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Nuclear magnetic resonance assignments and secondary structure of bovine S100β protein

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Abstract S100β is a neurite extension factor and has been implicated in Alzheimer's disease and Down's syndrome. It belongs to a group of low molecular weight calcium-binding proteins containing the helix-loop-helix calcium binding motif. The structure of only one S100 protein, calbindin D_{9k}, which has the lowest sequence similarity to the other members of the S100 group has been determined. We report the NMR assignments and secondary structure of calcium-free S100β. The secondary structure is similar to that of calbindin D_{9k}, determined using NMR, except that there is clear evidence for an additional well ordered 5-residue α-helix in S100β.

Key words: S100; Calcium-binding protein; Nuclear magnetic resonance; Secondary structure; S-100

1. Introduction

S100β is a member of a group of low molecular weight acidic calcium-binding proteins which contain two helix-loop-helix calcium binding motifs [1]. One of these calcium binding loops is unusual in that it is composed of 14 rather than the normal 12 amino acids and contains four positively charged amino acids. S100 proteins also contain hydrophobic regions at the N-and C-termini which are conserved among the members of the group even when their amino acid sequences are not.

A number of biochemical activities have been reported for S100β, including effects on cytoskeletal systems, protein phosphorylation and enzyme activities [1]. In addition to these intracellular functions, a disulphide-linked dimer of S100β has neurotrophic activity on selected neuronal populations and mitogenic and morphogenic activity on astrocytes [2,3]. The levels of S100β in the brain are elevated in Alzheimer's disease and Down's syndrome, and the S100β is localised primarily in activated astrocytes surrounding neuritic plaques [4]. The pattern of S100β overexpression in Alzheimer's disease correlates with the pattern of regional involvement by neuritic plaques [5], suggesting a possible relationship between S100β levels and the regional distribution of Alzheimer neuropathology.

Among the members of the S100 family of proteins, structural information exists only for calbindin D_{9k} for which X-ray [6] and NMR [7–9] structures are available. Calbindin D_{9k} is the protein with the lowest homology to the remainder of the S100 group, having only 27% identity to S100β. It is also somewhat smaller than the other proteins and lacks the C-terminal hydrophobic region. It is therefore not clear that calbindin D_{9k} provides a complete model for the structures of the other S100 proteins. The identification of a growing class of S100 proteins and the likely neurochemical importance of S100β make the determination of the structure of S100β of interest. Two preliminary crystallisation notes have appeared [10,11] but as yet no structure has been reported. Here we present NMR assignments and secondary structural data for this protein in the absence of calcium.

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2. Materials and methods

2.1. Cloning of S100β into pET vectors

DNA was prepared from a culture of the vector pVUSB-1 containing the synthetic gene for S100β [12]. The S100β gene was then amplified using the polymerase chain reaction with the oligonucleotides CCC GGG ATG TCT GAA CTC GAG AAA GC and CCC GAT CCT TAT TCA TGT TCG AAG AAC TC for forward and reverse strands, respectively. These oligonucleotides modified the 5′ and 3′ restriction sites to *Nde*I and *Bam*HI, respectively. The resulting DNA was cut with *Bam*HI and *Nde*I restriction enzymes and ligated into cut pET11a and pET12a vectors (Novagen) using T4 DNA ligase incubation overnight at 16°C. Each of the ligation mixtures was transformed into *E. coli* DH5α cells. Colonies of these cells were grown in LB ampicillin (100 μg/ml) liquid cultures. DNA was isolated, cut with *Xho*I and *Pst*I and run on a 1% agarose electrophoresis gel. The DNA from four clones (two in pET11a and two in pET12a) which had the correct sized inserts was isolated. Dyedeoxy terminator PCR sequencing reactions were carried out as described in the ABI instructions using both a forward primer (corresponding to 20 bp in the T7 promoter region) and a reverse primer (corresponding to 18 bp in the T7 terminator region), and the sequencing gels were run on an ABI Model 372A DNA sequencing system. This confirmed the correct gene sequence. DNA with the correct DNA sequence was transformed into *E. coli* BL21 DE3 pLysS competent cells and then single colonies from these plates plated onto LB ampicillin (100 μg/ml), chloramphenicol (20 μg/ml) agar plates. These colonies were used for protein expression.

