Journal of Dairy Research (2002) **69** 85–94. © *Proprietors of Journal of Dairy Research 2002*
DOI: 10.1017/S0022029901005271 *Printed in the United Kingdom*

Structural features of bovine caseinomacropeptide A and B by ^{1}H nuclear magnetic resonance spectroscopy

By MARK H. SMITH[1,2], PATRICK J. B. EDWARDS[2], KATE P. PALMANO[2] and LAWRENCE K. CREAMER[1]*

[1] *Food Science Section, New Zealand Dairy Research Institute, Palmerston North, New Zealand*

[2] *Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand*

(*Received 1 February 2001 and accepted for publication 24 October 2001*)

Summary. Samples of bovine caseinomacropeptide (CMP) were isolated from κ-casein A and κ-casein B and fractionated to give aglycosylated CMP A and CMP B and monoglycosylated CMP A. The secondary structures of these three peptides were compared under neutral and acidic (pH 4·2) conditions, using two-dimensional (2D) ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The differences between the spectra at pH 4·2 and 7·0 and the spectra of the aglycosylated and glycosylated CMP A were subtle, indicating little change in backbone conformation with these changes. These results suggest that differences in the coagulation properties of milks containing either κ-casein A or κ-casein B are more likely to be related to factors, such as micelle size or charge, than to structural differences arising from altered backbone conformation of the macropeptide segments of the κ-caseins.

Keywords: κ-Casein, CMP, NMR, protein conformation, genetic varients, peptides.

The caseins are nutritionally important milk proteins that exist in milk as colloidal particles known as micelles. These micelles are stabilized against coagulation and precipitation by a hydrated, negatively charged surface layer comprising the C-terminal region of κ-casein. The N-terminal two-thirds of this 169 amino acid protein has a high proportion of hydrophobic residues and buries itself in the micelle's interior. Chymosin cleaves κ-casein (in the first step of digestion by the calf and in the cheesemaking process) to yield caseinomacropeptide (CMP), which comprises the hydrophilic, 64 amino acid C-terminal segment of κ-casein, and para-κ-casein, which makes up the hydrophobic remainder.

There are at least six genetic variants of κ-casein (Creamer & Harris, 1997), of which the most common are A and B. The κ-casein genotype has been observed to influence the technological properties of milk and comparisons (Jakob, 1994; Horne *et al.* 1995; Walsh *et al.* 1998) of milks containing κ-casein A and κ-casein B showed that the B variant milks gave firmer cheese curds, increased the cheese yield and shortened renneting times. κ-Casein B was associated with higher contents of total protein, κ-casein and ionic calcium (Van Den Berg, 1994) and with smaller average micelle size. κ-Casein B genotype has also been suggested to confer better heat stability to milk (Robitaille, 1995).

* For correspondence; e-mail: Lawrie.creamer@nzdri.org.nz

The amino acid substitutions that differentiate variant A from variant B occur at residues 136 and 148 of κ-casein or about midway along the CMP. The polar residues, Thr^{136} and Asp^{148}, in κ-casein A are replaced in κ-casein B by the hydrophobic Ile and Ala respectively. The Thr residues at positions 131, 133 and 135 (and 136 for κ-casein A) have been identified as likely glycosylation sites on CMP and each Thr is able to form O-glycosidic links to oligosaccharide chains (Eigel *et al.* 1984). The pattern of glycosylation is different for κ-casein A and κ-casein B. Horne *et al.* (1995) suggested that κ-casein B probably provides less effective steric stabilization for the micelle than κ-casein A (due to the B variant's lower net charge), and therefore a smaller proportion of hydrolysed κ-casein B is required for the onset of coagulation.

Early structural studies on κ-casein and CMP have generally been limited to low resolution techniques such as circular dichroism (CD) spectroscopy (Ono *et al.* 1987) or Fourier transform infrared (FTIR) spectroscopy used in conjunction with structure prediction (Kumosinski *et al.* 1991) to build a three-dimensional model of κ-casein. Griffin & Roberts (1985) found κ-casein or CMP gave NMR signals that could be interpreted to show that the macropeptide Thr methyl groups were very mobile and thus the macropeptide probably had considerable freedom of movement. Creamer *et al.* (1998) took the approach that some very stable secondary structural elements in a protein may be partially retained in a peptide that was derived from that protein. Thus a helix that exists in the intact protein may be observable but sheets, which require a high degree of co-operativity, are unlikely to be observed. Plowman *et al.* (1997*a*) used various techniques to encourage secondary structure formation in a peptide corresponding to residues 130-153 of κ-casein B, and which contained a region that was predicted to have α-helical structure. They found that at low pH and at moderate concentrations of a structure-inducing solvent, trifluoroethanol, α-helix was formed.

