Solution conformation of a peptide corresponding to bovine κ-casein B residues 130–153 by circular dichroism spectroscopy and ^{1}H-nuclear magnetic resonance spectroscopy

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Summary. The peptide Pro^{130}–Thr–Ser–Thr–Pro–Thr–Ile–Glu–Ala–Val–Glu^{140}–Ser–Thr–Val–Ala–Thr–Leu–Glu–Ala–Ser–Pro^{150}–Glu–Val–Ile, which corresponds to residues 130–153 of κ-casein B, was synthesized and the conformation of the peptide in solution investigated by circular dichroism (CD) spectroscopy, structure prediction algorithms and ^{1}H-nuclear magnetic resonance spectroscopy. In a solution containing the structure-enhancing solvent trifluoroethanol the CD spectrum was typical of a peptide in the α-helical conformation and nuclear magnetic resonance showed that the amino acids between Ile^{136} and Ser^{149} (κ-casein numbering) were predominantly in the α-helical conformation, but that Pro^{130} to Thr^{135} and Pro^{150} to Ile^{153} were not. In addition, Thr^{133}–Pro^{134} and Ser^{149}–Pro^{150} were primarily in the *trans* conformation, the residues from Thr^{131} to Thr^{135} were in unordered structures and the residues from Glu^{151} to Ile^{153} were in an extended conformation. Residues Glu^{137} to Glu^{140} and Thr^{145} to Ala^{148} also displayed some 3_{10}-helix character. When the peptide was dissolved in 10 mM-cetyltrimethylammonium chloride solution at pH 6, the CD spectra indicated that the proportion of helical structure was comparable to that of the peptide in trifluoroethanol solution (400 ml/l), whereas when the peptide was dissolved in buffer alone or in 10 mM-SDS solution, the CD spectra were consistent with a low helical content. Acidification of these solutions to pH 2·85 resulted in a slight increase in the helical content of the peptide in buffer and more markedly in buffer containing SDS. When the peptide was in 5 mM-$CaCl_2$ solution at neutral pH, the CD spectrum indicated that some ordered structure was present. Taken together these results indicate that the ionizable residues Glu^{137}, Glu^{140}, Glu^{147} and Glu^{151} could be important in determining the stability of the putative helix. The structure predictions found that the sequence from Glu^{137} to Pro^{150} would be more likely to be in a helical than any other conformation in the intact bovine protein, but that pig, sheep and goat κ-caseins did not give a prediction of a strongly helical region in this part of the molecule.

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The important role of κ-casein in stabilizing the casein micelle in milk and during dairy manufacturing procedures is well known. The protein is considered to be on the surface of the native micelle and with the caseinomacropeptide (CMP), the hydrophilic C-terminal region of the molecule which carries many of the anionic residues, protruding from the surface of the micelle. By contrast the N-terminal region is more compact, relatively hydrophobic, contains all of the cationic (Lys and Arg) residues of κ-casein and is suggested to be the segment that is strongly associated with the other casein molecules of the micelle.

CMP, which can be cleaved from the N-terminal region (para-κ-casein) by aspartate proteinases, has been considered to contain very little of the α-helix or β-sheet secondary structures that comprise the essential skeleton of most proteins (Holt & Sawyer, 1988). Loucheux-Lefebvre *et al.* (1978) predicted the existence of two pieces of extended β-strand and one helix near residue 140 in the CMP regions of bovine and ovine κ-casein, and this has recently been confirmed by Kumosinski *et al.* (1991). Recent spectral studies (Griffin & Roberts, 1985; Ono *et al.* 1987) indicate that, whereas bovine CMP at neutral pH may contain some helix, there is likely to be substantial quantities of extended structure.

As a prelude to examining the intact CMP, an examination of the peptide corresponding to residues 130–153 of κ-casein has shown that helical structures are present in solution under some conditions.

EXPERIMENTAL

Structure predictions

The procedures outlined by Creamer *et al.* (1983) were initially applied to bovine κ-casein A and B. The improved version of the Garnier *et al.* (1978) algorithm was also used by applying the program PLOT.A/GOR (Luttke, 1990*a*), from the Mac Prot suite of programs, on a Macintosh SE computer. The directional window or moving average was set to eight and the decision constants for helix and sheet for all proteins examined were set to zero for comparison purposes.

The effects of post-translational substituents (phosphorylation and glycosylation) were not taken into account in the calculations. It seems likely that the effects on structure of glycosylation and phosphorylation, where known, would be comparable for the various species. The sequences were obtained from Mercier *et al.* (1976), Brignon *et al.* (1985) and Levine *et al.* (1992).

Peptide synthesis

The peptide Pro–Thr–Ser–Thr–Pro–Thr–Ile–Glu–Ala–Val–Glu–Ser–Thr–Val–Ala–Thr–Leu–Glu–Ala–Ser–Pro–Glu–Val–Ile was synthesized using a 4-hydroxymethyl-phenoxymethyl-copolystyrene–divinylbenzene (10 g/l) resin as a solid support with modified Fmoc (9-fluorenylmethyloxycarbonyl) solid phase peptide synthesis cycles (LKB, 1987; Atherton & Sheppard, 1989; Fields & Noble, 1990). An Applied Biosystems 431A peptide synthesizer (Applied Biosystems, Foster City, CA 94404, USA) was used for the peptide assembly; Fmoc-amino acids were activated for coupling using di-isopropylcarbodi-imide–*N*-hydroxybenzotriazole. The peptide was cleaved from the support using a mixture of 0·75 g phenol, 0·5 ml water, 0·5 ml thioanisole, 0·25 ml 1,2-ethanedithiol and 10 ml trifluoroacetic acid. The peptide was purified by HPLC using a Synchroprep C_{18} 15 μm bead column (Synchrom, Lincoln, IN 47955-9990, USA) and characterized by HPLC and amino acid analysis. The trichloroacetic acid was removed by dissolving the peptide in HCl (10 ml/l) followed by freeze drying. This was repeated three times. The peptide gave a single peak on

HPLC, had the expected amino acid ratios on an LKB 4151 Series 2 Alpha Plus amino acid analyser (Amrad-Pharmacia, S-751 82 Uppsala, Sweden) and gave a single peak of the expected molecular mass (2444 Da) by fast atom bombardment–mass spectroscopy on a VG70-250S double focusing magnetic sector mass spectrometer (Micromass UK Ltd, Altrincham WA14 5RZ, UK).

Chemicals used were all of analytical quality or the purest available.

Circular dichoism spectroscopy

Circular dichroism (CD) spectra were obtained using a Jasco model 720 spectropolarimeter (Jasco, Tokyo 192, Japan). The wavelength calibration was checked with benzene vapour (266·7 nm) and a neodymium filter (585·9 nm) using the absorbance mode, and the sensitivity and rotation at 290·5 nm were checked using pure ammonium D-10-camphorsulphonic acid from Katayama Chemical, Japan, and supplied by Jasco. Peptide solutions were measured in a 1 mm cell over the range 185–250 nm (scanned at 20 nm/min, 2 s time constant, 1 nm bandwidth, sensitivity at 10 millidegrees) and five scans were accumulated per sample.

The peptide concentrations were in the range 0·04–0·05 mM in 20 mM-NaCl solution. The molar concentration of the peptide in these solutions was calculated from the sample weight without correction for peptide content. The pH of the solution was adjusted using 0·1 M-NaOH or 0·1 M-HCl. On occasion $CaCl_2$ was added to a final concentration of 5 mM. On other occasions, SDS or cetyltrimethylammonium chloride (CTAC) was weighed and added to the existing peptide solution to give a final concentration of 10 mM in SDS or CTAC. CTAC was prepared from cetyltrimethylammonium bromide by passage through an Amberlite IR45 column previously equilibrated with 3 M-NaCl. For the studies involving the effect of trifluoroethanol (TFE), the peptide solution was divided into two aliquots. One of these was freeze dried and then redissolved in TFE–water (60:40, v/v). Portions of these two solutions were then mixed in the appropriate ratios to produce solutions containing 100, 200, 250, 300, 400 and 500 ml TFE/l with the same peptide concentration.

NMR spectroscopy

The peptide was dissolved in ^2H_2O–H_2O (10:90, v/v) at pH 5·7 to give a final peptide concentration of $\sim$ 40 mM. For the studies involving ^{2}H-labelled trifluoroethanol (99%; Cambridge Isotope Laboratories, Andover, MA 01810, USA) the concentration of labelled TFE was 400 ml/l. The uncorrected 'pH' of this solution, determined using a standard pH meter, was 5·0. There was no significant effect of peptide concentration on line widths or chemical shifts which were reported with respect to the standard 3-(trimethylsilyl)-1-propanesulphonic acid, sodium salt. Studies were carried out at 25 °C as determined using corrected probe readings from a BVT-2000 temperature control unit ($\pm$0·5 °C).

All NMR spectra were recorded using a Bruker DRX400 spectrometer (Bruker Analytik, D-76287 Rheinstetten-Karlsrute, Germany) fitted with a 2·5 mm microprobe. The assignment of the proton spectra of the peptide was made via the use of double-quantum filtered correlated spectroscopy (DQF-COSY; Piantini *et al.* 1982) and totally correlated spectroscopy (TOCSY; Braunschweiler & Ernst, 1983). Rotating frame nuclear Overhauser effect spectroscopy (ROESY; Bothner-By *et al.* 1984; Bax & Davis, 1985) and nuclear Overhauser effect spectroscopy (NOESY; Bodenhausen *et al.* 1984) were used to determine the nuclear Overhauser effect (NOE) between spatially proximate protons.

