¹³C-n.m.r. of the cyanylated β -lactoglobulins: evidence that Cys-121 provides the thiol group of β -lactoglobulins A and B

Paul PHELAN and J. Paul G. MALTHOUSE*

Department of Biochemistry, University College Dublin, Dublin 4, Ireland

The thiol groups of β -lactoglobulins A and B have been cyanylated using [¹³C]KCN. The samples of [cyanato-¹³C]-cyanylated- β -lactoglobulins A and B which we prepared had signals at 109.7 p.p.m and 114.4 p.p.m. We conclude that the thiocyanate carbon having a chemical shift of 109.7 p.p.m. is in an apolar environment similar to a cyclohexane solvent, whereas the thiocyanate carbon having a chemical shift of 114.4 p.p.m. is in a polar environment similar to water. The signals with chemical shifts of 109.7 p.p.m. are assigned to the thiocyanate carbons of the native [cyanato-¹³C]cyanylated- β -lactoglobulins A and B. We deduce that the signal at 114.4 p.p.m. is due to an irreversibly denatured/unfolded species produced by alkaline denaturation,

which is caused by intramolecular thiol/disulphide exchange occurring during our cyanylation procedure. We propose that Cys-119 is cyanylated in the irreversibly denatured species and Cys-121 is cyanylated in the native [cyanato-¹³C]cyanylated- β -lactoglobulins A and B. We suggest that the same intramolecular thiol-disulphide exchange reactions occurred when McKenzie and co-workers [McKenzie, Ralston and Shaw (1972) *Biochemistry* 11, 4539–4547] alkylated β -lactoglobulins with iodoacetamide. Therefore the one mol of thiol/mol of monomer in the native β -lactoglobulins is due to the thiol of Cys-121 and is not due to an equimolar mixture of Cys-119 and Cys-121 as they suggested.

INTRODUCTION

β -Lactoglobulin is a major whey protein in the milk of ruminants. Aschaffenburg and Drewry (1955, 1957) found that bovine milk could contain either a mixture of two different β -lactoglobulins, β -lactoglobulins A and B, or only one of these β -lactoglobulins. β -Lactoglobulin B has an identical amino acid sequence to β -lactoglobulin A except that while residues 64 and 118 are aspartate and valine respectively in β -lactoglobulin A they are glycine and alanine respectively in β -lactoglobulin B (Braunitzer et al., 1973). The bovine β -lactoglobulins A and B both contain five cysteine residues but the intact or denatured protein contains 1 mol of thiol/mol of monomer (McKenzie, 1971). One disulphide bond has been shown to occur between Cys-66 and Cys-160 (Hambling et al., 1992). McKenzie et al. (1972) reported that when β -lactoglobulin was incubated with [¹⁴C]iodoacetamide at pH 8.5 in 8 M urea equimolar amounts of Cys-119 and Cys-121 were alkylated. This suggests that the 1 mol of thiol/mol of monomer consists of 0.5 mol of thiol/mol of monomer from both Cys-119 and Cys-121. Therefore equimolar amounts of disulphide bonds involving cysteine residues 106–119 and 106–121 must also be present. However, X-ray crystallographic studies (Papiz et al., 1986; Monaco et al., 1987) have shown only one disulphide bond (between residues 106 and 119) involving Cys-106. This conflict between the X-ray crystallographic studies and the results of McKenzie et al. (1972) has not been resolved (Hambling et al., 1992).

In an earlier ¹³C-n.m.r. study of cyanylated β -lactoglobulins two signals from thiocyanate carbons in different environments were observed (Phelan and Malthouse, 1994). As a mixture of β -lactoglobulins A and B was used, it was not possible to determine whether the two thiocyanate signals were due to a single thiocyanate carbon being present on each of the cyanylated β -lactoglobulins A and B, or if they resulted from each protein having two thiocyanate carbons with different chemical shifts. In the present study we show that the same thiocyanate carbon

signals are detected when β -lactoglobulins A and B are cyanylated separately or as a mixture. We also show that one signal is due to a denatured cyanylated β -lactoglobulin while the other is due to an intact cyanylated β -lactoglobulin which we can isolate. Finally we discuss how our results could resolve the conflict between the X-ray crystallographic studies (Papiz et al., 1986; Monaco et al., 1987) and the results of McKenzie et al. (1972).

MATERIALS AND METHODS

Materials

Sephadex G-100 (superfine), 5,5'-dithiobis(2,2'-nitrobenzoic acid), 2-mercaptoethanol, urea, β -lactoglobulins A and B, and the mixture of β -lactoglobulins A and B (from bovine milk, crystallized three times) were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Trifluoroacetic acid (> 99%), formamide (> 99%), [¹³C]KCN (99 atom %) and ²H₂O (99.8 atom %) were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals used were obtained or synthesized as described by Doherty et al. (1992).