2.2. Expression and purification

S100β was expressed from the pET12a-S100β construct in both 2xYT media and 2xM9 media containing 1 g/l of [^{15}N]ammonium chloride as the sole nitrogen source. Cultures were grown in 500 ml batches of media in 2-litre flasks shaken at 37°C. Media contained 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. When the A_{600} reached 1.0 for the 2xYT cultures and 0.7 for the 2xM9 cultures expression was induced by the addition of 0.5 mM IPTG. After 3 h of further growth cells were harvested by centrifugation (8,000 × *g*, 8 min, 4°C). Cell pellets were frozen overnight at −20°C, thawed and resuspended in 100 ml of 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM β-mercaptoethanol and disrupted using a French press. After centrifugation as before, the supernatant was made 1% in streptomycin sulphate by the dropwise addition of a 20% solution and stirred at 4°C for a further 30 min. The solution was centrifuged as before, the supernatant made 80% in ammonium sulphate (591 g/l), stirred at 4°C for a further 30 min and then centrifuged (12,000 × *g*, 12 min, 4°C). The pH of the supernatant was reduced to 4.1 by the addition of 50% sulphuric acid solution. The solution was stirred at 4°C for one hour, centrifuged and the pellet resuspended in the original isolation buffer and dialysed overnight at

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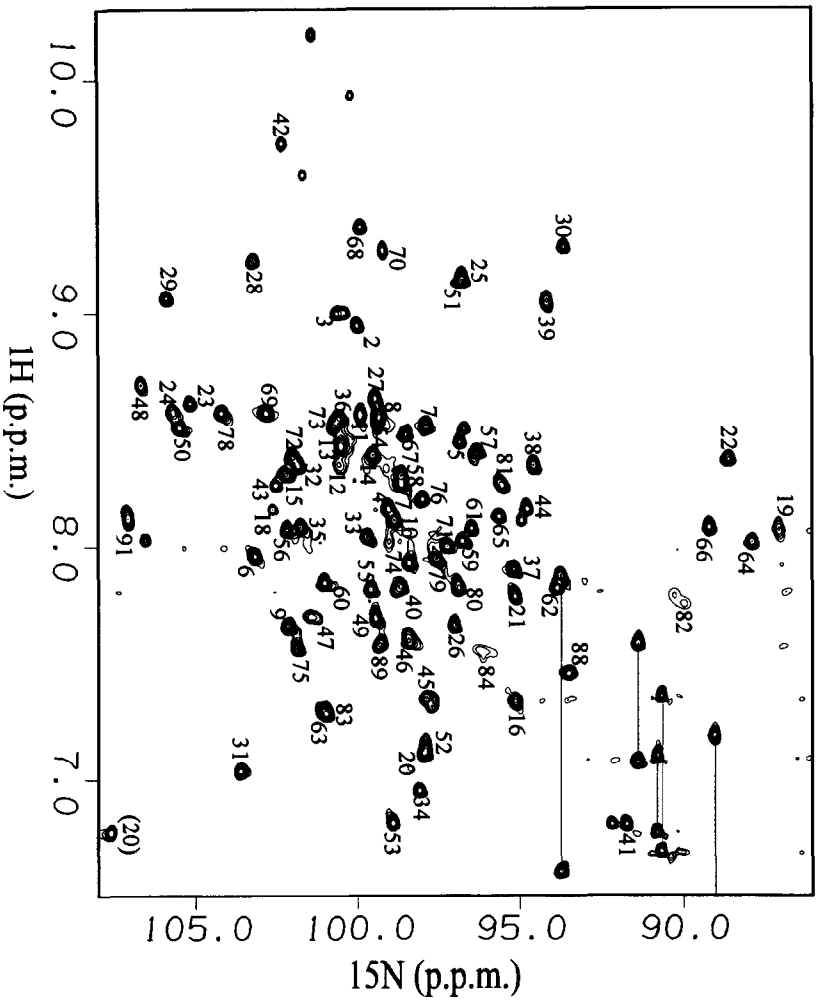


Fig. 1. HSQC spectrum of ^{15}N-labelled S100β Crosspeaks are labelled with the amino acid residue number. Sidechain amide pairs of crosspeaks are shown joined by horizontal lines. The resonance of the sidechain NH of Arg-20 is labelled (20) and is folded about the top of the spectrum.