We now report the application of 2D NMR to various CMPs, to determine (a) differences, if any, in the secondary structure of the CMP portion of the κ-caseins, which could then explain the disparity in the intermicellar interactions of either κ-casein A or κ-casein B, and (b) possible effects of glycosylation and pH on secondary structure.

Materials and methods

Sample preparation

Three peptides, monoglycoCMP A, aglycoCMP A and aglycoCMP B, were prepared using previously published methods (Coolbear *et al.* 1996). The glycosylated A variant fraction contained approximately one tetrasaccharide unit per peptide chain. The samples were dissolved in $^2H_2O/H_2O$ (10:90, v/v) to give a final CMP concentration of about 1·5 mM. pH measurements were made with a World Precision Instruments (Sarasota, FL 34240-9258, USA) micro pH probe calibrated against standard buffer solutions immediately prior to use. pH adjustments were made by adding small amounts of 0·1 M-NaOH or 0·1 M-HCl as appropriate. Sample degradation was assessed from 1D ^1H spectra recorded before and after the 2D experiments.

NMR spectroscopy

All NMR spectra were recorded at 25 °C on a Bruker Avance 400 MHz NMR spectrometer (Bruker Analytik, D-76287, Rheinstetten, Germany). Total correlation spectroscopy (TOCSY) spectra (Braunschweiler & Ernst, 1983) with WATERGATE

water suppression (Piotto *et al*. 1992) were recorded using a sweep width of 4000 Hz and a mixing time of 80 ms. The carrier frequency was set in the middle of the water peak (about 4·78 ppm). The spectra were acquired with a matrix size of 512 free induction decays of 2048 complex points. Each matrix was zero-filled to 2048 by 2048 complex points and a 90° shifted sinebell squared apodization was applied in each dimension prior to Fourier transformation. Spectra were referenced to internal 2,2-dimethylsilyl-2-silapentane-5-sulphonate, sodium salt (DSS) at 0 ppm.

Structure prediction

Three on-line structure prediction tools were used to obtain estimates of the secondary structures of CMP A and CMP B: the PredictProtein server at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany (Rost & Sander, 1993, 1994; Rost, 1996) (*http://www.embl-heidelberg.de/Services/sander/predictprotein/*), the nnpredict server (NNP) at the Department of Cellular & Molecular Pharmacology, University of California, San Francisco, CA 94143, USA (McClelland & Rumelhart, 1988; Kneller *et al*. 1990) (*http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html*) and the PSIpred V2.0 server at the Department of Biological Sciences, Brunel University, Uxbridge, UB8 3PH, UK (Altschul *et al*. 1997) (*http://insulin.brunel.ac.uk/psipred/*).

RESULTS AND DISCUSSION

The results of three secondary structure prediction algorithms applied to both CMP A and CMP B are compared and summarized in Fig. 1. The predictions indicated that the structures of CMP A and CMP B should be very similar but they disagreed on the details of the structures. All predicted that CMP is largely a disordered peptide with small sections of β-strand; however, two (NNP and PSIpred) predicted a segment of α-helix linked to a length of β-strand in the middle region of the peptide whereas the third (EMBL) predicted a continuous β-strand. Plowman *et al*. (1997*a*), using a different algorithm, found that α-helix was predicted in this region of the bovine protein but was not predicted for the closely related sheep or goat proteins. All of these prediction results should be treated with some caution because the protein structure databases from which they are derived contain, primarily, globular proteins and CMP is not a globular protein (Griffin & Roberts, 1985).

Backbone proton (H_N and H_α) chemical shifts tend to move upfield or downfield from their typical random coil values if their residues form part of a helix or a β-sheet respectively (Wishart *et al*. 1991). Therefore, structurally rich peptides are expected to have reasonably broad H_N and H_α regions. Figure 2 shows a 1D proton spectrum of CMP B at pH 7·0. The narrow chemical shift range across the H_N and H_α regions (marked A and C respectively) indicated that most of the structure was likely to be of a similar type. The number of H_α resonances clustered around 4·3 ppm suggested that a high proportion of the CMP backbone was neither sheet nor helix (Wishart *et al*. 1991). This is consistent with the structure prediction results, which indicated that less than half of the residues in CMP are likely to be incorporated into secondary structure elements. Thus, it is likely that CMP has little formal secondary structure in an aqueous environment.