Protein and thiol concentrations

Subunit concentrations of both the native and cyanylated proteins were determined spectrophotometrically using ϵ_{278} 17 500 M⁻¹·cm⁻¹ assuming an M_r value of 18 200 (Hambling et al., 1992). Thiol concentrations were determined using 5,5'-dithiobis(2,2'-nitrobenzoic acid) which was dissolved in 50 mM potassium phosphate buffer at pH 7.0. The concentrations of the 5,5'-dithiobis(2,2'-nitrobenzoic acid) solutions were determined spectrophotometrically using ϵ_{323} 18 000 M⁻¹·cm⁻¹ (Brocklehurst and Little, 1973). The concentrations of thiol-containing compounds were determined by incubating the thiol with a 20-fold excess of 5,5'-dithiobis(2,2'-nitrobenzoic acid) at pH 9.0 and calculating the amount of 2-nitro-5-thiobenzoic acid released using ϵ_{412} 13 600 M⁻¹·cm⁻¹ (Ellman, 1959).

* To whom correspondence and reprint requests should be addressed.

Preparation of cyanylated β -lactoglobulins

The thiol groups of β -lactoglobulin (approx. 0.5 mM) were titrated using a 20-fold excess of 5,5'-dithiobis(2,2'-nitrobenzoic acid) in 50 mM ammonium carbonate buffer at pH 8.5. When no further increase in the concentration of 2-nitro-5-thiobenzoic acid could be detected the pH was adjusted to 7.0 using 1 M HCl. 2-Nitro-5-thiobenzoic acid and excess 5,5'-dithiobis(2,2'-nitrobenzoic acid) were removed by dialysis against 4×2 litres of 10 mM potassium phosphate buffer at pH 7.0. The mixed disulphide (0.2–0.5 mM) was then incubated with a 100–200-fold excess of [^{13}C]KCN in 50 mM ammonium carbonate buffer at pH 9.2. No further increase in the concentration of 2-nitro-5-thiobenzoic acid was detected after 30 min. The pH of this solution was then adjusted to 7.0 using 1 M HCl. Low- M_r materials were removed by dialysis against 4×2 litres of 10 mM potassium phosphate buffer, pH 7.0. The cyanylated protein was then concentrated by ultrafiltration in an Amicon Diaflo cell fitted with a PM10 membrane.

Determination of the degree of cyanylation of β -lactoglobulin

The mixed disulphide of 2-nitro-5-thiobenzoic acid and β -lactoglobulin had absorption maxima at 278 nm and 330 nm. As the absorbance of β -lactoglobulin at 330 nm was negligible, the value at 330 nm can be assigned to the 2-nitro-5-thiobenzoic acid moiety of the mixed disulphide. We estimate that for 5,5'-dithiobis(2,2'-nitrobenzoic acid) ϵ_{278} is $9180 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Therefore the absorbance of the mixed disulphide at 278 nm is due to both β -lactoglobulin and the 2-nitro-5-thiobenzoic acid moiety. If we assume that the 2-nitro-5-thiobenzoic acid moiety of the mixed disulphide has absorbance coefficients (ϵ_{278} $4590 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and ϵ_{330} $9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) which are half those of 5,5'-dithiobis(2,2'-nitrobenzoic acid) then we can determine the amount of the 2-nitro-5-thiobenzoic acid moiety in the sample of the mixed disulphide and the contribution of the 2-nitro-5-thiobenzoic acid moiety to the absorbance at 278 nm. Therefore the amount of β -lactoglobulin present in the sample can be calculated, and so the number of mol of 2-nitro-5-thiobenzoic acid covalently bound/mol of β -lactoglobulin can be determined. From the decrease in the concentration of the mixed disulphide of 2-nitro-5-thiobenzoic acid) and β -lactoglobulin on cyanylation we estimated how many of the available thiol groups of the β -lactoglobulins were cyanylated by our procedure.

Removal of denatured protein from samples

Denatured cyanylated β -lactoglobulins were precipitated by adjusting the pH to 5.2. Potassium acetate buffer (3 M) at pH 5.2 was added to a 4.5–5.0 mM solution of cyanylated β -lactoglobulin in 10 mM potassium phosphate buffer at pH 7.0 to give a solution containing 0.1 M potassium acetate buffer at pH 5.2. After 4–5 h at 4 °C the precipitated protein was removed by centrifugation at $15000 g$ for 10 min. The acetate buffer was removed from 2 ml samples by dialysing them three times against solutions of 10 mM potassium phosphate at pH 7.0 (250 ml).

Gel filtration

Gel filtration of native and cyanylated β -lactoglobulins A and B was carried out using a column (1.6 cm $\times$ 67 cm) of Sephadex G-100 (superfine). The column was equilibrated and eluted with 0.01 M potassium phosphate buffer at pH 6.85 using a flow rate of 10 ml/h. The void volume of the column was determined

using Blue Dextran. BSA, ovalbumin, carbonic anhydrase and α -lactalbumin were used as M_r standards.

