.96*
N2	8.54	4.95	2.76,2.86	NH_2 6.77,7.49
I3	8.10	3.75	1.68	CγH 1.05,1.29
				$C\gamma H_3$ 0.57; $C\delta H_3$ 0.74
F4	7.65	4.22	3.06,3.09	CεH 7.02; CζH 7.02
				CδH 7.13
E5	7.85	3.97	2.00,206	CγH 2.40,2.42
M6	7.85	4.05	2.05,2.08	CγH 2.42,2.59
				SCH_3 1.98*
L7	7.88	4.01	1.48,1.76	CγH 1.76; $C\delta H_3$ 0.79
R8	7.88	3.86	1.62,1.79	CγH 1.42; CδH 3.02
				NHδ 7.03; NHε 6.53
I9	7.86	3.81	1.83	CγH 1.09,1.53
				$C\gamma H_3$ 0.81; $C\delta H_3$ 0.75
D10	8.32	4.43	2.84,2.89	
E11	7.84	4.34	1.88,219	CγH 2.30,2.39
G12	7.74	3.82		
L13	7.69	4.23	1.48–1.50	CγH 1.48–1.50;
				$C\delta G_3$ 0.78,0.80

*Assignments may be reversed

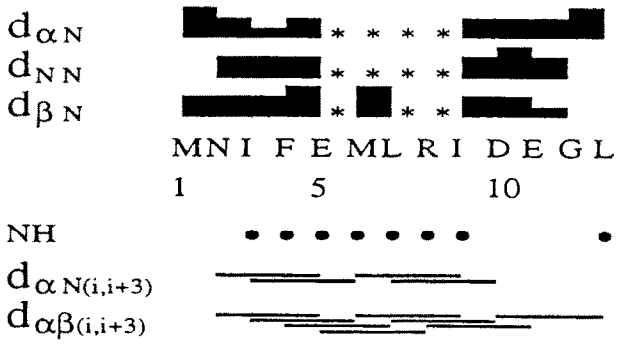

Fig. 2. Diagrammatic representation of sequential and medium range NOEs observed for LYS(1–13) in aqueous SDS_{d25}. NOE intensities for $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ connectivities are indicated by the height of the bars. Asterisks (*) represent connectivities that are not measurable due to peak overlap and filled (●) circles indicate residues for which slow exchange of the NH protons was observed.

when peak deconvolution techniques were attempted. Analysis of the $^3J_{NH-C\alpha H}$ coupling constants from the DQFCOSY spectrum of a 2 mM sample of LYS(1–13) in a 50% TFE/H_2O mixture gave values of 4–5 Hz for residues 3–10 (unpublished results). Although it cannot be assumed that small couplings will also be present for LYS(1–13) in SDS_{d25} micelles, it is likely that the inability to measure coupling constants in SDS is due to a combination of broad lines and low coupling constant values, supporting the hypothesis that a helix is present.

The NMR data provide strong evidence for the presence of an α-helix stretching from Ile-3 to at least Asp-10 and probably as far as Glu-11. The observation of 2 weak $C\alpha H–NH_{i+2}$ connectivities between residues 2 and 4 and between residues 10 and 12 indicates the possible presence of turn-like structures or 3_{10} helix [38]. The progressive increase in the strength of the $C\alpha H–NH_{i+1}$ connectivities at either end of the helix renders unlikely the possibility of 3_{10} helix formation and suggests that dynamic fraying at the helix termini is responsible for the two loosely defined turns indicated by the $C\alpha H–NH_{i+2}$ NOEs. This interpretation is supported by the simulated annealing calculations described below.

3.3 *Simulated annealing calculations of 3D structure*

The previous discussion provides an insight into the elements of secondary structure present in LYS(1–13). The self-consistency of the data and the large number of observed NOEs suggested that a more quantitative determination of the structure could be made by a simulated annealing protocol using the NOE data to provide interproton distance restraints.