4°C against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 50 mM sodium chloride. The pH of the dialysed protein was adjusted to 8.5 and the solution loaded onto a Hi-Load Q-Sepharose anion exchange column which had been equilibrated in 20 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol. The protein was eluted with a gradient of 0–0.6 M sodium chloride in the same buffer. The protein eluted at 0.4 M sodium chloride. The protein peak was reduced in volume to below 10 ml using a stirred ultrafiltration cell (Amicon) with a 3K cutoff membrane and then loaded onto a Hi-Load Superdex 75 gel filtration column equilibrated in 10 mM sodium phosphate buffer pH 7.0, 5 mM β-mercaptoethanol and 0.1 M sodium chloride. The S100β eluted after about 165 ml. Fractions containing S100β were buffer exchanged by ultrafiltration into water containing 5 mM [$^2H_4$]succinate, pH 6.5, 0.5 mM [$^2H_{10}$]dithiothreitol and then lyophilised.

2.3. Analysis

N-terminal amino acids were determined by automated Edman degradation on an ABI sequencer. SDS-polyacrylamide gel electrophoresis was carried out using the method of Shägger and Von Jagow[13] in the presence or absence of β-mercaptoethanol.

2.4. NMR spectroscopy

Samples for NMR contained 6.9 mM S100β (29.3 mg) dissolved in 400 μl of 20 mM [$^2H_4$]succinate (pH 6.3 for 90% H_2O/10% ^2H_2O samples and pH* 6.1 for ^2H_2O samples), 8 mM [$^2H_{10}$]dithiothreitol. The volume over the sample was purged with nitrogen and the tube sealed. Nmr spectra were acquired either on a Bruker AM500 spectrometer or a Bruker AMX600 spectrometer. For samples in ^2H_2O, spectra were acquired with presaturation of the residual H^2HO resonance for 1 s with about 2 W of rf power. In H_2O experiments weaker presaturation was combined with 'jump and return' [14] selective excitation using a 100 μs delay between the pulses. The acquisition used a 6024 Hz spectral width for spectra recorded in D_2O and 7200 Hz for spectra recorded in water, and a proton pulse width of about 8 μs (70°).

Two-dimensional homonuclear spectra were acquired with 2k data points in ω_2 and 512 increments in ω_1. Sine modulation was used in the acquisition of phase sensitive data using the TPPI method [15]. 64–128 scans were acquired per increment so that each spectrum took 12–24 h to acquire. All homonuclear spectra were acquired at 314k. Heteronuclear ^{1}H-^{15}N spectra were acquired at a temperature of 311k with a 1400 Hz (23 ppm) spectral width for ^{15}N, using GARP ^{15}N decoupling. NOESY (100 ms mixing time) and Double Quantum Filtered COSY experiments were acquired using standard methods [16,17]. TOCSY spectra were acquired using the MLEV-17 [18] mixing sequence and mixing times of 30–80 ms. 2D HSQC spectra were acquired with 256 increments in ω_1. 3D HMQC-NOESY (100 ms), 3D HMQC-TOCSY (45 ms) and 3D HMQC-NOESY-HMQC (125 ms) experiments were carried out using standard pulse sequences [19]; the spectra were acquired with 512 complex points in the ω_3 (acquisition) dimension, respectively 190, 160 and 64 points in the ω_1 dimensions and 72, 60 and 64 points in the ω_2 (^{15}N) dimensions.

Two-dimensional data were zero-filled to 1024 data points in the ω_1 dimension and multiplied by a Gaussian window function before two-dimensional Fourier transformation. Three-dimensional data were processed using Felix software (Biosym). The data were multiplied by