SDS/PAGE

SDS/PAGE was performed on a 15 % polyacrylamide resolving gel with a 3.75 % stacking gel using the buffer system described by Laemmli (1970) and a procedure adapted from that of Allore and Barber (1984). Proteins were denatured by boiling samples for 3 min at pH 2. Half of each denatured sample was then incubated for 20 min in the sample buffer at pH 6.8 containing 20 mM dithiothreitol, while the other half of each of these samples was incubated for 20 min in the same sample buffer but without the reducing agent. Aliquots of the reduced and non-reduced samples were then loaded into adjacent wells of the slab gel and co-electrophoresed. In the absence of the reducing agent a control sample of α -chymotrypsin migrated as a single band ($M_r \sim 27000$), whereas in the presence of the reducing agent two bands (M_r values of ~ 12000 and ~ 15000), corresponding to the B and C chains of α -chymotrypsin (Weber and Osborn, 1969), were observed. The mobilities of the bands due to cyanylated β -lactoglobulins A and B were unaffected by the presence or absence of reducing agents and were consistent with M_r values of ~ 18000 . This shows that cyanylated β -lactoglobulins A and B are not dimers ($M_r \sim 36000$) linked by disulphide bonds.

N.m.r. spectra

N.m.r. spectra were obtained using a Bruker WP 80 wide-gap spectrometer operating at 20.115 MHz for ^{13}C nuclei. The spectral conditions for the protein samples were: 8192 time-domain data points; spectral width 240 p.p.m.; acquisition time 0.852 s; 49152 transients were recorded per spectrum; 1W broadband ^1H decoupling; 45° pulse angle (the spin-lattice relaxation time of the thiocyanate carbons of the cyanylated protein were estimated to be 1.5 ± 0.4 s) and an exponential weighting factor of 5 Hz. All protein samples were at 28 ± 1 °C and they contained 25 % (v/v) $^2\text{H}_2\text{O}$. The sample size was 1.0–1.2 ml in 10-mm-diam. sample tubes. Protein subunit concentrations were 2.3–4.8 mM. The same spectral conditions were used for studies of [^{13}C]N-acetyl- β -thiocyanatoalanine except that a smaller pulse angle of 10° was used to allow for its large spin-lattice relaxation time (Doherty et al., 1992). For studies of [^{13}C]N-acetyl- β -thiocyanatoalanine in formamide and trifluoroacetic acid solvents a 2 ml sample in a 10-mm-diam. tube was used. For both samples a concentric tube (diam. = 4 mm) containing a deuterated solvent was used to give a deuterium lock signal. When formamide and trifluoroacetic acid were used as solvents [$^2\text{H}_6$]acetone and $^2\text{H}_2\text{O}$ (99.8 atom %) respectively, were used as lock compounds.

Referencing of n.m.r. signals

All ^{13}C chemical shifts are quoted relative to tetramethylsilane at 0 p.p.m. The fluorinated carbon of trifluoroacetic acid had a chemical shift of 116.42 p.p.m. when 10 % (v/v) tetramethylsilane was used as an internal standard. The chemical shift of the thiocyanate carbon of [^{13}C]N-acetyl- β -thiocyanatoalanine in trifluoroacetic acid was determined in the absence of tetramethylsilane using the signal at 116.42 p.p.m. as a secondary reference. The chemical shift of the thiocyanate carbon of [^{13}C]N-acetyl- β -thiocyanatoalanine in formamide was determined using the signal at 29.80 p.p.m. (Wehrli et al. 1988) due to the perdeuteriomethyl carbons of [$^2\text{H}_6$]acetone as an external reference. The value obtained was

corrected by +0.19 p.p.m. to allow for the differences in the magnetic susceptibilities of formamide and $[\text{H}_6]\text{acetone}$ (Martin et al., 1980).