Experimental conditions for the two dimensional NMR experiments were as follows: 1024 free induction decay of 4096 complex points were acquired for the DQF-COSY experiment and then zero-filled to a 4096×4096 matrix. A 90° skewed sinebell squared apodization was used in the first dimension followed by an exponential line broadening apodization in the second. For TOCSY, NOESY and ROESY, 512 free induction decay of 4096 complex points were acquired and then zero-filled to a 4096×4096 matrix. A 90° skewed sinebell squared apodization was used in both dimensions. For the two dimensional spectra, the spectral width was typically 3600 Hz in both dimensions. DQF-COSY, TOCSY, NOESY and ROESY experiments were performed in the phase-sensitive mode using the time proportional phase incrementation method (Marion & Wüthrich, 1983). TOCSY spectra were acquired with a mixing time of 80 ms using DIPSI isotropic mixing (Rucker & Shaka, 1989) (90° pulse calibrated at 28 μs). NOESY spectra were acquired with mixing times of 100 and 300 ms. ROESY spectra were acquired with a mixing time of 250 ms using a 4 kHz CW-spinlock pulse (90° pulse calibrated at 122 μs). All experiments used a weak presaturation pulse (2 s) to minimize the H_2O resonance. Two dimensional NMR results were processed using Felix 2.1 and 2.3 (Molecular Simulations Inc., San Diego, CA 92121-3752, USA) on Silicon Graphics workstations.

The spin–spin, $^3J_{\alpha NH}$, coupling constants of the peptide in TFE were determined by simulation of appropriate rows extracted from the DQF-COSY spectrum and zero-filled to 16 K using the program CC (Craik, 1994).

RESULTS

Structure predictions

The sequences of cow, sheep, goat and pig κ-casein are shown in Fig. 1. The alignment was made to maximize the similarities among the sequences and, when three or more amino acids were identical, they were highlighted. Clearly the cow, sheep and goat sequences are more similar to one another than to the pig sequence, particularly in the region from residue 1 to residue 85. Analysis of the κ-casein B sequence using the Chou & Fasman (1978) method as outlined by Creamer *et al.* (1983) gave results similar to those reported by Loucheux-Lefebvre *et al.* (1978) using the earlier criteria for helix, sheet and turn probabilities (Chou & Fasman, 1974*a*, *b*). This indicated that within the CMP the residues 135–154 were likely to be in the α-helical conformation even though a β-turn was also predicted for the tetrapeptide Ser^{149}–Val^{152}. The probability that the sequence from Pro^{130} to Ala^{138} was at the N-terminal end of a helix and that the sequences from Glu^{147} to Ser^{149} and Ile^{153} to Ser^{155} were at the C-terminal ends of helices was moderately high. The application of the algorithm to find likely sheet–turn–sheet regions indicated that these were unlikely in the CMP region of bovine κ-casein although a number were likely between residues 20 and 85 in the para-κ-casein region.

Despite the sequence differences (Fig. 1), the predicted structures (Fig. 2) are similar in this region, indicating the conservative nature of the substitutions. In the region (96–130 for the ruminants and 87–122 for the pig) that includes the chymosin-sensitive bonds (*) all four sequences are very similar. This is reflected in the strong similarity in the prediction curves (Fig. 2). There were also similarities in the patterns from 130 to the C-terminus (cow numbering) for the sheep and goat proteins, from 138 to the C-terminus for the pig protein and from residue 154 to the C-terminus for the cow protein. However, the pattern in region III of Fig. 2 (residues 122–138) for the pig is different from those of the other proteins. This is not

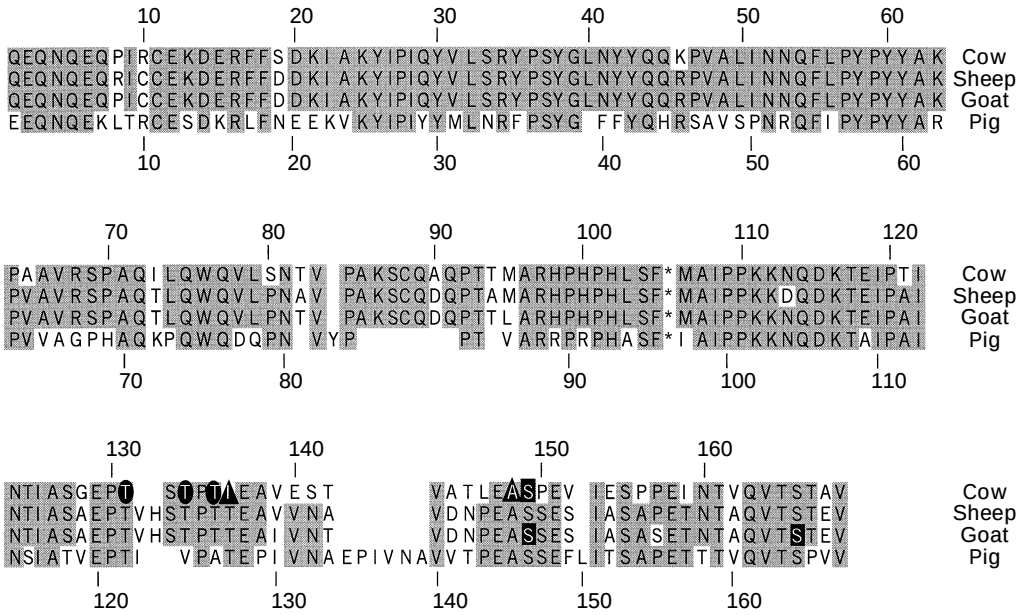

Fig. 1. Sequences of cow (B variant), sheep, goat and pig κ-caseins. The numbers above are for the cow sequence and the numbers below are for the pig sequence. The highlighted residues are those common for at least three sequences. The asterisks in the sequences show the major chymosin cleavage sites. The bovine A/B genetic variant sites are shown as dark triangles, the glycosylation sites (where known) are shown as black circles and the phosphorylation sites (where known) are shown as black squares.

surprising considering the duplication of residues EPIVNA (128–133 and 134–139 in Fig. 1, pig numbering). Consequently, the pattern displacement caused by the deletion of residues 85–91 (cow numbering) from the pig protein is made up by this duplication. The residues in the region from 136 to 152 of the cow protein were predicted to be largely in the helical conformation (Fig. 2) whereas helical structure was predicted to have a low probability for the human and rat proteins (not shown) as well as the sheep, goat and pig proteins (Fig. 2). This is not surprising given that Glu^{140}, Ser^{141}, Ala^{144} and Leu^{146} of the cow sequence are replaced by Val, Glu, Asp and Pro respectively in the sheep and goat proteins (Fig. 1); that is, amino acids with quite different structure-forming characteristics.

Circular dichroism spectroscopy

When the peptide κ-casein B 130–153 was examined in water at both pH 6·93 and 5·87, it showed an intense trough near 198 nm characteristic of a random coil (Greenfield & Fasman, 1969; Fig. 3*a*). However, there was also a shoulder near 220 nm, which indicated the presence of some secondary structure. On lowering the pH, the trough at 198 nm decreased in intensity and at pH 2·85 the shoulder at 220 nm showed a small but significant increase in intensity indicating that the peptide conformation was less random and more structured at the lower pH, with the most significant change occurring below pH 4. In the presence of 5 mM-$CaCl_2$, the spectra at pH 2·85 and 6·85 (Fig. 3*b*) were both similar to the spectrum of the peptide at pH 2·85 in the absence of $CaCl_2$ (Fig. 3*a*). These results suggest that the occupancy of ordered conformations by some amino acids in the peptide was increased by low pH or by calcium ions.