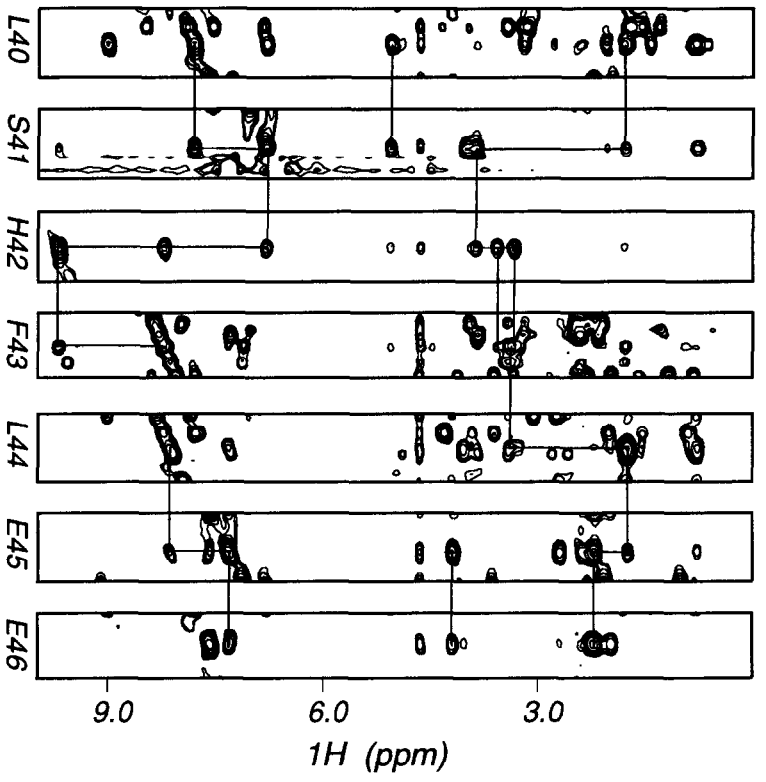


Fig. 2. Assignment of the α-helix running from Ser-41 to Glu-45. Slices from a 3D HMQC-NOESY (100 ms mixing time) experiment show the way in which assignments were established for this region of the protein using inter-residue nOes. Lines trace out the pathways used in the assignment through the strong NH_i-NH_{i-1} and NH_i-βH_{i-1} nOes. The pathway via the weak NH_i-αH_{i-1} nOes is also shown where this is visible. The NH_i-NH_{i+1} nOes between F43-L44 and E46-I47 were observed in an HMQC-NOESY-HMQC experiment (not shown).

a Gaussian function and zero filled to 1024 complex points in the ω_3 dimension before Fourier transformation. The ω_2 and ω_3 data were multiplied by a 70°-shifted sine bell squared function and zero filled to 512 real points if the dimension was for ^1H or 128 points if for ^{15}N. Proton chemical shifts are reported relative to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) and ^{15}N chemical shifts relative to 5 M $^{15}NH_4NO_3$ in 2 M nitric acid.

3. Results and discussion

3.1. Expression and purification of S100β

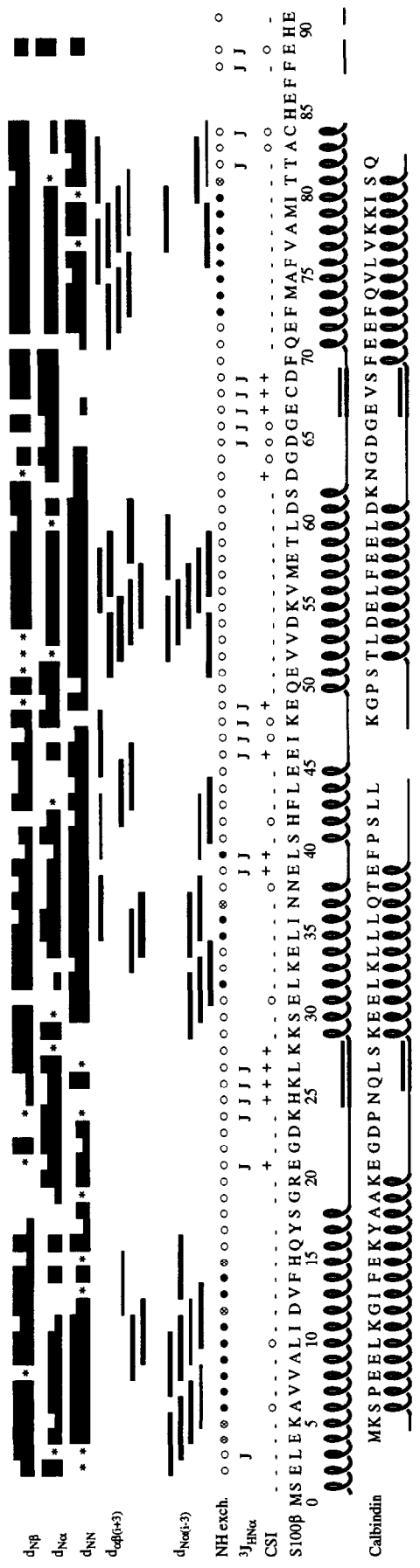
In order to obtain high-level expression of S100β for NMR spectroscopy, and to facilitate ^{15}N-labelling of the protein, the synthetic gene encoding the bovine S100β amino acid sequence [12] was subcloned into the T7-based expression vectors pET12a and pET11a. The pET12a system was used for expression in *E. coli* BL21 (DE3) pLysS cells. For isotopically normal protein, cells were grown in rich 2xYT medium, while for ^{15}N-labelling minimal 2xM9 medium containing $^{15}NH_4Cl$ was used. Yields of pure S100β protein were 57 mg/litre culture for 2xYT and 29 mg/litre culture for 2xM9 medium.