RESULTS AND DISCUSSION

Model compounds

The chemical shift of a thiocyanate carbon is minimal at 109.3 p.p.m. in cyclohexane, an apolar solvent which does not form hydrogen bonds and which has a dielectric constant of 2.0, whereas it is maximal at 115.7 p.p.m. in water, a polar solvent which is a good hydrogen bond donor and which has a dielectric constant of 78.5 (Doherty et al., 1992). The chemical shift of the carbonyl carbon of acetone is also minimal in cyclohexane but when chloroform, *t*-butanol, ethanol, methanol and water are used as solvents the solvent-induced changes in the chemical shift of the carbonyl carbon are 1.4–2.0 times larger than those of the thiocyanate carbon (Maciel and Ruben, 1963; Maciel and Natterstad, 1965; Doherty et al., 1992). The fact that the chemical shift of the carbonyl carbon of acetone is 3.9 p.p.m. larger in trifluoroacetic acid than in water has been attributed to more effective hydrogen bonding in trifluoroacetic acid (Maciel and Natterstad, 1965). In the present work we have shown that the thiocyanate carbon of $[\text{cyanato-}^{13}\text{C}]\text{N-acetyl-}\beta\text{-thiocyanato-alanine}$ has a chemical shift of 114.1 p.p.m. in trifluoroacetic acid. This chemical shift is larger than that of the same thiocyanate carbon (112.8 p.p.m.) in methyl formate whose dielectric constant of 8.5 (Doherty et al., 1992) is essentially the same as that of trifluoroacetic acid (dielectric constant 8.4; Harris and O'Konski, 1954). Therefore as methyl formate cannot act as a hydrogen bond donor to the thiocyanate group, this confirms that solvent-induced changes in the chemical shifts of thiocyanate carbons cannot be due only to changes in the dielectric constant of the solvent, and it suggests that hydrogen bonding does make a significant contribution to solvent-induced changes in the chemical shift of thiocyanate carbons. In the present work we have also shown that in formamide the chemical shift of the thiocyanate carbon of $[\text{cyanato-}^{13}\text{C}]\text{N-acetyl-}\beta\text{-thiocyanato-alanine}$ is 115.1 p.p.m. (dielectric constant = 109.5; Leader, 1951). This value is similar to its chemical shift in water (115.6 p.p.m.), which is also a good hydrogen bond donor with a high dielectric constant (Doherty et al., 1992). This confirms that the chemical shift of the thiocyanate carbon is maximal in solvents which have a high dielectric constant and which are good hydrogen bond donors.

Chemical modification of β -lactoglobulins

The thiol groups of β -lactoglobulin were titrated using a 20-fold excess of 5,5'-dithiobis (2,2'-nitrobenzoic acid) at pH 8.5 (Ellman, 1959). From the 2-nitro-5-thiobenzoic acid released we estimate that the samples of β -lactoglobulin A and β -lactoglobulin B, as well as the mixture of β -lactoglobulins A and B that we used, all contained 1.00 ± 0.01 mol of thiol/mol of subunit. Thus 93–98% of the available thiol groups of the β -lactoglobulins we examined were cyanylated using $[\text{C}^{13}]\text{KCN}$ as described in the Materials and methods section.

Evidence that both cyanylated β -lactoglobulins A and B contain more than one thiocyanate carbon

After cyanylation of a mixture of β -lactoglobulins A and B using $[\text{C}^{13}]\text{KCN}$ two signals were detected, one at 109.7 p.p.m. and the other at 114.4 p.p.m. (Figure 1b), which were not present in a control sample of the mixture of β -lactoglobulins A and B that

had not been cyanylated (Figure 1a). It has been suggested (Phelan and Malthouse, 1994) that these signals could result from the thiocyanate carbon of cyanylated β -lactoglobulin A having a different chemical shift from that of the thiocyanate carbon of β -lactoglobulin B. However, this possibility can be discounted because both of these signals were observed when a sample of β -lactoglobulin A (Figure 1c) was cyanylated and also when a sample of β -lactoglobulin B (Figure 1d) was cyanylated. These results show each sample of cyanylated β -lactoglobulin A and β -lactoglobulin B contains at least two thiocyanate carbons with different chemical shifts.

Figure 1 ^{13}C -n.m.r. spectra of cyanylated β -lactoglobulins

The acquisition parameters for the spectra were as described in the Materials and methods section. Sample conditions for spectra (a)–(i) were: (a) 4.5 mM mixture of the subunits of β -lactoglobulins A and B in 7.5 mM potassium phosphate; (b) 4.3 mM mixture of the subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulins A and B}$ in 7.5 mM potassium phosphate; (c) 4.2 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin A}$ in 7.5 mM potassium phosphate; (d) 4.3 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin B}$ in 7.5 mM potassium phosphate; (e) 2.1 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin B}$ in 7.5 mM potassium phosphate after denatured protein was removed as described in the Materials and methods section; (f) 2.2 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin B}$ in 4.0 mM potassium phosphate, 45 mM potassium acetate and 7.4 M urea; (g) same sample as (f) except that the urea has been removed by dialysis (three times) against 250 ml of 10 mM potassium phosphate at pH 7.0 and it contains 2.5 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin B}$ in 7.5 mM potassium phosphate; (h) 2.2 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin A}$ in 4.0 mM potassium phosphate and 7.4 M urea; (i) same sample as (h) except that the urea has been removed by dialysis against 3×250 ml of 10 mM potassium phosphate at pH 7.0 and it contains 2.2 mM subunits of $[\text{cyanato-}^{13}\text{C}]\text{cyanylated-}\beta\text{-lactoglobulin A}$ in 7.5 mM potassium phosphate. All samples had volumes in the range 1.1–2 ml, contained 25% (v/v) H_2O and were at pH 7.0 and $27 \pm 1^\circ\text{C}$.

β -lactoglobulin A and β -lactoglobulin B contains at least two thiocyanate carbons with different chemical shifts.