The lack of signal separation and the high proportion of the protein made up of a few residue types (i.e. Thr, Glx and Pro account for almost half of the residues)

```
CMP A
          106       115         125         135         145         155         165  169
           |         |           |           |           |           |           |    |
          MAIPPKKNQD KTEIPTINTI ASGEPTSTPT TEAVESTVAT LEDSPEVIES PPEINTVQVT STAV
(EMBL)    ---------- -EEE----EE EEEE------ EEEEEEEEE- ------EEE- -----EEEEE E---
(NNP)     ---------- ------EEE- ---------- -EEEHHHHE- ---------- -----EEEE- ----
(PSIpred) ---------- ---------- ---------- HHHHHHEEE- ------EE-- -----EE--- ----

CMP B
          106       115         125         135         145         155         165  169
           |         |           |           |           |           |           |    |
          MAIPPKKNQD KTEIPTINTI ASGEPTSTPT IEAVESTVAT LEASPEVIES PPEINTVQVT STAV
(EMBL)    ---------- -EEE--E-EE EEEE-----E EEEEEE-EE- -----EEEE- -----EEEEE E---
(NNP)     ---------- ------EEE- ---------- EEEEHHHHH- ---------- -----EEEE- ----
(PSIpred) ---------- ---------- ---------- HHHHHHEEE- ------EE-- -----EE--- ----
```

Fig. 1. Comparison of caseinomacropeptide A and caseinomacropeptide B using three on-line structure prediction algorithms. The server for each algorithm is shown in brackets (EMBL: the PredictProtein server at the European Molecular Biology Laboratory. NNP: the nnpredict server at the University of California. PSIpred: the PSIpred V2.0 server at Brunel University. Secondary structure predictions: H = α-helix; E = β-strand (or extended); – = no prediction. The initial M in the sequences corresponds to M^{106} in κ-casein.

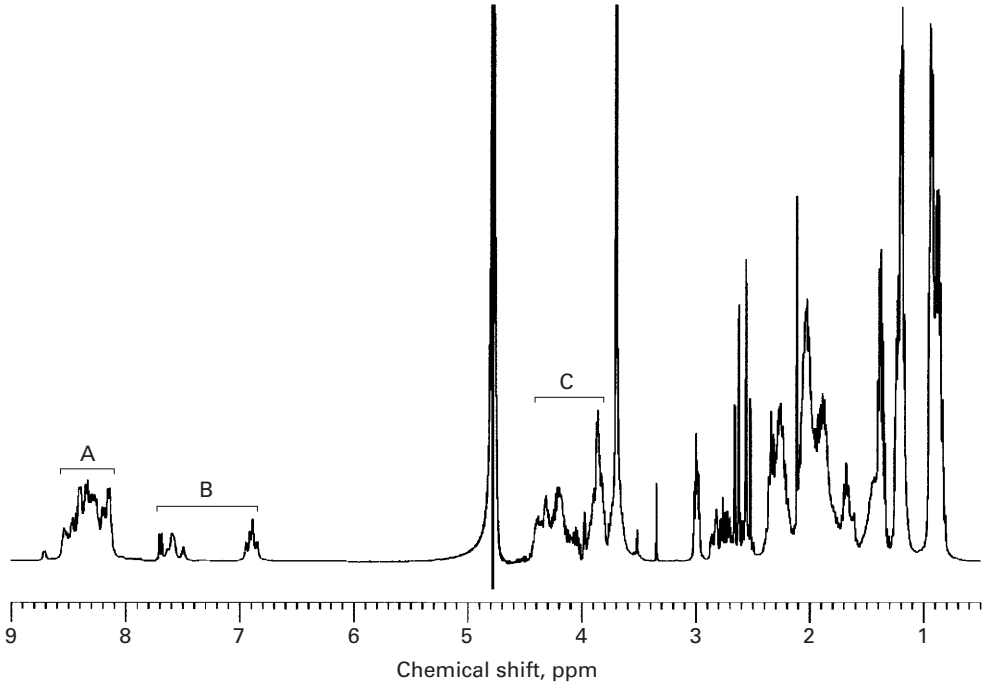

Fig. 2. 400 MHz ^{1}H NMR spectrum of caseinomacropeptide B measured at pH 7. Peaks marked with letters are as follows: A, the amide protons; B, the sidechain amides of Asn and Gln; C, all α-protons and the β-protons of Ser and Thr.

would have made the identification of individual H_α and H_N protons using 2D techniques extremely difficult. This precluded the determination of a full NMR assignment and structure.