3.2. Characterisation of protein

The expressed protein co-migrated with bovine brain S100β on SDS gels, confirming previous studies [12]. Electrospray mass spectrometry showed that the expressed protein had a molecular mass of 10671.77 ± 1.1 (calculated 10668.55). The sample contained another minor component with a molecular weight of 10541 arising from protein molecules lacking the N-terminal methionine. N-Terminal sequencing confirmed the sequence of the first six residues to be Met, Ser, Glu, Leu, Glu, Lys and confirmed that the sample was a mixture of molecules with and without the N-terminal methionine. The mass obtained from electrospray mass spectrometry indicates that the protein isolated from the *E. coli* expression system is fully reduced S100β and not the disulphide bridged dimer; this was confirmed by a non-reducing SDS gel. The linewidths of the NMR resonances of S100β, which are unchanged for protein concentrations of 1.7–6.9 mM, imply that the protein, although fully reduced, is not a monomer in solution. The exact state of self association remains to be established. Addition of up to 2 mM d_{10} EDTA to a sample caused no linewidth or chemical shift changes. Conversely the addition of calcium chloride solution caused both linewidth and chemical shift changes and allowed us to conclude that our preparation of S100β was calcium free.

3.3. Resonance assignment

The ^1H-^{15}N HSQC spectrum of ^{15}N-labelled S100β is shown in Fig. 1; it can be seen that there is excellent resolution of the backbone amide resonances in this spectrum. This facilitated the assignment of the proton and ^{15}N resonances of S100β, using three 3D ^1H-^{15}N experiments and the conventional approach of spin-system identification by through-bond connectivities and sequential assignment by through-space connectivities [20]. A 3D HMQC-TOCSY spectrum was used to assign the spin systems loosely into groups of possible amino acids on



the basis of the βH chemical shifts and γH/δH shifts if these were present. Due to the short spin–spin relaxation times of the protons of this protein, and the fact that many of the NH-αH scalar coupling constants are small (see below), many residues showed no connectivity beyond the βH, and the absence of further connections could not be used to rule out possible spin-system assignments.

The sequence-specific assignments relied on the identification, in the 3D HMQC-NOESY spectrum, of nOes from backbone NH protons to the NH, Cα or Cβ protons of the preceding residue. In this way short sequences of spin-systems were constructed which could only be fitted into one place in the amino acid sequence. The assignment of resonances of residues from Leu-40 to Glu-46 is shown in Fig. 2. In this process the 3D HMQC-NOESY-HMQC spectrum was particularly useful since nOes between NH protons with very similar chemical shifts could be detected provided that the shifts of the directly-bonded nitrogens were different. Assignments of 87 out of 91 backbone amides have been made in this way; these are shown in Fig. 1, and the nOe connectivities are summarised in Fig. 3. The four residues for which unambiguous assignments have not yet been possible are 85–87 and 90, all close to the C-terminus of the protein; in addition, the resonances of Met-0, present in most but not all of the molecules, have yet to be identified. Resonances of the C-terminal Glu-91 were tentatively assigned on the basis of four pieces of evidence: (i) its low field ^{15}N shift – the ^{15}N resonance of the C-terminal residue is often at low field and all the other resonances in this region of the spectrum have been assigned; (ii) this resonance has a long ^{15}N spin-lattice relaxation time compared to other residues in the protein; (iii) the chemical shifts of the other protons in this residue were close to those expected for glutamate; and (iv) the ^{15}N {^{1}H} nOe for this resonance was negative. The chemical shifts of all assigned proton and ^{15}N resonances are summarised in Table 1.