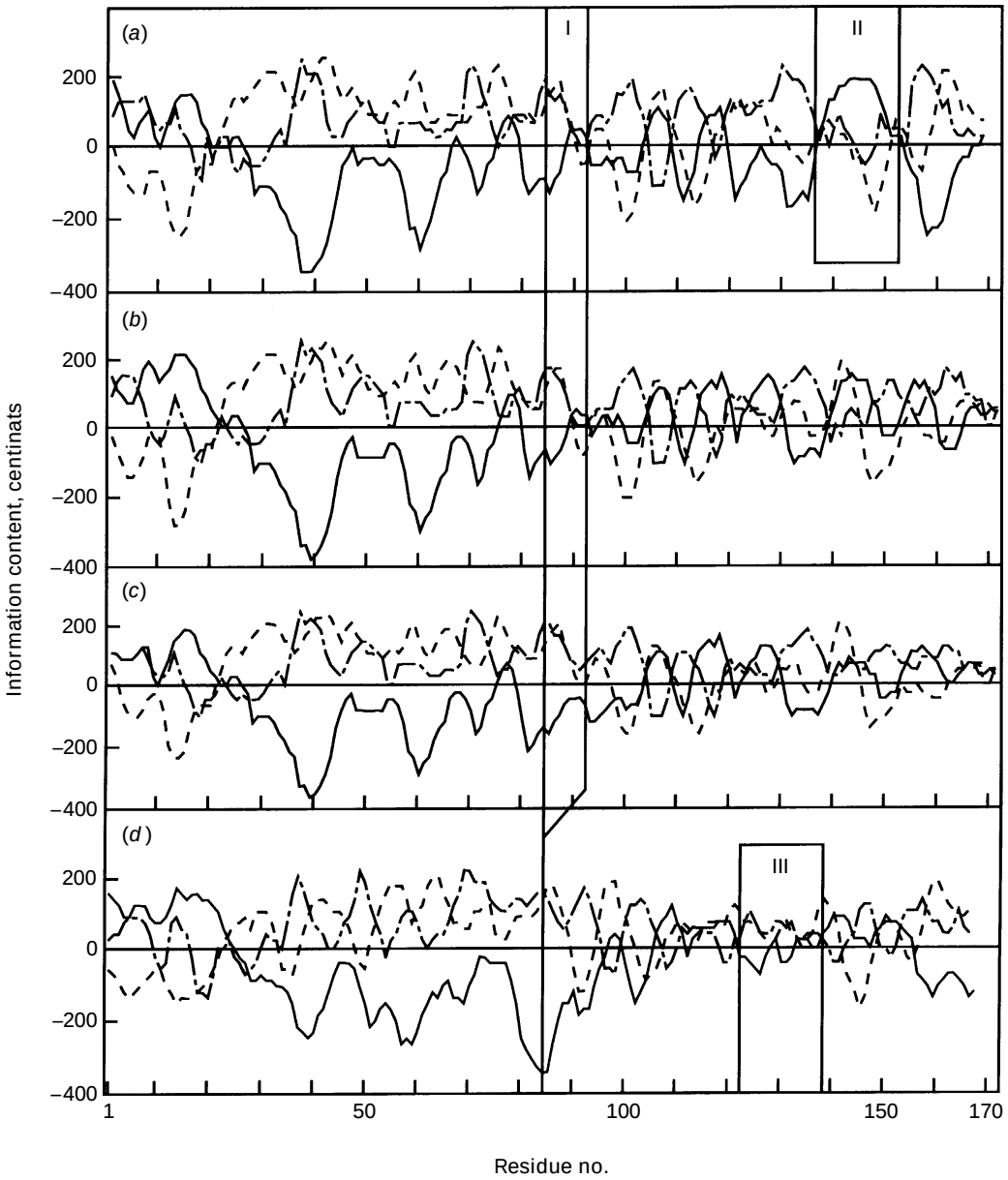

Fig. 2. Plots of the predicted secondary structure profiles for κ-casein: (*a*) cow, (*b*) sheep, (*c*) goat and (*d*) pig; ——, α-helix; – – – –, β-sheet and — · —, aperiodic structures respectively. The plots are similar except in the boxed regions which represent I, the region of cow, sheep and goat that is missing from the pig protein and hence shifts the chymosin-sensitive bond from between residues 105 and 106 in the cow protein to between residues 96 and 97 in the pig protein; II, the predicted helical region (residues 136–154) in the cow protein and III, the region from residues 124–138 that includes the six residue repeat in the pig protein.

The effect of TFE on the CD spectrum of the peptide at pH 5·5 is shown in Fig. 4. When the solution contained 100 ml TFE/l the spectrum was essentially unchanged from that in aqueous solution (Fig. 3*a*). Significant changes were present in the spectrum of the 600 ml TFE/l solution: the shoulder at 220 nm developed into an intense trough at 208 nm with a deep shoulder at $\sim$ 222 nm, whereas the trough at

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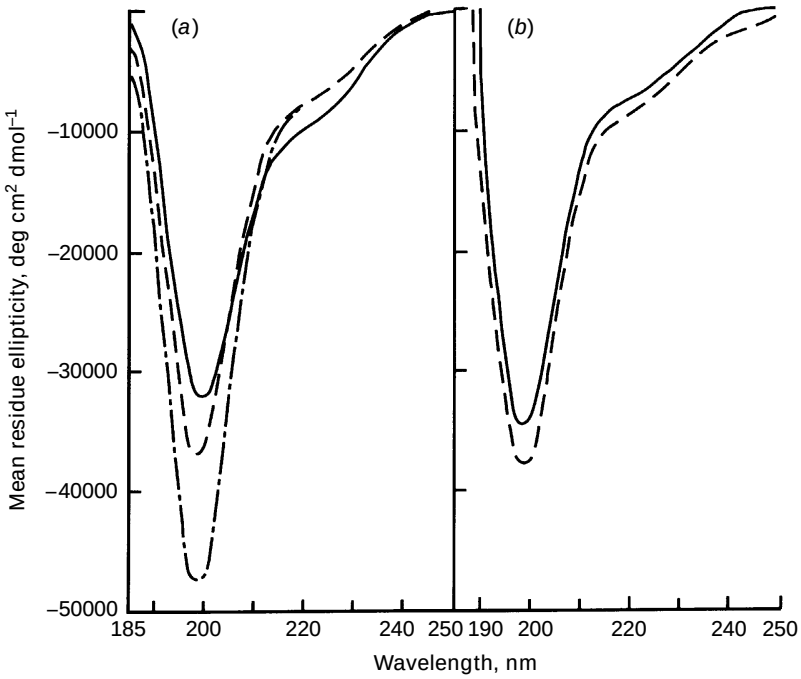

Fig. 3. Circular dichroism spectra of κ-casein B peptide 130–153 in aqueous solution: (*a*) water at pH ——, 2·85; – – – –, 4·45; —·—, 5·87 and 6·93 and (*b*) 5 mM-$CaCl_2$ at pH ——, 2·85 and – – – –, 6·85.

198 nm disappeared to be replaced by an intense peak at 190 nm. This result suggests (Yang *et al.* 1995) that in 600 ml TFE/l solution at pH 5·5 nearly all of the amino acids in the peptide had adopted an α-helical conformation. At pH 3, the peptide was observed to undergo a sharp transition from random coil to α-helix between 200 and 250 ml TFE/l (Fig. 4, inset). By contrast, at pH 5·5, the transition was less sharp and the α-helix was not fully established until a concentration of 400 ml TFE/l had been reached.

The addition of SDS (final concentration of 10 mM) to a peptide solution at pH 6·7 altered the resultant CD spectrum slightly (Fig. 5*a*). As the pH was lowered to 3, however, the development of a strong negative peak at 222 nm and a strong positive peak at 190 nm was consistent with the solution conformation of the peptide changing from mainly random coil to that of a more helical form (Greenfield & Fasman, 1969) though the lack of an isodichroic point suggests that this was not a simple random coil–helix transition. When the concentration of SDS was increased from 0 to 5 mM at pH 3, the transition from random coil to α-helix occurred over the concentration range 1–3 mM-SDS (Fig. 5*a*, inset). In contrast to SDS, the α-helical structure was maintained in 10 mM-CTAC solutions over the entire pH range from 2·95 to 6·8 (Fig. 5*b*).

Based on the assumption that the absorption at 222 nm is due entirely to α-helix the degree of α-helicity was calculated using the equation of Chen *et al.* (1974),

$$[\theta]_{\mathrm{mrw}\lambda} = (f_{\mathrm{H}} - ik/N)\,[\theta]_{\mathrm{H}\lambda_\infty},$$

where $[\theta]_{\mathrm{mrw}\lambda}$ is the observed mean residue ellipticity at wavelength λ, f_{H} is the fraction of helix, i is the number of helical segments (in this case 1), k is a wavelength-dependent constant, N is the number of residues and $[\theta]_{\mathrm{H}\lambda_\infty}$ is the maximum mean residue ellipticity for a helix of infinite length. On this basis, the $[\theta]_{\mathrm{mrw}222}$ for the

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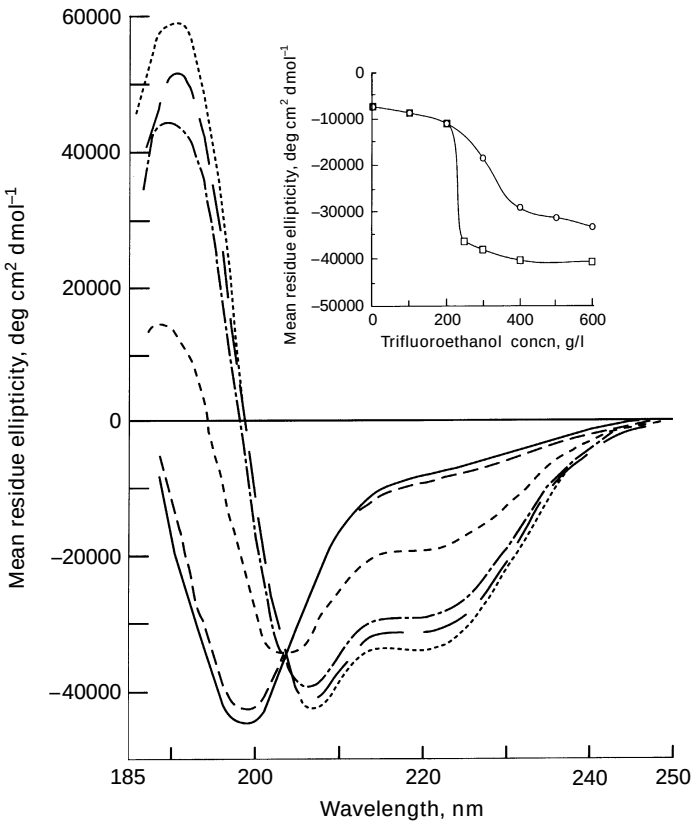

Fig. 4. Circular dichroism spectra of κ-casein B peptide 130–153 at pH 5·5 in ——, 0; – – – –, 100; ----, 300; —·—, 400; ——, 500 and · · · · · ·, 600 ml/l in trifluoroethanol. Inset: plot of molar ellipticity at 222 nm *v.* trifluoroethanol concentration for the peptide at pH □, 3·0 and ○, 5·5.

130–153 peptide in a 100% helical conformation was calculated to be -35270 deg cm^2 dmol^{-1}. The helicity of the peptide in water, SDS and TFE, expressed as a percentage, determined on the basis of the mean residue ellipticity at 222 nm, is shown in Table 1. These calculations indicate that the helicity of the peptide was the same in both 10 mM-SDS at pH 3 and TFE (400 ml/l) at pH 5·5.

NMR spectroscopy

NMR spectra were obtained both in aqueous solution at pH 5·7 and in ^{2}H-labelled TFE (400 ml/l) at pH 5·0. These pH were selected because at lower pH the peptide (40 mM) began to aggregate and precipitate in the NMR tube. The concentration of 400 ml/l for labelled TFE was selected for the NMR study as the CD studies (Fig. 4) indicated that this was an appropriate concentration of TFE to establish significant helical content at this pH.