Earlier studies sought to overcome these difficulties by examining peptides representing segments of κ-casein (Plowman *et al.* 1997*a*; Creamer *et al.* 1998). In these

https://doi.org/10.1017/S0022029901005271 Published online by Cambridge University Press

studies a peptide spanning the region from Pro^{130} to Ile^{153} was shown to have a predominantly α-helical structure from Ile^{136} to Ser^{149} in the helix-inducing trifluoroethanol (TFE)/water solvent system (Dyson & Wright, 1993). The portion of the 2D nuclear Overhauser (NOESY) spectrum (Bodenhausen *et al.* 1984) correlating the spatially proximate protons showed many strong $d_{NN}(i, i+1)$ cross-peaks typical of α-helical structure. In contrast, this region of the NOESY spectra of the intact CMPs lacked these strong cross-peaks and therefore CMP probably did not contain a significant amount of α-helix under the conditions used in our experiments.

H_α chemical shifts are expected to change from their random coil values by, on average, $-0{\cdot}39$ ppm on incorporation of these protons into a helix and $+0{\cdot}37$ ppm when involved in β-strand or extended structures (Wishart *et al.* 1991). Therefore the sensitivity of H_α chemical shifts to changes in secondary structure can be used to give a global view of the conformational changes in the macropeptide with environment (or sequence) in much the same way as low resolution techniques such as CD and FTIR spectroscopy. We could, therefore, determine whether there were any major differences in the structural elements of various CMP samples as a result of the variant substitution from A to B, with glycosylation or on changing the pH, by comparison of the respective 2D spectra.

Comparison of caseinomacropeptides A and B at pH 7

The interactions of κ-casein variants with chymosin and therefore the renneting of milk is influenced by substitutions close to the cleavage site. For example, the substitution of Arg^{97} for His in the C variant leads to a reduction in the rate of κ-casein cleavage (Delacroix-Buchet *et al.* 1993; Lodes *et al.* 1996), most probably because of a reduction in the stability of the enzyme-substrate complex (Plowman *et al.* 1997*b*). However, the observation of differences in the renneting times in κ-casein A and κ-casein B milks has been largely attributed to differences in the extent of expression of the variants and differences in the relative abilities of the variants to provide steric stabilization to the micelle (Horne *et al.* 1995).

Figure 3*a* compares the fingerprint regions of TOCSY spectra of CMP A and CMP B at pH 7. The results of the TOCSY experiment are shown because this technique yields a single absorptive peak for each correlation. The H_α correlations were clustered between 4·1 and 4·7 ppm which is characteristic of a random conformation. It has been pointed out that any structural features of the κ-caseins are likely to be transient (Holt & Sawyer, 1988, 1993; Sawyer & Holt, 1993). The existence of a relatively small amount of structured peptide undergoing rapid exchange with peptides in random conformations would cause the positions of H_N-H_α correlations to move to the population weighted averages of the H_N and H_α chemical shifts in the different environments. Therefore the location and narrow chemical shift dispersion of the H_α correlations suggested that at any particular instant the majority, if not all, of the peptide was unstructured. The high degree of overlap in the spectra indicated little or no structural difference between the two variants at this pH and in the absence of calcium ions.

Even though the CMP variant substitutions at positions 136 and 148 are distant from the hydrolysis site (bond 105-106), if the enzyme-substrate interaction is altered it is possible that the rate of hydrolysis of κ-casein by chymosin could be affected. However, it has been reported that a peptide spanning the chymosin-sensitive region of κ-casein, His^{98} to Lys^{112}, has the same rate of chymosin cleavage as the native protein (Visser *et al.* 1980). This implies that all the elements required for the