3.4. Secondary structure determination

The available NMR data which provide information on the secondary structure of S100β are summarised in Fig. 3; these include short- and medium-range nOes, NH exchange rates, NH-CαH coupling constants and the chemical shift index of Wishart et al. [21]. The slowly-exchanging NH protons were identified by obtaining an HSQC spectrum within one hour of dissolving the protein in ^2H_2O and again after 24 h. NH protons which had not exchanged fell into three regions of the sequence, as shown in Fig. 3. An HMQC-J experiment was carried out to determine the values of the NH-CαH coupling constants. For most residues, the coupling constant was too small to lead to an observable splitting of the peak; those residues which do

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Fig. 3. NMR data for S100β protein. The neighbouring residue nOe connections are shown by vertical bars. NOe intensity is indicated by the height of the bar. Where overlap prevents observation of nOes between neighbouring residues * is shown. Medium range nOes are denoted by horizontal lines where the width of the line indicates nOe intensity. Amide proton exchange is shown by ●, ⊗ and ○ for slow, medium and fast exchange, respectively. Large (~7–8 Hz) NH-CαH couplings are shown by J. Chemical shift indices are shown as +, ○ and − for values of +1, 0 and −1. Predicted secondary structure is shown with the sequence and secondary structure for calbindin D_{9k} aligned under those for S100β.