Chemical shift assignments

The DQF-COSY and TOCSY spectra for the peptide in water showed poor peak dispersion. Furthermore the NOE in the NOESY spectra were weak and there were too few peaks in the $d_{\alpha N(i,i+1)}$ cross peak region to make unambiguous chemical shift assignments. The NOE in the NOESY spectrum were also weaker than the NOE in the ROESY spectra at comparable mixing times. No $d_{NN(i,i+1)}$ cross peaks were

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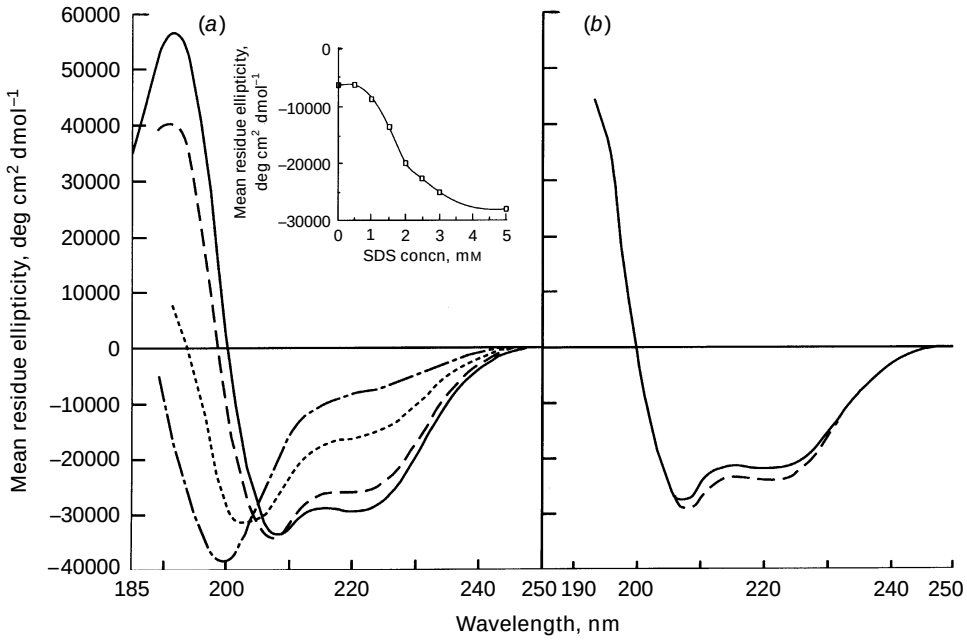

Fig. 5. Circular dichroism spectra of κ-casein B peptide 130–153 (*a*) in 10 mM-SDS at pH ——, 2·85; – – –, 4·65; · · · · · ·, 5·80 and —·—, 6·70 and (*b*) in 10 mM-cetyltrimethylammonium chloride at pH – – –, 2·95 and ——, 6·8. Inset: plot of molar ellipticity at 222 nm *v.* SDS concentration for the peptide at pH 3·0.

Table 1. *Circular dichroism determination of α-helix content* (%) *for peptide κ-casein B 130–153 at pH 3·0 and 5·5 in different solvents*

Solvent	pH 3·0	pH 5·5
Water	27	20
10 mM-SDS	83	45
Trifluoroethanol (400 ml/l)		82

observed in the NOESY spectra and only a few weak $d_{NN(i,i+1)}$ cross peaks were observed in the ROESY spectra.

In contrast, good peak dispersions were obtained for the peptide in 400 ml TFE/l and as a result the resonance assignments were relatively straightforward using the techniques of Wüthrich (1986), with the exception of Thr[131] and the prolines, which have no protons on the backbone nitrogens. Moreover, the NOE in the NOESY spectra were more intense than those in the ROESY spectra at comparable mixing times and were well resolved. There were only two overlaps in the fingerprint region: the $d_{\alpha N(i,i+1)}$ cross peaks of Thr[135] and Ala[148] were partly overlapped, whereas those of Ala[144] and Leu[146] were coincident. This latter peak appeared between Ser[141] and Ala[138] in Fig. 6 and is shown in Fig. 7*b*. The protons of Pro[130] were difficult to assign as the $d_{\alpha\delta(i,i+1)}$ cross peak was obscured by a T1 stripe due to residual water, and the cross peaks of Pro[134] and Pro[150] were very similar. However, they were easily resolved on the basis of the $d_{\alpha N(i,i+1)}$ cross peaks. Using the DQF-COSY and TOCSY spectra in conjunction with the NOESY spectrum, it was possible to make resonance assignments for all residues in the peptide with the exception of Thr[131]. With this complete, two peaks still remained: a $d_{\alpha N(i,i)}$ cross peak below the diagonal at 4·7 and 8·05 ppm (the corresponding connectivity above the diagonal was obscured by a T2

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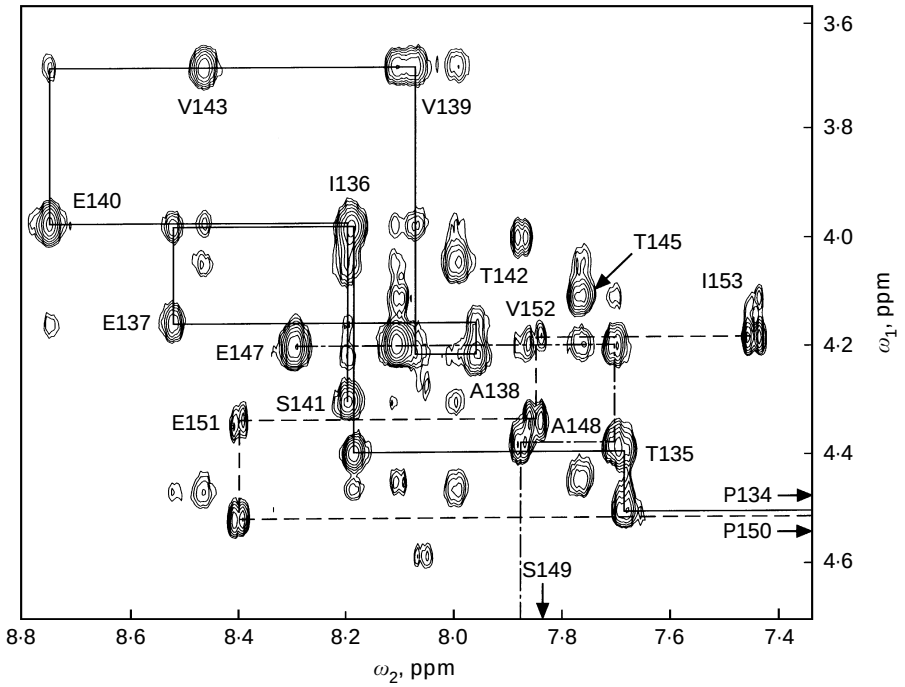

Fig. 6. The αH–NH region of the nuclear Overhauser effect spectrum for κ-casein B peptide 130–153 in trifluoroethanol (400 ml/l) at pH 5·0 and 25 °C. The mixing time was 300 ms. A partial sequential assignment is given for residues ——, 135–141; —·—, 147–149 and – – –, 151–153. One letter symbols are used for amino acids and numbers indicate their position.

stripe due to the residual water peak) and an additional cross peak at 4·46 and 1·33 ppm in the vicinity of the other threonine $d_{\beta\gamma(i,i)}$ cross peaks of Thr^{133}, Thr^{135}, Thr^{142} and Thr^{145}. Assuming that these two cross peaks were due to the same spin system, it was apparent that the corresponding $d_{\alpha\beta(i,i+1)}$ cross peak would be so close to the residual water peak as to be obscured by it. On this basis, these cross peaks at 4·7 and 8·05 ppm and 4·46 and 1·33 ppm were assigned to the Thr^{131} spin system. The complete resonance assignments of the peptide are given in Table 2.

After all the resonance assignments had been made for the peptide in TFE, some additional weak $d_{\alpha N(i,i)}$ cross peaks were observed in the DQF-COSY spectrum (Fig. 7*b*). Comparison with the equivalent region in the DQF-COSY spectrum for the peptide in water (Fig. 7*a*, Table 3) suggests that the positions of these peaks were similar to those of the peptide in water and suggests the presence of another conformational form in TFE. These peaks are unlikely to be due to impurities in the synthetic peptide. The intensities of these peaks are $\sim 25\%$ of the intensities of the peaks in the helical form and this would be consistent with the proportion of peptide in the helical form as determined by CD and NMR methods (Tables 1 and 4). This other conformational form was most probably random coil as this appeared to be the predominant conformation in water; for instance peak I (Fig. 7*b*) was tentatively assigned to Ile^{153} and peak II to Thr^{145} in the random coil structure. Any differences between the chemical shifts of these resonances between the two solvents most probably arose from minor differences in pH between the two experiments.