Evidence for the signal at 114.4 p.p.m. being due to denatured cyanylated β -lactoglobulins

β -Lactoglobulin A is more susceptible to alkaline denaturation than β -lactoglobulin B (Roels et al., 1971). Therefore as the cyanylated β -lactoglobulins A and B were both prepared by the same procedure, involving exposure to alkaline pH values (see the Materials and methods section), the fact that the signal at 114.4 p.p.m. is larger in the sample of cyanylated β -lactoglobulin A (Figure 1c) than in the sample of cyanylated β -lactoglobulin B (Figure 1d) suggests that the signal at 114.4 p.p.m. could be due to a denatured cyanylated β -lactoglobulin species. The fact that all of the three samples of cyanylated β -lactoglobulin A that we prepared contained more of the signal at 114.4 p.p.m. than was present in the four samples of cyanylated β -lactoglobulin B that we prepared using the same procedure supports this suggestion. Likewise, the fact that one of the thiocyanate carbons of denatured [*cyanato*- ^{13}C]cyanylated apoflavodoxin from *Megasphaera elsdenii* has a very similar chemical shift of 114.5 p.p.m. is also consistent with the signal at 114.4 p.p.m. being due to the thiocyanate carbons of denatured [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B (Doherty et al., 1992).

Denatured β -lactoglobulins A and B produced by alkaline denaturation can be precipitated at pH 5.2 (Groves et al., 1951; Roels et al., 1971). Therefore a sample of cyanylated β -lactoglobulin B (Figure 1d) was adjusted to pH 5.2, centrifuged to remove precipitated protein and re-adjusted to pH 7.0 (Figure 1e) as described in the Materials and methods section. This procedure removed the signal at 114.4 p.p.m. (Figure 1e) which confirms that this signal is due to denatured cyanylated β -lactoglobulin B. But when the precipitated protein was dialysed against 10 mM potassium phosphate buffer at pH 7.0, the dissolved protein had a signal at 114.4 p.p.m. and a smaller signal at 109.7 p.p.m., which was approximately half the intensity of the signal at 114.4 p.p.m. (spectra not shown). Therefore this procedure could not be used to prepare samples containing only the signal at 114.4 p.p.m. Adjusting a sample of cyanylated β -lactoglobulin A to pH 5.2 preferentially precipitated protein with a thiocyanate signal at 114.4 p.p.m. (spectra not shown) confirming that this signal was due to denatured cyanylated β -lactoglobulin A.

Effect of urea on the n.m.r. signals from the thiocyanate carbons of cyanylated β -lactoglobulins

Urea concentrations > 7 M have been shown to denature/unfold β -lactoglobulin (Pace and Tanford, 1968; Alexander and Pace, 1971; McKenzie and Ralston, 1973a,b) and several of its mixed disulphide derivatives (Cupo and Pace, 1983) as well as cyanylated β -lactoglobulins (Ralston, 1972). Therefore in order to determine the chemical shift of the thiocyanate carbon when [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin B is denatured/unfolded, 7.4 M urea was added to a sample of [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin B (Figure 1f; the signal at 163.4 p.p.m. is due to urea) which had been prepared in the same way as the sample of β -lactoglobulin B in Figure 1(e). In the presence of 7.4 M urea the signal at 109.7 p.p.m. was replaced by a single narrow signal (linewidth = 4.6 Hz) at 114.4 p.p.m. (Figure 1f) which supports our suggestion that the signal at 114.4 p.p.m. (Figures 1b, 1c and 1d) is due to the thiocyanate carbon of denatured/unfolded cyanylated β -lactoglobulins A and/or B. When the urea was removed by dialysis the signal at 114.4 p.p.m.

was replaced by a signal at 109.7 p.p.m. (Figure 1g) showing that the urea-induced denaturation/unfolding was reversible. Likewise, when 7.4 M urea was added to a sample of β -lactoglobulin A having signals at 109.7 p.p.m. and 114.4 p.p.m. (Figure 1c) a signal at 114.6 p.p.m. with a linewidth of 14.6 Hz was observed (Figure 1h). This signal is broader than the other signals that we have observed because it is composed of at least two species, reversibly unfolded [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin A and irreversibly unfolded [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin A produced by alkaline denaturation. However, when the urea was removed by dialysis the spectrum contained both signals at 109.7 p.p.m. and at 114.4 p.p.m. (Figure 1i), showing that alkaline denaturation is not reversible. Increasing the concentration of urea from 0 to 6 M in 1 M increments at pH 6.8 produced a progressive decrease in the magnitude of the signal at 109.7 p.p.m. and a concomitant increase in the magnitude of the signal at 114.4 p.p.m. (spectra not shown). The mid-point of the increase in magnitude of the signal at 114.4 p.p.m. occurred at a concentration of approx. 4.3 M urea. This mid-point is the same as that obtained from urea-unfolding experiments with unmodified β -lactoglobulins at this pH (Kella and Kinsella, 1988), which suggests that cyanylation does not reduce the stability of the protein with respect to urea denaturation at this pH.