Table 1
NMR assignments of S100β protein at pH 6.3 and 311 K

	N[a]	NH[b]	αH	βH[c]	Other
Met-0					
Ser-1		9.00	4.82	4.28	
Glu-2	100.1	8.95	4.04	2.07	γ2.45
Leu-3	100.5	8.97	4.04	1.83	γCH_3 1.56, δCH_3 0.77, 1.04
Glu-4	99.07	8.17	3.35	2.25	
Lys-5	96.88	8.45	3.89	1.87	
Ala-6	103.2	7.96	4.29	1.71	
Val-7	97.9	8.52	3.67	2.32	γCH_3 1.19, γCH_3 0.95
Val-8	99.4	8.57	3.50	2.23	γCH_3 0.99, γCH_3 1.25
Ala-9	102.2	7.65	4.30	1.62	
Leu-10	98.8	8.11	4.10	2.56	1.77
Ile-11	99.9	8.59	3.39	1.75	CH_3 0.10, CH_3 0.15
Asp-12	100.9	8.40	4.43	2.88	
Val-13	100.6	8.46	3.82	2.22	γCH_3 1.29, γCH_3 1.23
Phe-14	99.6	8.39	3.50	3.15	Hδ 6.23, Hε 7.04, Hζ 7.30
His-15	102.3	8.32	3.85	2.39, 2.17	
Gln-16	95.1	7.34	4.13	2.61	
Tyr-17	100.6	9.56	4.00		
Ser-18	102.5	8.17			
Gly-19	87.0	8.03	3.71, 3.96		
Arg-20	98.0	7.15	4.17	1.82	γ 1.59 δ 3.17, NH_ε 6.76, ^{15}N 65
Glu-21	95.2	7.80	4.45	1.90, 2.12	
Gly-22	88.6	8.37	3.67, 3.85		
Asp-23	105.2	8.59	4.44	2.65	
Lys-24	105.5	8.57	4.25	1.54	
His-25	96.6	9.15	4.91	3.65, 3.24	
Lys-26	97.1	7.66	5.28	1.72	γ 1.27, δ 1.50
Leu-27	99.6	8.62	4.80	1.37, 1.47	γ 1.38, δ 0.55
Lys-28	103.2	9.20	4.87	1.67, 2.33	
Lys-29	105.9	9.06	3.79	2.28	
Ser-30	93.8	9.28	4.15	3.11, 2.85	
Glu-31	103.6	7.05	4.33	2.52, 2.02	
Leu-32	101.9	8.34	3.81	2.39, 2.12	
Lys-33	99.6	8.04	3.47	1.95	
Glu-34	98.1	6.96	3.98	2.28	
Leu-35	101.7	8.09	3.42	1.60	γ 0.81, δCH_3 0.40, 0.62
Ile-36	100.5	8.55	3.20	1.76	γCH_3 0.77
Asn-37	95.2	7.89	4.19	2.65	NH_2 7.19, 6.33, ^{15}N 88.9
Asn-38	94.6	8.36	4.65	2.77, 3.05	NH_2 7.57, 7.07, ^{15}N 94.4
Glu-39	94.2	9.04	4.89	1.69	2.40
Leu-40	98.0	7.83	5.06	2.03, 1.81	γ 1.45, δCH_3 0.63, 0.84
Ser-41	91.8	6.84	3.83	4.02	
His-42	102.3	9.74	4.65	3.31, 3.56	
Phe-43	102.4	8.25	4.51	3.37	Hδ 7.14, Hε 7.34, Hζ 7.41
Leu-44	95.0	8.18	4.02	1.71	
Glu-45	97.79	7.38	4.18	2.22	
Glu-46	98.2	7.59	4.55	1.97, 2.19	
Ile-47	101.4	7.68	3.95	1.79	γCH_3 0.74, δCH_3 1.12, 0.90
Lys-48	106.6	8.78	4.37	1.68	γ 1.47
Glu-49	99.44	7.69	4.74	2.18, 1.95	
Gln-50	105.4	8.49	3.68	1.94	γ 2.37
Glu-51	96.9	9.13	4.09	2.03	
Val-52	97.89	7.18	3.64	2.07	γCH_3 0.81, 1.01
Val-53	98.9	6.86	3.34	2.11	γCH_3 0.91, 0.91
Asp-54	99.47	8.52	4.25	2.65	
Lys-55	99.7	7.80	4.13	1.98	γ 1.49
Val-56	101.9	8.07	3.60	2.31	γ 0.83, 1.18
Met-57	96.4	8.43	4.08	2.25	
Glu-58	98.7	8.32	4.03	2.11	
Thr-59	96.7	7.99	3.95	4.48	γCH_3 1.36
Leu-60	101.0	7.85	3.89	1.69	γ 1.67, δCH_3 0.23, 0.43
Asp-61	96.5	8.07	4.47	2.61	
Ser-62	93.9	7.82	4.30	3.99	
Asp-63	101.1	7.31	4.98	2.52	
Gly-64	87.9	8.00	3.84, 3.89		
Asp-65	95.8	8.13	4.86	2.57, 2.80	
Gly-66	89.2	8.08	3.83, 4.12		
Glu-67	98.6	8.47	5.00	2.34, 1.93	
Cys-68	100.0	9.37	5.15	2.68, 3.02	
Asp-69	102.3	8.57	5.80	3.55, 2.74	

Table 1 (*continued*)

	N[a]	NH[b]	αH	βH[c]	Other
Phe-70	99.2	9.23			7.04, 7.28
Gln-71	97.2	8.02	3.42	2.10	
Glu-72	102.0	8.39	3.94	2.31	
Phe-73	100.7	8.51	4.34	3.50, 3.22	H_δ 6.85, H_ε 7.05
Met-74	98.4	7.92	3.12	1.72	
Ala-75	101.9	7.58	3.99	1.21	
Phe-76	98.1	8.20	4.28	3.04, 2.79	
Val-77	98.64	8.25	3.21	1.67	γCH_3 0.30, 0.59
Ala-78	104.2	8.58	3.92	1.39	
Met-79	97.6	7.96	3.86	2.15, 1.86	
Ile-80	97.0	7.81	3.56	2.00	γCH_3 0.91
Thr-81	95.48	8.21	3.63	4.30	γCH_3 0.91
Thr-82	89.8	7.72	3.31	3.92	γCH_3 0.68
Ala-83	101.1	7.30	4.45	1.60	
Cys-84	96.3	7.59	4.70	2.87, 2.75	
His-85					
Glu-86					
Phe-87					
Phe-88	93.7	7.46	4.03	3.24, 2.92	
Glu-89	99.2	7.60	4.20	2.15, 1.79	
His-90					
Glu-91	107.1	8.09	4.02	2.13, 1.85	