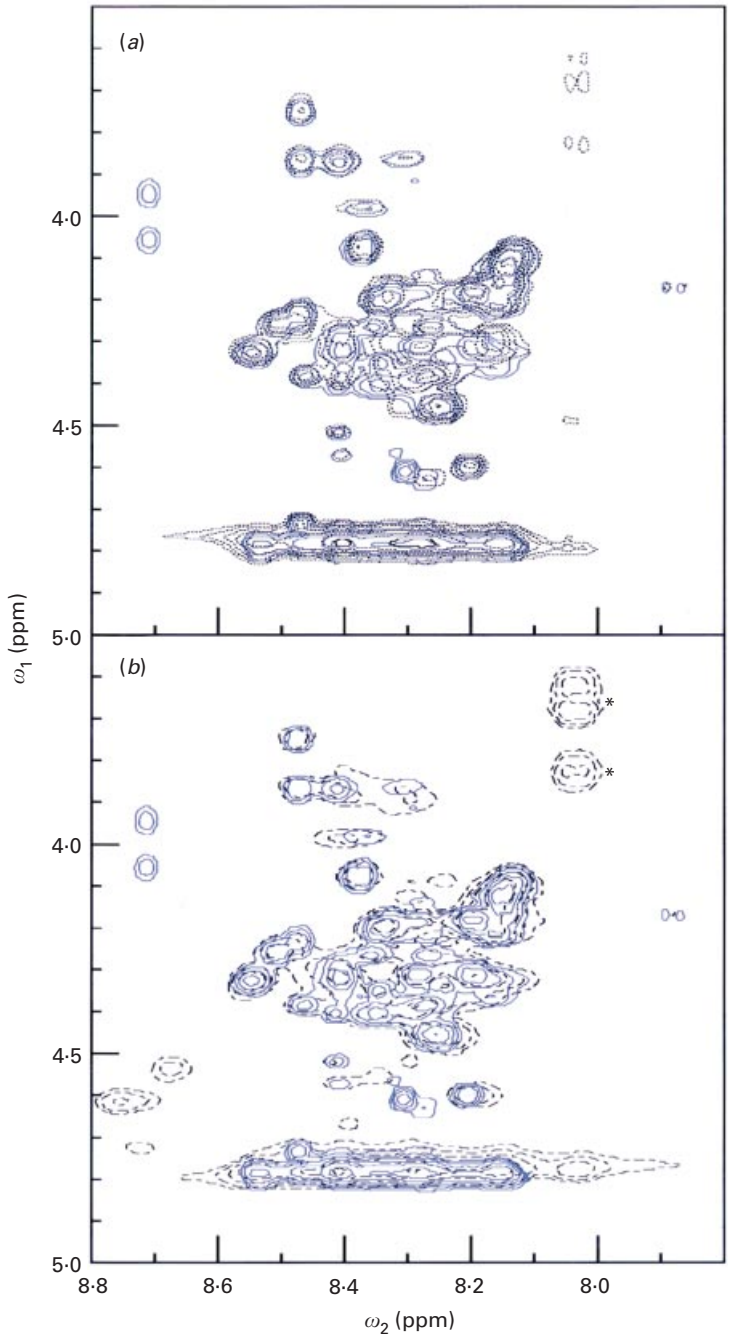

Fig. 3. For legend see facing page.

recognition of the substrate by the enzyme are included in this region. Substitutions further along the sequence should play no part unless they introduce a conformational change that would limit the access of the enzyme. The similarity of the spectra and therefore of the conformations of the variants would make such a dramatic change seem unlikely.

https://doi.org/10.1017/S0022029901005271 Published online by Cambridge University Press