Determination of whether inter- or intra-molecular thiol-disulphide exchange reactions have occurred during the preparation of cyanylated β -lactoglobulins

There is evidence that alkaline denaturation can involve inter-molecular thiol-disulphide exchange producing covalently bonded dimers, trimers, tetramers, etc. (McKenzie and Sawyer, 1967; Bon Hoa et al., 1973; McKenzie and Ralston 1973c; Roels et al., 1971, 1973). The signals at 109.7 p.p.m. (Figures 1b–e1, and 1g, 1i) and at 114.4 p.p.m. (Figures 1b, 1c, and 1i) from [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B had linewidths of 4.8 ± 0.8 Hz (mean of 12 values) and 5.7 ± 1.0 Hz (mean of six values) respectively. Similar signals from the thiocyanate carbons of [*cyanato*- ^{13}C]cyanylated flavodoxin (M_r 15000) from *Megasphaera elsdenii* had linewidths of 1.7–2.4 Hz (Malthouse et al., 1989). If the thiocyanate carbons in both of these intact proteins relax by equivalent mechanisms then their linewidths will be proportional to their M_r values (Malthouse, 1986), and if this assumption is correct, then these linewidths suggest that the [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B exist as dimers with M_r values of 36000 (Hambling et al., 1992) at pH 7.0. As the linewidths of the signals from the thiocyanate carbons of intact (109.7 p.p.m.) and alkali-denatured (114.4 p.p.m.) [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B were both similar, there was no evidence for significant amounts of trimers, tetramers, etc. being present in our samples. However, the signal at 114.4 p.p.m. (Figure 1f) due to the thiocyanate carbon of [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin B, which was unfolded in 7.4 M urea, was 4.6 Hz, which is essentially the same as the linewidths of the signals due to native and alkaline-denatured [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B. Consequently if the polymers are also unfolded then their linewidths will not reflect their M_r values and so we cannot use linewidth measurements to dismiss the possibility that inter-molecular thiol-disulphide interchange has produced covalently linked trimers, tetramers, octamers, etc. But the rate of loss of the β -lactoglobulin dimers due to polymerization reactions (Roels et al., 1971) is slow (half life approx. 72 h at pH 8.9). Therefore as the reaction of the β -lactoglobulins with 5,5'-dithiobis(2,2'-nitrobenzoic acid) was completed within 4 h at pH 8.5 (see the

Figure 2 Structures of some β -lactoglobulin monomers and dimers which could be formed by intramolecular and intermolecular thiol-disulphide exchange respectively

Materials and methods section), we estimate that in our samples less than 5% of the [cyanato-¹³C]cyanylated β -lactoglobulin dimers will have polymerized to form trimers, tetramers, octamers, etc. Gel-filtration experiments at pH 6.85 (see the Materials and methods section) confirmed this prediction and showed that at concentrations of 50–75 μ M the native and cyanylated β -lactoglobulins A and B exist predominantly as dimers. Therefore the thiol-disulphide exchange which has occurred in our samples must be either intramolecular or intermolecular, leading to the formation of non-covalently or covalently linked dimers respectively.

We assume that in native β -lactoglobulins A and B disulphide bridges only occur between Cys-66–Cys-160 and Cys-106–Cys-119 with Cys-121 providing 1 mol of thiol/mol of monomer (structure A; Figure 2) as observed by Papiz et al. (1986) and Monaco et al. (1987). However, we suggest that when the β -lactoglobulins A and B are incubated with 5,5'-dithiobis(2,2'-nitrobenzoic acid) at pH 8.5 inter- and/or intramolecular thiol-disulphide exchange could occur between the thiol group of Cys-121 and the disulphide of Cys-106–Cys-119. Intramolecular thiol-disulphide exchange should produce denatured β -lactoglobulin monomers containing disulphide bridges between Cys-66–Cys-160 and Cys-106–Cys-121 with Cys-119 providing 1 mol of thiol/mol of monomer (structure B; Figure 2). However, intermolecular thiol-disulphide exchange between the thiol group of Cys-121 of one monomer and the disulphide

of Cys-106–Cys-119 of another monomer could produce two denatured β -lactoglobulin dimers (structures C and D; Figure 2). One of these dimers will have an intermolecular disulphide bond between Cys-121–Cys-106 and it will contain thiol groups due to Cys-119 and Cys-121 (structure C; Figure 2). The other dimer will have an intermolecular disulphide bond between Cys-121–Cys-119 and it will contain thiol groups due to Cys-106 and Cys-121 (structure D; Figure 2).

Analysis by SDS/PAGE of samples of cyanylated β -lactoglobulins A and B confirmed that the denatured proteins had M_r values of 18000 both in the presence and absence of dithiothreitol (which reduces disulphide bonds) (see the Materials and methods section). Therefore we conclude that any thiol-disulphide exchange which has occurred during the preparation of our samples of cyanylated β -lactoglobulins A and B must have been intramolecular.

pH dependence of the chemical shift of the signal due to the thiocyanate carbon of non-denatured cyanylated β -lactoglobulin

The chemical shift of the thiocyanate carbon of the non-denatured [cyanato-¹³C]cyanylated- β -lactoglobulin was 109.7 ± 0.1 p.p.m. (mean of eight values) from pH values of 1.6–9.1.