[a] ^{15}N chemical shifts are referenced to 5 M $^{15}NH_4NO_3$ in 2 M HNO_3 at 0 ppm.
[b] ^1H shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) at 0 ppm.
[c] Where only one βH chemical shift is shown the shifts for both β protons are degenerate. Blanks are left for unassigned atoms.

have resolved NH-CαH couplings are indicated in Fig. 3. Examination of the short and medium range nOes indicate a substantial, approximately 60%, α-helical content of this protein as would be expected from its sequence similarity to calbindin D_{9k}, and this is supported by using the chemical shift index method of Wishart et al. [21]. The deduced secondary structure of S100β is compared to that seen for calcium free calbindin D_{9k} in solution [22] in Fig. 3, the two sequences having been aligned using the Cameleon program (Oxford Molecular Ltd.). With this alignment, Met-0 of calbindin corresponds to residue 4 of S100β; the first α-helix of S100β includes the 'extra' N-terminal residues, and extends to approximately residue 18. The majority of this helix is very well-defined by NH_i-NH_{i+1}, $H\alpha_i$-NH_{i+3} and $H\alpha_i$-$H\beta_{i+3}$ nOes, and slow exchange is observed for the NH protons in the central part of the helix. Towards its C-terminus, the helix is less clearly defined by nOes; its exact end is hard to define using the present data, but it does appear to stop two or three residues short of the corresponding helix in calbindin. The lack of definition of the end of this helix is caused in part by resonance overlap but mainly by the weak resonances of Ser-18 and Tyr-17 in the HSQC spectra which prevented the observation of medium range nOes to or from these residues. The second helix, from Lys-29 to Asn-38, is also well-defined by nOes, and corresponds closely to the second helix in calbindin. There then appears to be a gap of two residues, suggested by the chemical shift index and by the observation of large NH-CαH coupling constants.

This is followed by an additional five residues of helix, Ser-41 to Glu-45, well defined by NH_i-NH_{i+1}, $H\alpha_i$-NH_{i+3} and $H\alpha_i$-$H\beta_{i+3}$ nOes. This α-helix is not observed in the solution secondary structure of calbindin [22] but is in a similar location to the 'irregular helix' from residues 36 to 41 seen in the crystal structure of calbindin [6]. In a recent solution structure of calbindin this region is described as an 'ill-defined ten residue loop', and in calbindin medium range $H\alpha_i$-NH_{i+3} and $H\alpha_i$-$H\beta_{i+3}$ nOes are not observed in this region [22,23]. Preliminary measurements of ^{15}N (^1H) nOes demonstrate that residues 41–46 have similar large nOe values as the residues in the surrounding helices, supporting the conclusion that this region is a well-ordered α-helix in S100β.

The helix from Gln-50 to Asp-62 again corresponds to a helix in calbindin, but is perhaps slightly longer. The C-terminal helix begins at Glu-71, and is well-defined by nOes up to Cys-84. Between this point and the C-terminus are several residues whose resonances have not yet been assigned, due to a lack of clear nOes. This may be due to a lack of well-defined structure in this region of the protein, and the observation of broad HSQC peaks for Thr-82 and Cys-84 would be consistent with a looser, only partially helical conformation towards the C-terminus of the protein. In addition to the extensive α-helical structure, there also appears a four residue β-strand involving residues His-25 to Lys-28, and another three residue β-strand involving residues Glu-67 to Asp-69, residues at the ends of the two calcium binding loops. These are clearly indicated by the chemical shift index method and by the observation of large NH-CαH couplings, but only in the latter case is there very clear nOe evidence. The only inter-strand nOe yet observed is an NH-NH nOe between Leu-27 and Cys-68.

The results presented here show that S100β has a generally similar secondary structure to calbindin D_{9k}. However, a three residue insert compared to calbindin has resulted in the formation of a new helix not seen in calbindin. A more detailed comparison must await completion of the side-chain resonance assignments, which is currently in progress with the aid of combined ^{15}N and ^{13}C labelling, and a full tertiary structure determination.

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