In calculating quantitative 3D structures from NOE data, caution must be exercised if there is the possibility that multiple conformers may be present. In those cases the observed NOEs will represent averages over two or more conformers. For LYS(1–13) an anomalous weak connectivity was observed between the CαH proton of Asp-10 and one of the side chain amide protons of

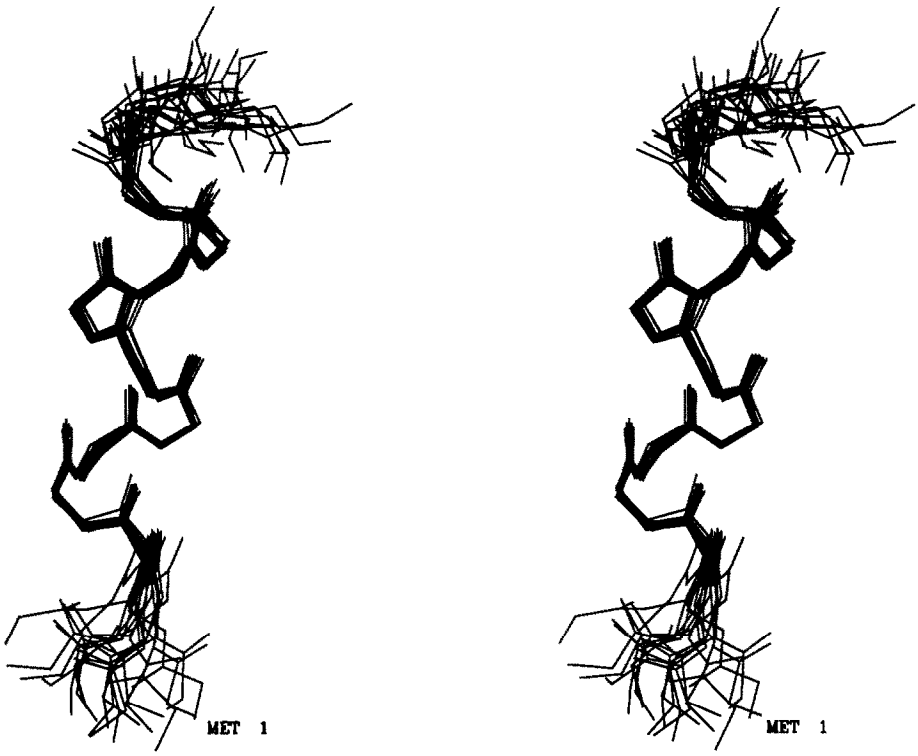

Fig. 3. Stereoview showing the best fit superposition of the backbone Cα, NH and C=O atoms of the 20 final SA structures (residues 3–11) of LYS(1–13) in SDS_{d25}.

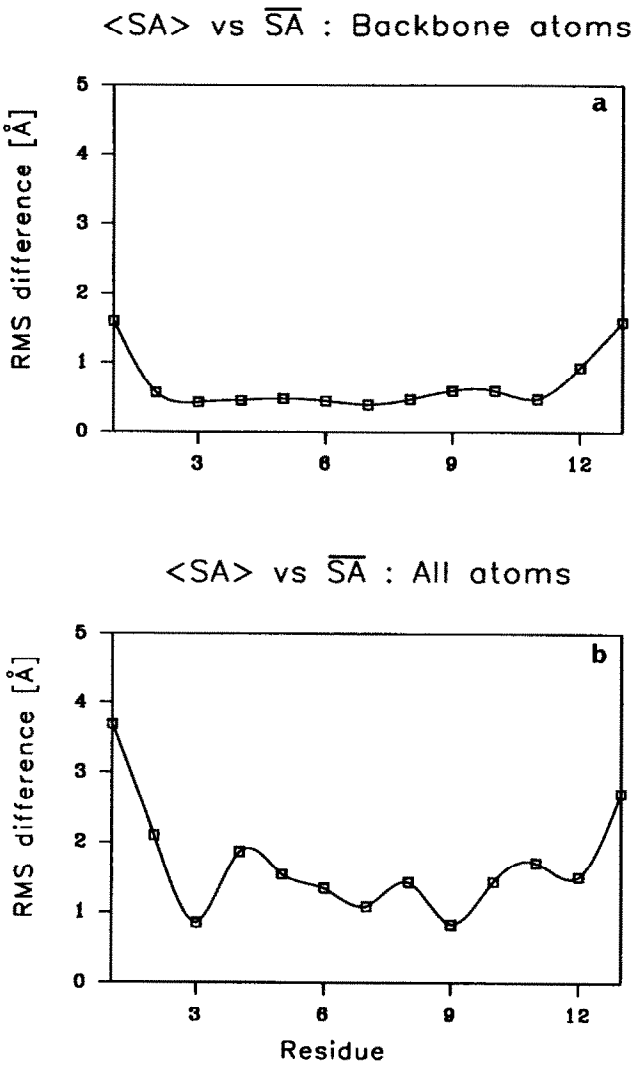

Fig. 4. Atomic RMS distribution of the 20 individual SA structures about the mean SA structure (a) for backbone atoms and (b) all atoms.