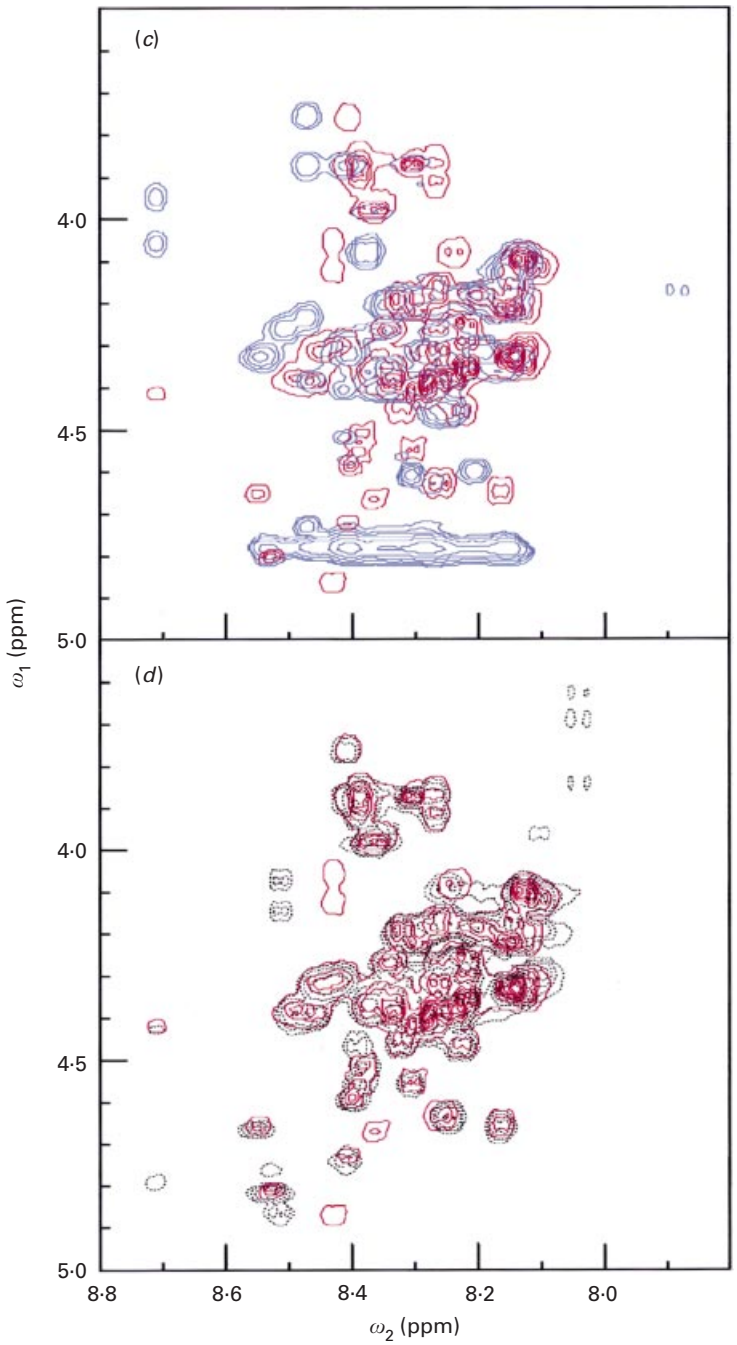

Fig. 3. Fingerprint region of 2D TOCSY spectra of caseinomacropeptide A (solid lines), glycosylated-caseinomacropeptide A (dashed lines) and caseinomacropeptide B (dotted lines). The pH is indicated by the colour of the caseinomacropeptide A contours (pH 4·2 = red, pH 7 = blue). The spectra show correlations between amide proton (H_N) resonances (along ω_2) and their corresponding α-proton (H_α) resonances (along ω_1). (*a*) Caseinomacropeptide A and caseinomacropeptide B at pH 7; (*b*) caseinomacropeptide A and glycosylated caseinomacropeptide A at pH 7; (*c*) caseinomacropeptide A at pH 7 and pH 4·2; (*d*) caseinomacropeptide A and caseinomacropeptide B at pH 4·2. Carbohydrate signals are marked with an *. The large stripe at $\omega_1 = 4{\cdot}78$ ppm is due to chemical exchange between H_N and water.

https://doi.org/10.1017/S0022029901005271 Published online by Cambridge University Press

Comparison of aglycosylated and glycosylated caseinomacropeptide A

Presence of carbohydrate moieties does not appear to affect the rate of cleavage of isolated κ-casein by chymosin (Horne *et al.* 1995), but it does affect the stability of the micelle and its susceptibility to coagulation on the addition of rennet to milk. The presence of sialic acid groups increases the hydrophilic nature of CMP and increases its ability to solvate the casein micelle. The increase in the hydrodynamic size of the CMP portion of the protein would increase the steric repulsion between the micelles and would increase the proportion of κ-casein that needed to be hydrolysed by chymosin before coagulation could occur. It has been noted that the influence of glycosylation on the rate of rennet coagulation is less than that of the variant substitution (Horne *et al.* 1995). It is not clear whether the presence of a sialic acid moiety influences the steric repulsion by altering the conformation of the CMP region in conjunction with an increase in hydrodynamic size.

Fig. 3*b* shows a comparison of the fingerprint regions of TOCSY spectra of CMP A and glycosylated CMP A at pH 7·0. Apart from the peaks due to sialic acid (marked with an *), the spectra were remarkably similar, indicating a high degree of similarity in the backbone conformations of the glycosylated and non-glycosylated forms. The three peaks at the bottom left hand corner of Fig. 3*b* were present in the spectrum of CMP B at pH 7, but at an intensity too low to be displayed in Fig. 3*a*. The protons giving rise to these peaks did not demonstrate correlations with any other protons and probably arose from an impurity in the sample. The similarity of the spectra suggests that the sialic acid moieties do not influence the conformational structure of the macropeptide. Thus, the change in overall volume of the CMP region probably determines the micellar properties. This result is as expected because post-translational modifications usually occur on the surface of a protein and are therefore less likely to cause a change in the overall conformation of the underlying peptide chain.

Effect of lower pH on caseinomacropeptides A and B

A comparison of the fingerprint regions of TOCSY spectra of CMP A at pH 7 and pH 4·2 is shown in Fig. 3*c*. The differences between the spectra obtained at neutral and acidic pHs were small. In the spectrum at pH 4·2, there was a small overall shift of the H_α resonances with chemical shifts above 4·5 ppm. A downfield displacement of H_α resonances is consistent with a move from random to extended conformation. Nevertheless, it appeared that, on reducing the pH, no new structures were formed and the random coil still dominated. Finally, Fig. 3*d* shows a comparison between CMP A and CMP B at pH 4·2. Here again there was great similarity between the spectra, which indicated that the conformation of CMP B was equally insensitive to the increase in acidity.