General discussion

Bovine β -lactoglobulins A and B undergo (Hambling et al., 1992) three pH-dependent conformational transitions (eqn. 1).

The first two steps are reversible. The first step ($Q \leftrightarrow N$) occurs between pH values of 4–6 (Timasheff et al., 1966; McKenzie and Sawyer, 1967) and the second step ($N \leftrightarrow R$) occurs between pH values of 6–8 (Tanford et al., 1959). The fact that no significant change in the chemical shift of the thiocyanate carbon at 109.7 p.p.m. was detected from pH values of 1.6–9.1 shows that either cyanylated β -lactoglobulins do not undergo the reversible pH-dependent conformational transitions ($Q \leftrightarrow N \leftrightarrow R$), or that these conformational transitions do not affect the chemical shift of the thiocyanate carbon. As the cyanylated β -lactoglobulins have been shown (Ralston, 1972) to undergo the second conformational transition ($N \leftrightarrow R$) then the chemical shift of the thiocyanate carbon must be unaffected by this transition. The third step ($R \rightarrow D$) is an irreversible denaturation step, the rate of which increases as the pH increases (Groves et al., 1951; Tanford et al., 1959; Roels et al., 1971, 1973). The irreversible denaturation step is thought to involve thiol-disulphide exchange reactions (Schellman, 1958; McKenzie and Sawyer, 1967; Roels et al., 1971, 1973; McKenzie and Ralston, 1973a,b). The rate of thiol-disulphide exchange depends on the concentration of the thiolate anion and so, while exchange is expected at alkaline pH values, it should be minimal at acid values (McKenzie et al., 1972; Creighton, 1980). At pH 3 the urea denaturation of β -lactoglobulins A and B has been shown to be reversible (Alexander and Pace, 1971). However, while irreversible denaturation is slow at pH 3.5 it is faster at pH 5.2 and much more rapid at pH 8.9 (McKenzie and Ralston, 1973a,b). This irreversible denaturation was reduced when the thiol groups were chemically modified to prevent thiol-disulphide exchange (Roels et al., 1968; Ralston, 1972; McKenzie and Ralston, 1973a,b). At pH 7 the urea denaturation of [cyanato-¹³C]cyanylated- β -lactoglobulin B (Figures 1e–g) and A (Figures 1c, 1h, and 1i) was reversible, which shows that cyanylation of the thiol group can prevent irreversible denaturation in 7.4 M urea at pH 7.0.

We propose that in native cyanylated β -lactoglobulins Cys-121 is cyanylated (structure A; Figure 2), whereas in our samples of alkali-denatured β -lactoglobulins Cys-119 is cyanylated (structure B; Figure 2). The chemical shift of 109.7 p.p.m. for the thiocyanate carbons of native [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B (Figures 1a–1e, and 1h) shows that the thiol group of Cys-121 is in an apolar environment equivalent to cyclohexane (Doherty et al., 1992). This result is consistent with the X-ray crystallographic data of Papiz et al. (1986) which shows that the thiol group of Cys-121 is buried at a sheet–helix interface. The chemical shift of 114.4 p.p.m. for the thiocyanate carbon of [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin B (Figure 1f) in 7.4 M urea shows that when [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins are unfolded/denatured the thiocyanate carbon is transferred to a polar environment similar to that of water (Doherty et al., 1992). Papiz et al. (1986) have reported that the distance between the sulphur atoms of Cys-119 and Cys-121 is 10.5 Å, so that a significant rearrangement is required to allow formation of a disulphide bond between Cys-106 and Cys-121. It appears that this rearrangement irreversibly denatures/unfolds [*cyanato*- ^{13}C]cyanylated- β -lactoglobulins A and B such that the thiocyanate carbon of Cys-119 is in a polar environment similar to water and essentially identical with that experienced by the thiocyanate carbon of cyanylated Cys-121 when [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin A or B is unfolded in 7.4 M urea. We propose that when McKenzie et al. (1972) alkylated β -lactoglobulin using iodoacetamide in the presence of urea at pH 7.9, they produced a mixture of native and alkali-denatured alkylated β -lactoglobulins equivalent to the native (structure A; Figure 2) and alkali-denatured [*cyanato*- ^{13}C]cyanylated- β -lactoglobulin species (structure B; Figure 2) giving signals at 109.7 p.p.m. and 114.4 p.p.m. respectively. This explanation removes the conflict between results of the X-ray crystallographic studies (Papiz et al., 1986; Monaco et al., 1987) and the covalent modification studies of McKenzie et al. (1972).

We thank EOLAS (The Irish Science and Technology Agency) for a Basic Research Award for P.P.