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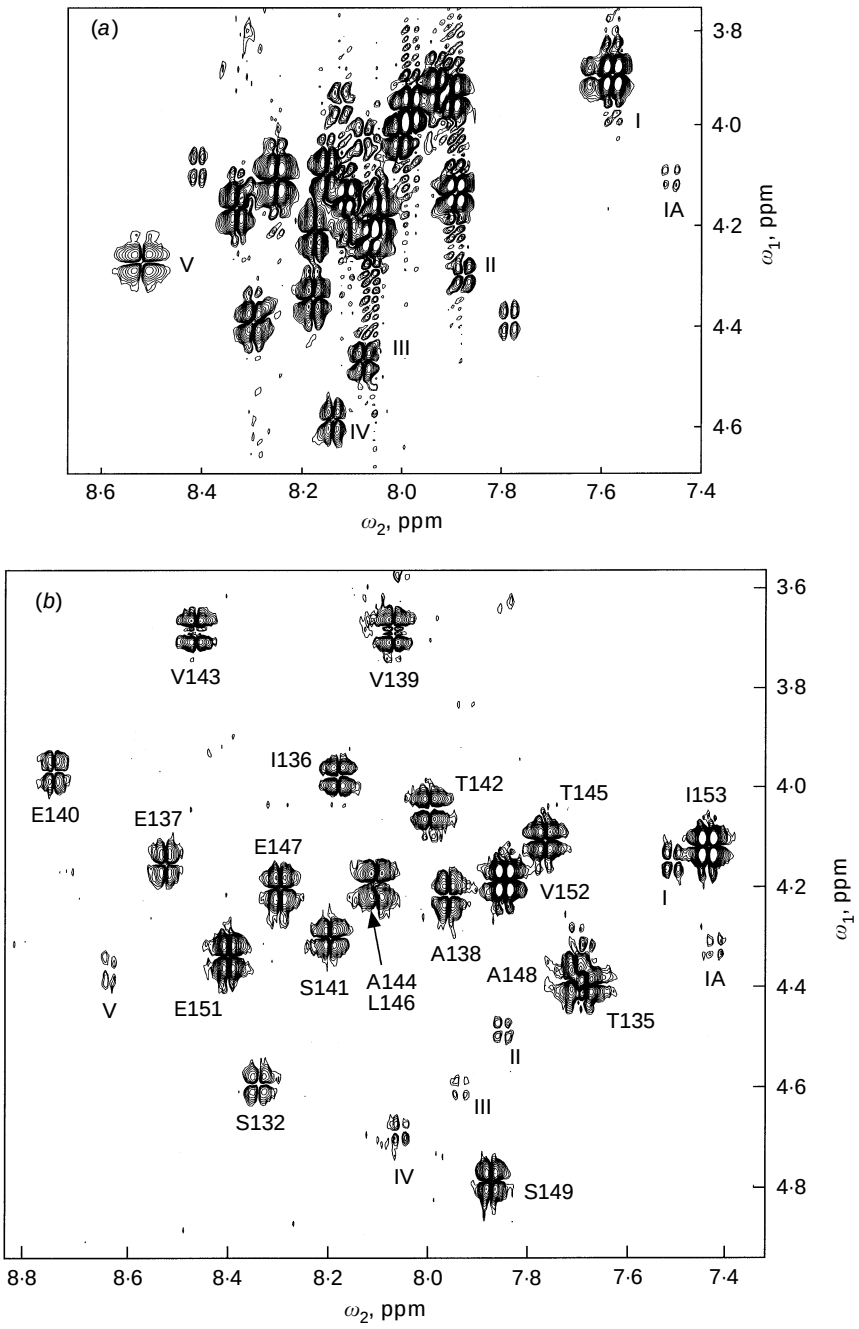

Fig. 7. The αH–NH region of the double-quantum filtered correlated spectra of κ-casein B peptide 130–153 in (*a*) water at pH 5·7 and 25 °C and (*b*) trifluoroethanol (400 ml/l) at pH 5·0 and 25 °C. One letter symbols are used for amino acids. In addition, a series of unassigned peaks in the trifluoroethanol spectrum and their counterparts in the water spectrum are indicated by Roman numerals. Of these, peak IA was present in both and may indicate the presence of a third conformation.

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Table 2. *^{1}H NMR chemical shifts (ppm) and resonance assignments for the assigned residues of κ-casein B peptide 130–153 in trifluoroethanol (400 ml/l) at 25 °C and pH 5·0*

Residue	NH	αH	βH	Others
Pro^{130}	—	4·45	2·55	γCH$_2$, 2·14, 2·1; δCH$_2$, 3·49, 3·44
Thr^{131}	8·05	4·7	4·46	γCH$_3$, 1·33
Ser^{132}	8·34	4·6	3·97, 3·93	
Thr^{133}	8·06	4·49	4·28	γCH$_3$, 1·31
Pro^{134}	—	4·51	2·33	γCH$_2$, 2·07, 2·01; δCH$_2$, 3·86, 3·77
Thr^{135}	7·67	4·4	4·29	γCH$_3$, 1·35
Ile^{136}	8·18	3·98	1·94	γCH$_2$, 1·2; γCH$_3$, 1·0; δCH$_2$, 1·0
Glu^{137}	8·53	4·16	2·08	γCH$_2$, 2·4
Ala^{138}	7·96	4·22	1·58	
Val^{139}	8·07	3·69	2·29	γCH$_3$, 1·08, 0·97
Glu^{140}	8·75	3·97	2·30, 2·16	γCH$_2$, 2·53
Ser^{141}	8·2	4·3	4·06, 4·02	
Thr^{142}	8·0	4·05	4·47	γCH$_3$, 1·27
Val^{143}	8·46	3·69	2·19	γCH$_3$, 1·09, 0·97
Ala^{144}	8·11	4·19	1·56	
Thr^{145}	7·77	4·11	4·46	γCH$_3$, 1·32
Leu^{146}	8·11	4·19	1·96	γCH, 0·96; δCH$_3$, 0·91
Glu^{147}	8·3	4·2	2·12	γCH$_2$, 2·45, 2·33
Ala^{148}	7·7	4·37	1·52	
Ser^{149}	7·87	4·79	4·03, 3·98	
Pro^{150}	—	4·52	2·33	γCH$_2$, 2·07, 2·0; δCH$_2$, 3·85, 3·83
Glu^{151}	8·4	4·34	2·13, 2·0	γCH$_2$, 2·34
Val^{152}	7·84	4·19	2·19	γCH$_3$, 1·0
Ile^{153}	7·44	4·12	1·87	γCH$_2$, 1·2; γCH$_3$, 0·94; δCH$_2$, 0·94

Table 3. *^{1}H NMR chemical shifts (ppm) for the unassigned NH–αH resonances for κ-casein B peptide 130–153 in trifluoroethanol (400 ml/l) and related peaks in water*

	αH		NH	
Peak	Trifluoroethanol	Water	Trifluoroethanol	Water
I	4·15	3·9	7·51	7·58
IA	4·33	4·1	7·42	7·46
II	4·49	4·29	7·85	7·88
III	4·61	4·46	7·93	8·08
IV	4·69	4·59	8·05	8·14
V	4·37	4·27	8·64	8·52

Secondary structure

Summaries of the sequential and medium-range NOE for the peptide in TFE are depicted in Fig. 8. Whereas a strong set of $d_{\mathrm{NN}(i,i+1)}$ cross peaks was observed in TFE (Fig. 9) only a few weak $d_{\mathrm{NN}(i,i+1)}$ cross peaks were observed when the peptide resonances were determined in aqueous solution. This provides further support for the idea that the peptide exists largely in a series of rapidly interconverting disordered structures in the latter solvent at near neutral pH. The occurrence of strong $d_{\alpha\beta(i,i+3)}$ and $d_{\mathrm{NN}(i,i+1)}$ cross peaks as well as weaker $d_{\alpha\mathrm{N}(i,i+3)}$ cross peaks in 400 ml TFE/l is good evidence for the existence of an α-helix involving residues Ile^{136}–Ser^{149}. Not all of the expected $d_{\alpha\beta(i,i+3)}$ and $d_{\alpha\mathrm{N}(i,i+3)}$ cross peaks were observed. This was because of stronger overlapping intraresidue NOE connectivities in the spectrum. Weak $d_{\alpha\mathrm{N}(i,i+4)}$ cross peaks are also diagnostic of α-helices. Whereas one NOE was observed between Glu^{140} and Ala^{144}, at least one other NOE in the fingerprint region could have been due to either a $d_{\alpha\mathrm{N}(i,i+4)}$ cross peak between Thr^{142} and Leu^{146} or a $d_{\alpha\mathrm{N}(i,i+2)}$ cross peak between Thr^{142} and Ala^{144}.

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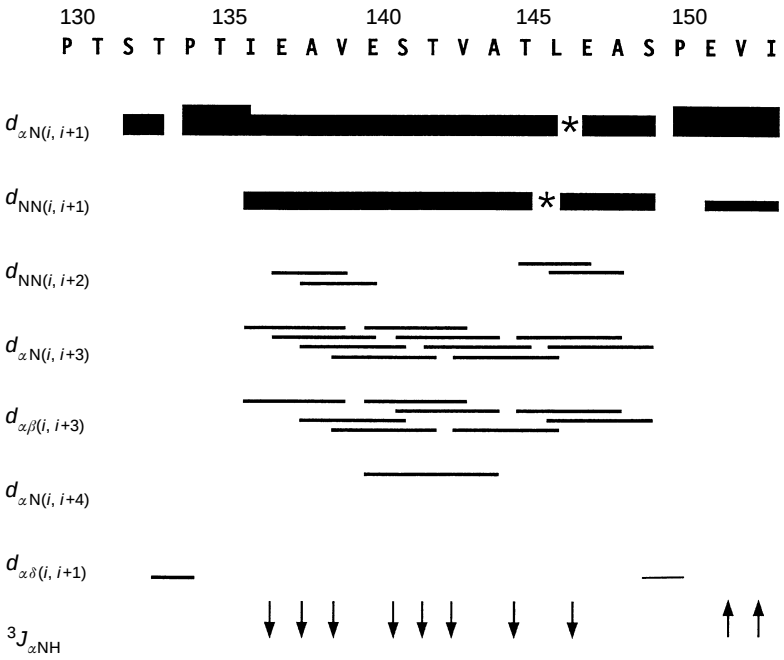

Fig. 8. Summary of nuclear Overhauser effect (NOE) connectivities for κ-casein B peptide 130–153 in trifluoroethanol (400 ml/l) at 25 °C. Approximate NOE intensities are indicated by the height of the bars. Medium range cross peaks are shown as lines connecting residues. *, Overlapping NOE; ↓, $^3J_{\alpha NH}$ coupling constants ⩽ 6 Hz; ↑ $^3J_{\alpha NH}$ coupling constants ⩾ 8 Hz.

The presence of several weak $d_{NN(i, i+2)}$ cross peaks involving some N- and C-cap residues was indicative of some populations of 3_{10}-helix. This suggests some degree of partial unfolding at either end of the helix as 3_{10}-helices have been identified on the unfolding pathways of helical peptides (Sundaralingam & Sekharudu, 1989; Tirado-Rives & Jorgensen, 1991). In addition, $d_{\alpha\delta(i, i+1)}$ cross peaks were observed for Thr[133]–Pro[134] and Ser[149]–Pro[150], indicating that the Xaa–Pro peptide bond in each instance was primarily in the *trans* configuration, although the presence of a T1 stripe in this region made the observation of any potential $d_{\alpha\alpha(i, i+1)}$ cross peaks uncertain.