Asn-2. This seemed inconsistent with the remaining NOEs and was suggestive of a long-range interaction similar to that seen by Osterhout et al. for peptide RN-24 [42]. In that case the presence of several NOEs not consistent with helix formation was interpreted as indicating the presence of small populations of partially unfolded helix and extended structures, in rapid exchange (on the NMR time scale) with the helical conformer. This conformational ensemble has been termed 'nascent helix' [9] and is often observed, in aqueous solution, for small linear peptides with an intrinsic propensity towards helix formation [7]. The environment provided by structure inducing solvents such as TFE or SDS is normally sufficient to bring about stable helix formation for such peptides.

In the current case, the NOEs from the SDS-bound peptide will be much stronger than those arising from the presence of any small population of unfolded or partially folded conformers that may be present in the free state. To assess the contribution of unfolded populations to overall NOE strengths a procedure developed by Bradley et al. [43] was applied to LYS(1–13). In this procedure the strength of the CαH–NH_{i+1} and NH–NH NOE connectivities are used to provide an estimate of the fraction of molecules possessing an helical conformation. For LYS(1–13) the method was applied only to those residues where there was no peak overlap, namely 3–4, 4–5, 9–10 and 11–12. The average contribution of these suggests that 96% of the time averaged population is helical. This result suggests that an analysis of the observed NOEs can be made in terms of a single, predominant conformer. The lone, weak, long-range NOE observed for LYS(1–13) may result from a small population of unfolded peptide (in the free state) or, alternatively, from spin diffusion. In either case it would not be appropriate to include it in the structure calculations and it was therefore omitted from further consideration.

A total of 144 distance constraints derived from 85 intra-residue, 34 sequential and 28 medium range, unique, NOEs was used to calculate 3D structures consistent with the input NOE data. This was done using a simulated annealing protocol starting from a random coordinate set. In order to ensure full sampling of coordinate space for the starting conformation, a set of 25 structures was calculated. All simulated annealing runs converged to a common family of structures in which a helical arrangement of amino acids was readily apparent. The Φ dihedral angles were measured in this initial set of structures and it was found that for residues 3–10 all Φ angles consistently were within $-65 \pm 25°$, corresponding to $^3J_{NH-C\alpha H}$ values of 4 Hz. NH to C = O distances were also measured to identify possible H-bonding acceptors. Based on these observations and the slowly exchanging amide protons, 5 hydrogen bonding constraints were added to the initial distance constraints to calculate a further set of 25 SA structures. These were subsequently subjected to restrained energy minimization. All resultant structures satisfied the experimental constraints with no interproton distance violations greater than 0.1 Å. All of the residues in the ordered region of the structure lie within allowed regions of the Ramachandran plot.

A superposition of the backbone atoms of residues 3–11 for the best 20 structures, shown in Fig. 3, displays a well-defined α-helical region with some disorder indicative of dynamic fraying in the termini. The backbone pairwise RMSD for all residues is 1.11 Å with a standard deviation of 0.33 Å and, for residues 3–11 the corresponding values are 0.96 Å and 0.30 Å, respectively. The average RMSD to the mean structure for the backbone is 0.77 Å. Fig. 4 shows the atomic RMS distribution of the 20 individual SA structures about the mean structure and it can be seen from this that the RMS values are around 0.5 Å for residues 3–11. The low RMS values support the previous assumption that the

NOE data reflects a single predominant conformation in SDS micelles and conformational averaging, at least in the region 3–11, is either absent or marginal. The RMS values for all atoms fluctuate much more than those of the backbone atoms alone, reflecting the lack of NOEs involving side chain atoms and the fact that the side chains have greater mobility than does the backbone of the peptide.

4. CONCLUSIONS

The results of this study indicate that, in SDS micelles, the peptide fragment LYS(1–13) forms a well-ordered α-helix from residues 3–10 consistent with the region seen in previous studies of the crystal structure [39,40] and NMR studies [41] of native T4 lysozyme. This provides a further example of native sequence in a short peptide showing α-helical structure. However, what makes this peptide unusual is that it does not contain alanine, which is generally accepted as being the most helix forming of the 20 naturally occuring amino acids, particularly in short peptides [44,45]. The fact that this region, when isolated, can form secondary structure that is not stabilized by extensive tertiary interactions provides support for the hypothesis that helix A is formed early during re-folding, and conceivably provides a nucleation site for the folding of the remainder of the protein [18].

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