REFERENCES

- Alexander, S. S. and Pace, C. N. (1971) *Biochemistry* **10**, 2738–2743
- Allore, R. J. and Barber, B. H. (1984) *Anal. Biochem.* **137**, 523–527
- Aschaffenburg, R. and Drewry, J. (1955) *Nature (London)* **176**, 218–219
- Aschaffenburg, R. and Drewry, J. (1957) *Nature (London)* **180**, 376–378
- Bon Hoa, G. H., Guinand, S., Douzou, P. and Pantaloni, C. (1973) *Biochimie* **55**, 269–276
- Braunitzer, G., Chen, R., Schrank, B. and Stangl, A. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 867–878
- Brocklehurst, K. and Little, G. (1973) *Biochem. J.* **133**, 67–80
- Creighton, T. E. (1980) *J. Mol. Biol.* **137**, 61–80
- Cupo, J. F. and Pace, N. C. (1983) *Biochemistry* **22**, 2654–2658
- Doherty, G. M., Motherway, R., Mayhew, S. G. and Malthouse, J. P. G. (1992) *Biochemistry* **31**, 7922–7930
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Groves, M. L., Hipp, N. J. and McMeekin, T. L. (1951) *J. Am. Chem. Soc.* **73**, 2790–2793
- Hambling, S. G., McAlpine, A. S. and Sawyer, L. (1992) in *Advanced Dairy Chemistry* (Fox, P. F., ed.), vol. 1: Proteins, pp. 141–190, Elsevier Science Publishers, U.K.
- Harris, F. E. and O'Konski, C. T. (1954) *J. Am. Chem. Soc.* **76**, 4317–4318
- Kella, N. K. D. and Kinsella, J. E. (1988) *Int. J. Peptide Protein Res.* **32**, 396–405
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leader, G. R. (1951) *J. Am. Chem. Soc.* **73**, 856–857
- Maciel, G. E. and Natterstad, J. J. (1965) *J. Chem. Phys.* **42**, 2752–2759
- Maciel, G. E. and Ruben, G. C. (1963) *J. Am. Chem. Soc.* **85**, 3903–3904
- Malthouse, J. P. G. (1986) *Prog. Nucl. Magn. Reson. Spectrosc.* **18**, 1–60
- Malthouse, J. P. G., Motherway, R. and Mayhew, S. G. (1989) *Biochem. Soc. Trans.* **17**, 395–396
- Martin, G. L., Martin, G. J. and Delpuech, J. J. (1980) in *Practical NMR Spectroscopy*, pp. 171–179, Heydon and Son Ltd., London
- McKenzie, H. A. (1971) in *Milk Proteins: Chemistry and Molecular Biology* (McKenzie, H. A., ed.), vol. 2, pp. 257–330, Academic Press, New York
- McKenzie, H. A. and Ralston, G. B. (1973a) *Biochemistry* **12**, 1025–1034
- McKenzie, H. A. and Ralston, G. B. (1973b) *Aust. J. Biol. Sci.* **26**, 851–857
- McKenzie, H. A. and Ralston, G. B. (1973c) *Aust. J. Biol. Sci.* **26**, 859–870
- McKenzie, H. A. and Sawyer, W. H. (1967) *Nature (London)* **214**, 1101–1104
- McKenzie, H. A., Ralston, G. B. and Shaw, D. C. (1972) *Biochemistry* **11**, 4539–4547
- Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L. and Eliopoulos, E. E. (1987) *J. Mol. Biol.* **197**, 695–706
- Pace, N. C. and Tanford, C. (1968) *Biochemistry* **7**, 198–208
- Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E. and Kraulis, P. J. (1986) *Nature (London)* **324**, 383–385
- Phelan, P. and Malthouse, J. P. G. (1994) *Biochem. Soc. Trans.* **22**, 31
- Ralston, G. B. (1972) *C. R. Trav. Lab. Carlsberg* **38**, 499–512
- Roels, H., Preaux, G. and Lontie, R. (1968) *Arch. Int. Physiol. Biochim.* **76**, 198–200
- Roels, H., Preaux, G. and Lontie, R. (1971) *Biochimie* **53**, 1085–1093
- Roels, H., Preaux, G. and Lontie, R. (1973) *Biochimie* **55**, 421–430
- Schellman, J. A. (1958) *C. R. Trav. Lab. Carlsberg Ser. Chim.* **30**, 395–414
- Tanford, C., Bunville, L. G. and Nozaki, Y. (1959) *J. Am. Chem. Soc.* **81**, 4032–4035
- Timasheff, S. G., Mescanti, L., Basch, J. J. and Townend, R. (1966) *J. Biol. Chem.* **241**, 2496–2501
- Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wehrli, F. W., Marchand, A. P. and Wehrli, S. (1988) in *Interpretation of Carbon-13 NMR Spectra*, 2nd edn., p. 34, John Wiley and Sons, Chichester