As further confirmation of the presence of a helix in the region from Ile[136] to Ser[149], several $^3J_{\alpha NH}$ coupling constants were measured at ⩽ 6 Hz (residues Glu[137]–Val[139], Ser[141]–Val[143], Thr[145] and Glu[147]) indicating that extended or β-strand structures were not present in significant amounts in this region (Fig. 8). Unfortunately, the strong overlap between Ala[144] and Leu[146] meant that the coupling constants for these residues could not be measured with the same degree of accuracy as the others. The residues at the N- and C-caps of the helix were found to have $^3J_{\alpha NH}$ coupling constants of 6–8 Hz, providing further support for partial unfolding at either end of the helix. In contrast, the $^3J_{\alpha NH}$ coupling constants between Val[152] and Ile[153] were observed to be ⩾ 8 Hz indicating that extended structures were likely to be present in significant amounts near the C-terminus.

The deviations from random coil values in aqueous solution (Wishart *et al.* 1992) for the αH, NH and βH chemical shifts of the peptide in 400 ml TFE/l are shown in Fig. 10. For the residues from 136 to 147, the series of αH shifts upfield from random coil values were consistent with these residues being involved in an α-helix. The deviations from random coil values were greater in the middle of the putative helix and less at either end, particularly the C-cap where the deviation for Ala[148] was close

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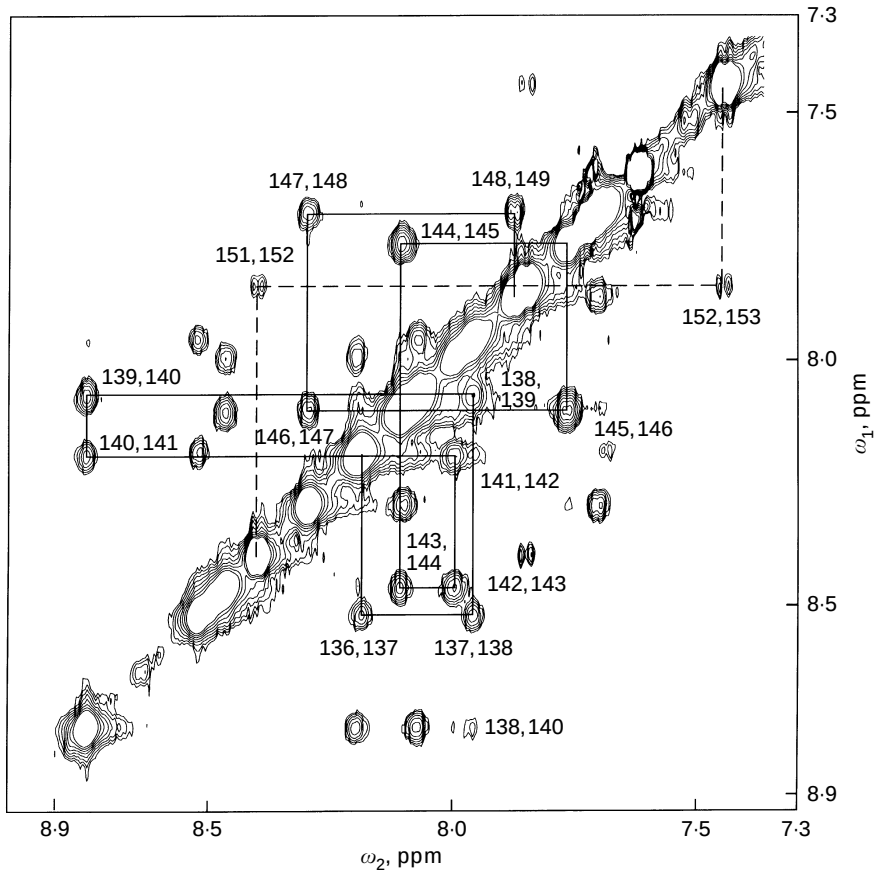

Fig. 9. The NH–NH region of nuclear Overhauser effect spectra of κ-casein B peptide 130–153 in trifluoroethanol (400 ml/l) at pH 5·0 and 25 °C. The mixing time was 300 ms. Solid lines connect $d_{\mathrm{NN}(i,i+1)}$ cross peaks for the residues involved in the α-helix, whereas dashed lines connect $d_{\mathrm{NN}(i,i+1)}$ cross peaks in the region of extended structure at the C-terminus.

to random coil values; the value for Ser^{149} showed a strong downfield shift, indicating that, whereas residues Ile^{136} to Glu^{147} had a high α-helix occupancy, residues Ala^{148} and Ser^{149} had a lower α-helix occupancy and a significant 3_{10}-helix occupancy. The residues Thr^{131}–Thr^{133} all showed downfield shifts $> 0{\cdot}1$ ppm, whereas those for Glu^{151}–Ile^{153} were close to random coil values, which is consistent with these residues being in random coil or extended chain conformation in solution.

Amide proton chemical shifts are generally sensitive to solvent conditions, pH and temperature and are generally less useful than α-proton shifts in indicating the secondary structural environment of the amino acid residues. However, a periodicity in amide proton chemical shifts has been identified in amphipathic helices (Dyson & Wright, 1993; McLeish *et al.* 1994). Whereas most residues in the helix showed strong upfield NH shifts, both Glu^{137} and Glu^{140} showed strong downfield shifts, Val^{143} showed a weak downfield shift, and Glu^{147} showed a weak upfield shift, though one that was close to random coil values (Fig. 10). This mild periodic fluctuation in the amide protons of the peptide is indicative of some amphipathic character in the helix. It was previously noted by McLeish *et al.* (1994) that the βH chemical shifts show increasing downfield shifts with an increase in helical character. The βH chemical shifts for the peptide all showed pronounced downfield shifts particularly in the

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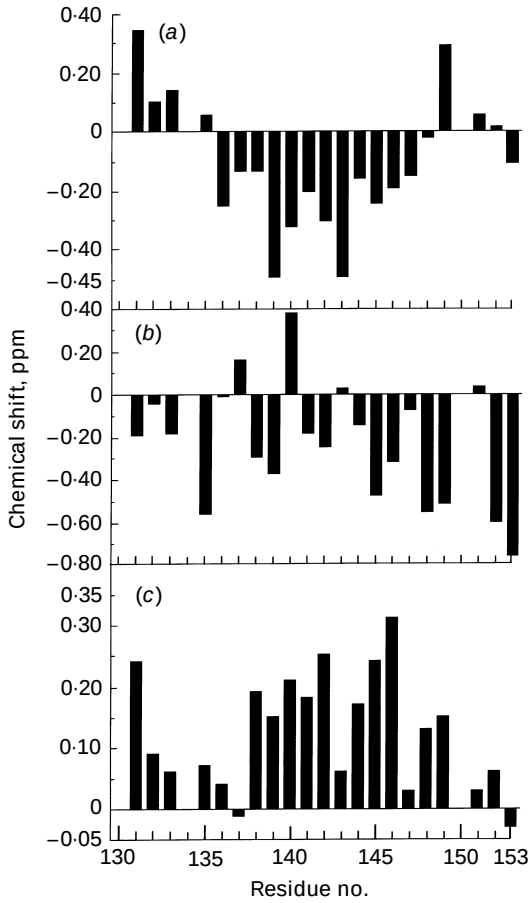

Fig. 10. Deviations from aqueous random coil values axis *v*. residue number axis for the (*a*) αH, (*b*) NH and (*c*) βH chemical shifts of κ-casein B peptide 130–153 in trifluoroethanol solution (400 ml/l).

Table 4. *Helix population estimates for selected inter-residue cross peaks in κ-casein peptide 130–153 in trifluoroethanol (400 ml/l) at 25 °C and pH 5·0*

Cross peak	Helical content, %
Ile^{136}–Glu^{137}	83
Glu^{137}–Ala^{138}	86
Val^{139}–Glu^{140}	96
Ser^{141}–Thr^{142}	94
Thr^{142}–Val^{143}	93
Glu^{147}–Ala^{148}	75
Ala^{148}–Ser^{149}	79
Glu^{151}–Val^{152}	44
Val^{152}–Ile^{153}	47

region involving the residues from 136 to 149, which supports the other observations of the presence of a helix in this region.

Helical population estimates were determined by the NOE-based method of Bradley *et al*. (1990) for the peptide in 400 ml TFE/l using results from the 100 ms NOESY and are reported in Table 4. It was not possible to obtain estimates for all

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the $i, i+1$ NOE in the NOESY spectrum largely because of overlaps between a number of $d_{\alpha N(i,i+1)}$ and $d_{NN(i,i+1)}$ NOE. However, based on results from those NOE where it was possible, it would appear for the residues from Val[139] to Val[143] in the centre of the helix that the helical occupancy was 95%, whereas for those residues at either end of the helical region the helical occupancy was 70–85%. This lower helical occupancy at either end of the helical region supports previous observations of fraying at both ends of a helix (Dyson & Wright, 1993). At the C-terminus of the peptide, the helical occupancy was $< 50\%$. A semiquantitative estimate of the population of the helical region when the peptide was in TFE could be obtained by applying the method of Rozek *et al.* (1995). This involves dividing the average αH chemical shift by 0·35, which is the average upfield αH shift observed in the amino acid residues of proteins in an α-helical conformation. Based on this approach, the population of α-helical state between residues Ile[136] and Ser[149] in the peptide would be at least 60%. However, this is only an approximation as the αH chemical shifts approach zero at each end of the helix as a consequence of helical fraying (Fig. 10).