It was noted by Kawasaki *et al.* (1993) that the molecular mass of CMP indicated by gel filtration was pH-dependent and that at a neutral pH the measured hydrodynamic volume and estimated molecular mass of CMP was significantly higher than the actual value. (Similar behaviour has been reported for all the caseins (Green & Pastewka, 1976; Creamer & Richardson, 1984) and was linked to the extended structures of certain regions of each casein.) Kawasaki *et al.* (1993) proposed two mechanisms to explain this phenomenon: the formation of oligomers and hydration. It is also possible that the observed behaviour is due to structural changes in the CMP brought about by a reduction in overall charge on the peptide. The number of acidic sidechains in CMP A (eight Glu and two Asp) and the large

number of Pro residues (eight) would suggest that aggregation at neutral pH is the less likely explanation for the higher than expected hydration volume (molecular weight). More likely explanations would be a high degree of hydration, a high axial ratio for the peptide in solution, or, possibly, a more disordered main chain.

Plowman *et al.* (1997*a*) used CD to show that peptide 130-153 of κ-casein B showed a slight tendency to develop secondary structure in the presence of $CaCl_2$ or at low pH and that it definitely showed helical structure in 10 mM anionic or cationic detergent solutions and in 300 g/kg trifluoroethanol solutions. This led them to suggest that there could be transient formation of helical structures within native κ-casein. The present results do not preclude this possibility, but do make it seem less likely.

Results shown in Fig. 3 indicated that variant substitutions and the addition of carbohydrate groups had little effect on the overall solution conformation of the amino acid side-chains of the CMP. In an earlier study, it was shown that the sharp lines in the spectrum of intact casein micelles closely resembled the spectrum of CMP (Griffin & Roberts, 1985), indicating that the CMP portion of κ-casein attached to the casein micelle is quite flexible. This supports the present findings, which indicate that neither glycosylation nor the Thr^{136} to Ile and Asp^{148} to Ala (A to B) mutations that distinguish the A and B variant proteins have a major effect on the backbone conformation. This conclusion supports Horne *et al.* (1995), who suggest that the dominant interaction controlling the rate of coagulation is the intermicellar interaction rather than the interaction between enzyme and substrate. Hence most of the differences in the behaviour of the micelles in milks with the A and B variants of κ-casein can be attributed to the charge difference caused by the Asp^{148} to Ala (A to B) mutation.

In order to make further progress with the structure of κ-casein in its natural environment, a more extensive NMR study would be required. The three-dimensional technique using ^{13}C and ^{15}N labelled protein expressed in a microbial system, e.g. Cavanagh *et al.* (1996), would assist in making definitive assignments. However, the difficulties posed by the high frequencies of the Pro, Glx and Thr residues in the CMP portion of κ-casein remain. Using NMR to obtain important structural information about the para-κ-casein portion of κ-casein, particularly the interaction with other caseins within the core of the micelle, may well be hampered by the broad signals expected from the large aggregates that exist under physiological conditions.

We are grateful to Mike Boland, Geoff Jameson and Wayne Thresher for valuable discussions during the preparation of this paper and to the Foundation for Research, Science and Technology, New Zealand for support via Contract Nos. DRI 403 and 801.

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25** 3389–3402

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

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

Cavanagh, J., Fairbrother, W. J., Palmer, A. G. & Skelton, N. J. 1996 *Protein NMR Spectroscopy*. San Diego, CA: Academic Press

Coolbear, K. P., Elgar, D. F. & Ayers, J. S. 1996 Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. *International Dairy Journal* **6** 1055–1068