DISCUSSION

Based on the relative intensities of the $d_{\alpha N(i,i+1)}$ and $d_{NN(i,i+1)}$ cross peaks in the NOESY spectrum of the peptide in 400 ml TFE/l, there were three distinct regions in the peptide (Table 5). At the N-terminus, the presence of only $d_{\alpha N(i,i+1)}$ cross peaks for residues Thr[131]–Thr[133] would be consistent with a random coil conformation (Millhauser, 1995). Residues Ile[136]–Ser[149] were characterized by strong $d_{NN(i,i+1)}$ cross peaks and weak $d_{\alpha N(i,i+1)}$ cross peaks which is typical of α-helical structure (Bradley *et al.* 1990). The presence of a series of $d_{\alpha\beta(i,i+3)}$ and $d_{\alpha N(i,i+3)}$ cross peaks for the residues in this region of the peptide provides additional support for the existence of an α-helix involving these residues, as do the $^3J_{\alpha NH}$ coupling constants. The $^3J_{\alpha NH}$ coupling constants, α-proton random coil chemical shifts and $d_{NN(i,i+2)}$ NOE for the residues at the N- and C-caps of the helix support the idea that this helix has frayed ends, which is in line with other studies of helices in isolated peptides (Dyson & Wright, 1993). In contrast, residues Glu[151]–Ile[153] were characterized by strong $d_{\alpha N(i,i+1)}$ cross peaks and weak $d_{NN(i,i+1)}$ cross peaks, which is typical of extended structure (Bradley *et al.* 1990). For Val[152] and Ile[153], the $^3J_{\alpha NH}$ coupling constants were once again more consistent with extended structure.

From the NMR studies, it was apparent that the maximum number of residues involved in the helix in the peptide in TFE at pH 5·0 was 14 (Figs 6 and 8). This is in contrast to the CD studies of the peptide in SDS at pH 3 and TFE at 5·5 (Figs 4 and 5) which indicated that the helicity under both conditions was 83% (Table 1) or involving ~ 19 residues. As CD ellipticity values are generally lowered by helix fraying and non-ideal helix geometry (Dyson & Wright, 1991), this result is somewhat surprising and may be partly attributed to the presence of other structural forms in equilibrium with the random coil and helical conformations of the peptide. It may also be partly due to the presence of the additional folded region at the C-terminus of the peptide.

Earlier spectral studies indicated that under physiological conditions CMP may contain segments that have a moderate occupancy of the helical conformation (Ono *et al.* 1987). Griffin *et al.* (1986) showed that TFE and other alcohols induce helical structure in κ-casein and that most of the induced helix is probably located in the CMP rather than the para-κ-casein region of the molecule. This was supported by sequence prediction studies (Table 5; Loucheux-Lefebvre *et al.* 1978; Raap *et al.*

Table 5. *Structural propensities of the residues in κ-casein B peptide 130–153 from the NMR spectra and prediction algorithms*

Residue	Conformation in trifluoroethanol (400 ml/l)†	Prediction‡	
		GOR	C&F
Pro^{130}	r	r	r, N–cap
Thr^{131}	r	r	r, N–cap, b–t
Ser^{132}	r	r	r, N–cap
Thr^{133}	r	b–t	r, N–cap
Pro^{134}	r	r	r, N–cap
Thr^{135}	r	r	h, N–cap
Ile^{136}	h	e	h, N–cap
Glu^{137}	h, 3–10	e	h, N–cap
Ala^{138}	h, 3–10	h	h, N–cap
Val^{139}	h, 3–10	h	h
Glu^{140}	h, 3–10	h	h
Ser^{141}	h	h	h
Thr^{142}	h	h	h
Val^{143}	h	h	h
Ala^{144}	h	h	h
Thr^{145}	h, 3–10	h	h
Leu^{146}	h, 3–10	h	h
Glu^{147}	h, 3–10	h	h, C–cap
Ala^{148}	h, 3–10	h	h, C–cap
Ser^{149}	h	h	h, C–cap, b–t
Pro^{150}	r	h	h, b–t
Glu^{151}	e	h	h, b–t
Val^{152}	e	h	h, b–t
Ile^{153}	e	h	h, C–cap

† h, helix; e, extended; r, random coil; b–t, β-turn; 3–10, 3_{10}-helix.
‡ See Fig. 2(*a*) and text; GOR, Garnier, Osguthorpe & Robson (1978); C&F, Chou & Fasman (1978).

1983; Kumosinski *et al.* 1991) and the present study indicates that the peptide corresponding to residues 130–153 of κ-casein was strongly helical under favourable, but non-physiological conditions. It is well recognized that small peptides derived from proteins often lose the structure they had in the intact protein. The use of structure-inducing solutes such as TFE often restore secondary structure (in particular helix) to a peptide presumably by providing an environment comparable to that within the protein, with the proviso that TFE induces helical structure only in sequences that have some intrinsic tendency for helical formation (Dyson & Wright, 1993). TFE has no tendency to stabilize turns or β-structure, and it has been observed to stabilize helical structure in a peptide isolated from a β-structure region in a native protein (Sönnichsen *et al.* 1992). For that particular peptide, sequence structure prediction studies had indicated that both helical and β-structure were favoured and consequently the isolated peptide was not expected to show propensity to form β-structures in TFE as it lacked the stabilizing effect of the adjacent strand. The present study showed that the peptide 130–153 contained a helical structure under favourable conditions and structure prediction supported the proposition that the most likely region for helix in bovine κ-casein would be residues 136–149.

Fig. 11 shows the κ-casein sequence for both A and B variants displayed as helical wheels. This display mode shows the residues most likely to be in juxtaposition in putative helices. There are three discernible regions in the figures, corresponding to stripes along the helix. The lower right-hand side is hydrophobic, indicated by a continuous stripe of hydrophobic residues (Ile^{136} in B; Val^{139}; Val^{143}; Leu^{146}) and the left-hand side is largely acidic. It is possible to speculate that, when these residues

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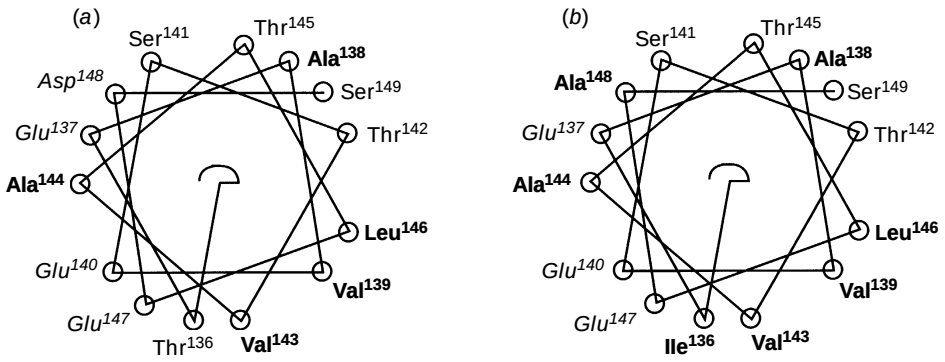

Fig. 11. Helical wheel plots of the region from residues 136–149 in κ-casein (*a*) A and (*b*) B generated by PLOT.A/HEL (Luttke, 1990*b*). Hydrophilic residues are roman, hydrophobic residues bold and acidic residues italic. Potential sites of glycosylation are at Thr^{136} (A variant) and Ser^{141} and the phosphorylation site is at Ser^{149} (Kawakami *et al.* 1992).

(136–149) form a helix, two such helices could associate via the hydrophobic stripes, or one helix could associate with some other hydrophobic surface. Such an association may explain the tendency for the peptide to precipitate below pH 5·0 in aqueous solution. The strongly acidic stripe would be expected to have some inter-residue repulsion leading to lower stability. There is some evidence for this as helical content is higher at low pH (Fig. 3), where some of the carboxylates are protonated, lowering the charge, and in the presence of calcium (Fig. 3), which can also lower net negative charge. Although the primary sites on the caseins for the binding of calcium are the organic phosphate groups (Ho & Waugh, 1965) evidence has been obtained from Fourier transform i.r. studies for the interaction between the carboxylate groups of aspartate and glutamate residues and calcium (Byler & Farrell, 1989). Phosphorylation and glycosylation are expected to have little effect on the amphipathic character of this helix as the phosphorylation site lies at one end of the helix and the primary glycosylation sites, specifically Thr^{131}, Thr^{133} and Thr^{135} (Kanamori *et al.* 1980), lie outside the helical region.

Detergents such as SDS are monomeric at low concentrations and cooperatively form detergent micelles at higher concentration (Creamer & Richardson, 1984). Hydrophobic molecules, including segments of a protein, can be incorporated into such micelles and one such model of protein structure during SDS-PAGE is of a series or necklace of detergent micelles linked by the polypeptide chains. At low SDS:peptide molar ratios, a β form can exist if a segment has a β-forming potential, but excess SDS usually disrupts the β form and can convert it to a helix if the segment has a helix-forming potential (Wu *et al.* 1981). In contrast, protein segments with no structure-forming potential remain unordered regardless of the SDS concentration used. At pH 3 and in low concentrations of SDS, the peptide had a CD spectrum characteristic of random coil conformation, supporting the idea that the β-structure-forming potential in this sequence was low. For peptides with a negative charge, peptide–SDS aggregates are favoured at low pH because the electrostatic repulsion between the acidic groups of the peptide and SDS will hinder clustering of the detergent anions on to the polypeptide chain at higher pH values. CTAC is a detergent that also forms micelles but differs from SDS in that the net charge is positive. Consequently peptides that contain negative charges would be expected to be incorporated more readily into CTAC than into SDS micelles; the results (Fig. 5) suggest that this was so in the present instance.

Most studies tend to support the suggestion that the proportion of helical structure in κ-casein is low and of the order of 7% (Ono *et al.* 1987; Kumosinski *et al.* 1991). According to Ono *et al.* (1987) using the method of Provencher & Glöckner (1981), the helical structure was largely confined to CMP where it was estimated to be 22%, or ∼ 15 residues, which is very similar in length to the 14-residue helix we have found in the region involving residues 130–153 in κ-casein. Thus, these results tend to support the idea that CMP can exist in solution in a number of conformations one of which would involve an α-helix between residues 136 and 149. Although this is in line with the results from a number of structure prediction studies for bovine κ-casein (Loucheux-Lefebvre *et al.* 1978; Raap *et al.* 1983; Kumosinski *et al.* 1991), the absence of a similarly predicted region in human, rat, sheep, goat and pig κ-caseins when the same algorithm was applied to them would suggest that the presence of a helical region in this part of κ-casein could be specific to the bovine protein. This difference could have occurred by chance, although there are six substitutions in the region 139–146 between the cow and the goat or the sheep proteins. This is also the region (positions 136 and 148) where the A and B genetic variant substitutions occur, where glycosylation takes place (Thr^{131}, Thr^{133}, Thr^{135} and Thr^{136}; A variant) and where Ser^{149} but not Ser^{127} is phosphorylated. It could be argued that the transient formation of a helical conformation may influence some of these post-translational modifications, but clearly more results are necessary to verify such a possibility. It could be speculated that for the cow there could have been a congenital advantage in having a transient helix in this segment of κ-casein.

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REFERENCES

Atherton, E. & Sheppard, R. C. 1989 *Solid Phase Peptide Synthesis. A Practical Approach.* Oxford: IRL Press

Bax, A. & Davis, D. G. 1985 Practical aspects of two-dimensional transverse NOE spectroscopy. *Journal of Magnetic Resonance* **63** 207–213

Bodenhausen, G., Kogler, H. & Ernst, R. R. 1984 Selection of coherence-transfer pathways in NMR pulse experiments. *Journal of Magnetic Resonance* **58** 370–388

Bothner-By, A. A., Stephens, R. L., Lee, J., Warren, C. D. & Jeanloz, R. W. 1984 Structural determination of a tetrasaccharide. Transient nuclear Overhauser effects in the rotating frame. *Journal of the American Chemical Society* **106** 811–813

Bradley, E. K., Thomason, J. F., Cohen, F. E., Kosen, P. A. & Kuntz, I. D. 1990 Studies of synthetic helical peptides using circular dichroism and nuclear magnetic resonance. *Journal of Molecular Biology* **215** 607–622

Braunschweiler, L. & Ernst, R. R. 1983 Coherence transfer by isotropic mixing. Application to proton correlation spectroscopy. *Journal of Magnetic Resonance* **53** 521–528

Brignon, G., Chtourou, A. & Ribadeau-Dumas, B. 1985 Preparation and amino acid sequence of human κ-casein. *FEBS Letters* **188** 48–54

Byler, D. M. & Farrell, H. M. 1989 Infrared spectroscopic evidence for calcium ion interaction with carboxylate groups of casein. *Journal of Dairy Science* **72** 1719–1723

Chen, Y.-H., Yang, J. T. & Chau, K. H. 1974 Determination of the helix and β form of proteins in aqueous solution by circular dichroism. *Biochemistry* **13** 3350–3359

Chou, P. Y. & Fasman, G. D. 1974*a* Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. *Biochemistry* **13** 211–222

Chou, P. Y. & Fasman, G. D. 1974*b* Prediction of protein conformation. *Biochemistry* **13** 222–245

Chou, P. Y. & Fasman, G. D. 1978 Prediction of the secondary structure of proteins from their amino acid sequence. *Advances in Enzymology* **47** 45–148

Craik, D. J. 1994 *DQF-COSY Cross Peak Simulation Program CC*. Melbourne: Victorian College of Pharmacy

Creamer, L. K., Parry, D. A. D. & Malcolm, G. N. 1983 Secondary structure of bovine β-lactoglobulin B. *Archives of Biochemistry and Biophysics* **227** 98–105

Creamer, L. K. & Richardson, T. 1984 Anomalous behavior of bovine α_{s1}- and β-caseins on gel electrophoresis in sodium dodecyl sulfate buffers. *Archives of Biochemistry and Biophysics* **234** 476–486

Dyson, H. J. & Wright, P. E. 1991 Defining solution conformations of small linear peptides. *Annual Review of Biophysics and Biophysical Chemistry* **20** 519–538

Dyson, H. J. & Wright, P. E. 1993 Peptide conformation and protein folding. *Current Opinion in Structural Biology* **3** 60–65

Fields, G. B. & Noble, R. L. 1990 Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *International Journal of Peptide and Protein Research* **35** 161–214

Garnier, J., Osguthorpe, D. J. & Robson, B. 1978 Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* **120** 97–120

Greenfield, N. & Fasman, G. D. 1969 Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8** 4108–4116

Griffin, M. C. A., Price, J. C. & Martin, S. R. 1986 Effect of alcohols on the structure of caseins: circular dichroism studies of κ-casein A. *International Journal of Biological Macromolecules* **8** 367–371

Griffin, M. C. A. & Roberts, G. C. K. 1985 A ^{1}H-n.m.r. study of casein micelles. *Biochemical Journal* **228** 273–276

Ho, C. & Waugh, D. F. 1965 Interactions of bovine caseins with divalent cations. *Journal of the American Chemical Society* **87** 889–892

Holt, C. & Sawyer, L. 1988 Primary and predicted secondary structures of the caseins in relation to their biological functions. *Protein Engineering* **2** 251–259

Kanamori, M., Kawaguchi, N., Ibuki, F. & Doi, H. 1980 Attachment sites of carbohydrate moieties to peptide chain of bovine κ-casein from normal milk. *Agricultural and Biological Chemistry* **44** 1855–1861

Kawakami, H., Kawasaki, Y., Dosako, S., Tanimoto, M. & Nakajima, I. 1992 Determination of κ-casein glycomacropeptide by high performance liquid chromatography without trichloroacetic acid pretreatment. *Milchwissenschaft* **47** 688–693

Kumosinski, T. F., Brown, E. M. & Farrell, H. M. 1991 Three-dimensional molecular modeling of bovine caseins: κ-casein. *Journal of Dairy Science* **74** 2879–2887

Levine, W. B., Alexander, L. J., Hoganson, G. E. & Beattie, C. W. 1992 Cloning and sequencing of the porcine κ-casein cDNA. *Animal Genetics* **23** 361–363

LKB 1987 *LKB Biolynx 4175 Peptide Synthesiser User's Manual*. Uppsala: LKB Biochrom

Loucheux-Lefebvre, M.-H., Aubert, J.-P. & Jollès, P. 1978 Prediction of the conformation of the cow and sheep κ-caseins. *Biophysical Journal* **23** 323–336

Luttke, A. 1990*a* PLOT.A/GOR Koln, Germany

Luttke, A. 1990*b* PLOT.A/HEL, Koln, Germany

McLeish, M. J., Nielsen, K. J., Najbar, L. V., Wade, J. D., Lin, F., Doughty, M. B. & Craik, D. J. 1994 Conformation of a peptide corresponding to T4 lysozyme residues 59–81 by NMR and CD spectroscopy. *Biochemistry* **33** 11174–11183

Marion, D. & Wüthrich, K. 1983 Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of ^{1}H–^{1}H spin–spin coupling constants in proteins. *Biochemical and Biophysical Research Communications* **113** 967–974

Mercier, J.-C., Chobert, J.-M. & Addeo, F. 1976 Comparative study of the amino acid sequences of the caseinomacropeptides from several species. *FEBS Letters* **72** 208–214

Millhauser, G. L. 1995 Views of helical peptides: a proposal for the position of 3_{10}-helix along the thermodynamic folding pathway. *Biochemistry* **34** 3873–3877

Ono, T., Yada, R., Yutani, K. & Nakai, S. 1987 Comparison of conformations of κ-casein, para-κ-casein and glycomacropeptide. *Biochimica et Biophysica Acta* **911** 318–325

Piantini, U., Sørensen, O. W. & Ernst, R. R. 1982 Multiple quantum filters for elucidating NMR coupling networks. *Journal of the American Chemical Society* **104** 6800–6801

Provencher, S. W. & Glöckner, J. 1981 Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* **20** 33–37

Raap, J., Kerling, K. E. T., Vreeman, H. J. & Visser, S. 1983 Peptide substrates for chymosin (rennin): conformational studies of κ-casein and some κ-casein-related oligopeptides by circular dichroism and secondary structure prediction. *Archives of Biochemistry and Biophysics* **221** 117–124

Rozek, A., Buchko, G. W. & Cushley, R. J. 1995 Conformation of two peptides corresponding to human apolipoprotein C-1 residues 7–24 and 35–53 in the presence of sodium dodecyl sulfate by CD and NMR spectroscopy. *Biochemistry* **34** 7401–7408

Rucker, S. P. & Shaka, A. J. 1989 Broadband homonuclear cross polarization in 2D N.M.R. using DIPSI-2. *Molecular Physics* **68** 509–517

Sönnichsen, F. D., van Eyk, J. E., Hodges, R. S. & Sykes, B. D. 1992 Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. *Biochemistry* **31** 8790–8798

Sundaralingam, M. & Sekharudu, Y. C. 1989 Water-inserted α-helical segments implicate reverse turns as folding intermediates. *Science* **224** 1333–1337

Tirado-Rives, J. & Jorgensen, W. L. 1991 Molecular dynamics simulations of the unfolding of an α-helical analogue of ribonuclease A S-peptide in water. *Biochemistry* **30** 3864–3871

Wishart, D. S., Sykes, B. D. & Richards, F. M. 1992 The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* **31** 1647–1651

Wu, C.-S. C., Ikeda, K. & Yang, J. T. 1981 Ordered conformation of polypeptides and proteins in acidic dodecyl sulfate solution. *Biochemistry* **20** 566–570

Wüthrich, K. 1986 *NMR of Proteins and Nucleic Acids*. New York: Wiley

Yang, J. J., Buck, M., Pitkeathy, M., Kotik, M., Haynie, D. T., Dobson, C. M. & Radford, S. E. 1995 Conformational properties of four peptides spanning the sequence of hen lysozyme. *Journal of Molecular Biology* **252** 483–491