Creamer, L. K. & Harris, D. P. 1997 Relationship between milk protein polymorphism and physico-chemical properties. In *Milk Protein Polymorphism*, International Dairy Federation Special Issue **9702** pp. 110–123
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
Creamer, L. K., Plowman, J. E., Liddell, M. J., Smith, M. H. & Hill, J. P. 1998 Micelle stability: κ-casein structure and function. *Journal of Dairy Science* **81** 3004–3012
Delacroix-Buchet, A., Lefier, D. & Nuyts-Petit, V. 1993 [κ-Casein polymorphism of three French cattle breeds and milk rennet coagulation ability.] *Lait* **73** 61–72
Dyson, H. J. & Wright, P. E. 1993 Peptide conformation and protein folding. *Current Opinion in Structural Biology* **3** 60–65
Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M. Jr., Harwalkar, V. R., Jenness, R. & Whitney, R. McL. 1984 Nomenclature of proteins of cow's milk: fifth revision. *Journal of Dairy Science* **67** 1599–1631
Green, M. R. & Pastewka, J. V. 1976 Molecular weights of three mouse milk caseins by sodium dodecyl sulfate polyacrylamide gel electrophoresis and κ-like characteristics of a fourth casein. *Journal of Dairy Science* **59** 1738–1745
Griffin, M. C. A. & Roberts, G. C. K. 1985 A ^{1}H-n.m.r. study of casein micelles. *Biochemical Journal* **228** 273–276
Holt, C. & Sawyer, L. 1988 Primary and predicted secondary structures of the caseins in relation to their biological functions. *Protein Engineering* **2** 251–259
Holt, C. & Sawyer, L. 1993 Caseins as rheomorphic proteins: interpretation of primary and secondary structures of the α_{s1}-, β- and κ-caseins. *Journal of the Chemical Society, Faraday Transactions* **89** 2683–2692
Horne, D. S., Muir, D. D., Leaver, J., Maccreight, T. A. & Banks, J. M. 1995 Effects of κ-Cn phenotype on rennet coagulation time. *International Dairy Federation Bulletin* no. 304 11
Jakob, E. 1994 Genetic polymorphism of milk proteins. *International Dairy Federation Bulletin* no. 298 17–27
Kawasaki, Y., Kawakami, H., Tanimoto, M., Dosako, S., Tomizawa, A., Kotake, M. & Nakajima, I. 1993 pH-dependent molecular weight changes of κ-casein glycomacropeptide and its preparation by ultrafiltration. *Milchwissenschaft* **48** 191–196
Kneller, D. G., Cohen, F. E. & Langridge, R. 1990 Improvements in protein secondary structure prediction by an enhanced neural network. *Journal of Molecular Biology* **214** 171–182
Kumosinski, T. F., Brown, E. M. & Farrell, H. M. Jr, 1991 Three-dimensional molecular modeling of bovine caseins: κ-casein. *Journal of Dairy Science* **74** 2879–2887
Lodes, A., Buchberger, J., Krause, I., Aumann, J. & Klostermeyer, H. 1996 The influence of genetic variants of milk proteins on the compositional and technological properties of milk. 2. Rennet coagulation time and firmness of the rennet curd. *Milchwissenschaft* **51** 543–548
McClelland, J. L. & Rumelhart, D. E. 1988 *Explorations in Parallel Distributed Processing*. Cambridge, MA: MIT Press
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
Piotto, M., Saudek, V. & Sklenar, V. 1992 Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *Journal of Biomolecular NMR* **2** 661–665
Plowman, J. E., Creamer, L. K., Liddell, M. J. & Cross, J. J. 1997*a* 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. *Journal of Dairy Research* **64** 377–397
Plowman, J. E., Creamer, L. K., Smith, M. H. & Hill, J. P. 1997*b* Restrained molecular dynamics investigation of the differences in association of chymosin to κ-caseins A and C. *Journal of Dairy Research* **64** 299–304
Robitaille, G. 1995 Influence of κ-casein and β-lactoglobulin genetic variants on the heat stability of milk. *Journal of Dairy Research* **62** 593–600
Rost, B. 1996 PHD: predicting one-dimensional protein structure by profile based neural networks. *Methods in Enzymology* **266** 525–539
Rost, B. & Sander, C. 1993 Prediction of protein secondary structure at better than 70% accuracy. *Journal of Molecular Biology* **232** 584–599
Rost, B. & Sander, C. 1994 Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins: Structure, Function and Genetics* **19** 55–77
Van Den Berg, G. 1994 Genetic polymorphism of κ-casein and β-lactoglobulin in relation to milk composition and cheesemaking properties. In *Cheese Yield and Factors Affecting its Control*, International Dairy Federation Special Issue **9402** 123–133
Visser, S., Van Rooijen, P. J. & Slangen, C. J. 1980 Peptide substrates for chymosin (rennin). Isolation and substrate behaviour of two tryptic fragments of bovine κ-casein. *European Journal of Biochemistry* **108** 415–421
Walsh, C. D., Guinee, T. P., Reville, W. D., Harrington, D., Murphy, J. J., O'Kennedy, B. T. & Fitzgerald, R. J. 1998 Influence of κ-casein genetic variant on rennet gel microstructure, Cheddar cheesemaking properties and casein micelle size. *International Dairy Journal* **8** 707–714
Wishart, D. S., Sykes, B. D. & Richards, F. M. 1991 Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *Journal of Molecular Biology* **222** 311–333