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Location of Sulfhydryl and Disulfide Groups in Bovine β-Lactoglobulins and Effects of Urea†

H. A. McKenzie, G. B. Ralston,‡ and D. C. Shaw*

ABSTRACT: As part of a study to understand the role that the single cysteine residue and two cystine residues (per single chain, monomer) play in the unfolding and aggregation reactions of bovine β-lactoglobulins, both in the presence and absence of urea, it is important to locate these residues in the native molecule. It is shown here that alternate positions for the free sulfhydryl group occur in equal proportions in the isolated protein of each β-lactoglobulin variant examined. The cysteine residue is located at position 68 with a disulfide bridge from residues 57 to 70, *or* at position 70 with residue 57 bridged to 68, both forms having a disulfide bridge between residues 123 and 160. These positions have been determined by diagonal peptide mapping, by a method for quantitatively determining the ratio of multiple positions of a free sulfhydryl group if such exist, and by making use of the partial sequence, with corrections indicated by the present work. The significance of the findings is discussed.

It is becoming increasingly evident that one of the keys to the behavior of the ruminant β-lactoglobulins lies in the single sulfhydryl group present in each monomer chain (for reviews, see McKenzie, 1970, 1971). There are also present two disulfide bridges in each chain. The bovine proteins exhibit genetic polymorphism. Near the isoionic point (pH ~

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5.2) each of the variants exists in solution primarily as a dimer consisting of two noncovalently linked identical monomer units (for differences in the variants considered here, see Figure 7). It is now well known that polymerization of the A, B, and C variants in alkaline solution is partly due to intermolecular linkages involving –SH oxidation and –SH/–SS– interchange reactions. We have shown how they are important in the overall reactions in urea solution, even at low pH (Ralston, 1969; McKenzie and Ralston, 1971, 1973). However, it is less frequently realized that the sulfhydryl itself can play a direct role in conformational changes and in the dissociation of β-lactoglobulin to the monomer. When certain monofunctional organic reagents for –SH groups are present in stoichiometric amounts at pH 6–8, β-lactoglobulin undergoes a change in conformation and an increase in dissociation to levels that are far greater than in the absence of the reagent (see, *e.g.*, Lontie and Préaux, 1966; Lontie *et al.*, 1964; Roels *et al.*, 1968a,b; and Zimmerman *et al.*, 1969).

Thus it is important to determine the conformation of the native protein and the position of the cysteine and cystine residues in the polypeptide chain using carefully chosen conditions such that interchange is at a minimum. When our work on urea denaturation was initiated, the amino acid sequence of the β-lactoglobulins was not known, although the nature of the amino acid substitutions in the variants and the N- and C-terminal amino acid residues were known. During the course of the investigation Frank and Braunitzer (1967) published a partial amino acid sequence for the bovine A and B variants. Their results for the numbers of residues were in complete agreement with the amino acid composition considered most likely by McKenzie (1967) from published amino acid compositions. Although Frank and Braunitzer determined the positions of the half-cystine residues in the protein they did not locate the disulfide linkages or the –SH group. We have determined their position in the isolated protein and have studied the effect of urea denaturation on the position of the free sulfhydryl group and the disulfide bridging. In earlier reports (Ralston, 1969; McKenzie 1970, 1971; McKenzie and Ralston, 1971), we concluded that there were disulfide bridges from Cys-57 to -70 and -123 to -160 with the cysteine residues at 69 (new numbering 68). As the work progressed it became evident that the simple assumption that the free sulfhydryl group occurs in the same position in all molecules of the native protein was incorrect and hence a rationale for the quantitative determination of the ratio if more than one position exists was developed and applied (McKenzie and Shaw, 1972). Details of the work are presented here. Since completing it, three short communications by Préaux and coworkers have been published (Mainferme *et al.*, 1971; Martial *et al.*, 1971; Pérez Gómez *et al.*, 1971). Their conclusions will be considered in the Discussion.

Experimental Procedures

Materials. Bovine β-lactoglobulins A and B were prepared by method Ia of Armstrong *et al.* (1967), β-lactoglobulin AB from the milk of a single heterozygous cow by the chromatographic method of Armstrong *et al.* (1970) and the A and Droughtmaster variants separated from the heterozygote by the method of Bell *et al.* (1970). Caprid β-lactoglobulin was prepared by method IA as for bovine samples, but crystallized from ammonium sulfate by the method of Askonas (1954).

Pepsin was obtained from Worthington Biochemical Corp., Freehold, N. J., subtilisin "Novo" from Novo, Copenhagen, Denmark, and [^{14}C]iodoacetamide from Radiochemical Centre, Amersham, Great Britain. Iodoacetamide, from Sigma Chemical Co., St. Louis, Mo., was recrystallized from ethyl acetate–petroleum ether (bp 30–60°).

Amino acid analyses were performed as described by Spackman *et al.* (1958) and Bell *et al.* (1968). The citrate–HCl buffers used in the analyses were prepared to the formulation of Spackman (1962). Thiodiglycol (~0.05 ml) was added to the 6 M HCl (2 ml) used for hydrolysis of peptides containing CMCys which had been eluted off paper, and good recovery of the derivative was obtained. The addition of the reducing reagent was not necessary in the case of carboxymethylated proteins, the evacuation procedure of Crestfield *et al.* (1963) being used routinely; in fact if the reducing agent was present in protein hydrolyses extra peaks due to mixed disulfides were observed.

The specific activity of [^{14}C]CMCys was obtained by using a Nuclear-Chicago scintillation counter flow cell in conjunction with the amino acid analyzer.

Calculation of Percentage ^{14}C Carboxymethylation and Recovery of Radioactivity. In the calculation of the percentage carboxymethylation with ^{14}C-labeled reagent for the determination of the recovery of radioactivity in a particular residue, the most consistent results were obtained by integrating the peak for CMCys from analyses of the fully carboxymethylated protein, totalling the counts over the peak and using the specific activity of [^{14}C]CMCys derived from cysteine and a sample of the [^{14}C]iodoacetamide which had been used in the reaction on that protein. The use of the reduced and fully carboxymethylated protein which was defined as containing 5.0 residues of CMCys provides a direct correction for any loss of CMCys during hydrolysis in the particular analysis and any inaccuracy in the color constant for the derivative.

Diagonal Peptide Mapping. The method of Brown and Hartley (1963) was used. Bovine β-lactoglobulins A, B, and C (40 mg) were digested separately at 37° for 4 hr with pepsin (0.4 mg) in 5 ml of 0.01 M HCl, after adjustment of the pH value to 2.0 with 0.1 M HCl. Each lyophilized sample of peptic digest was dissolved in water (0.2 ml). Aliquots of these solutions (containing *ca.* 2 mg of original protein) were subjected to high-voltage electrophoresis (40 V/cm, 1 hr) as a 2-cm band on Whatman No. 3MM paper. After drying, the paper strips were exposed to performic acid vapor for 2 hr. The strips were then dried *in vacuo* over KOH pellets, stitched on to a full sheet of No. 3MM paper, and subjected to electrophoresis at right angles to the original direction under the same conditions as before. Two diagonal maps were prepared for each digest: one at pH 1.9 and one at pH 4.7. The pH 1.9 buffer was the acetic acid–formic acid system of Atfield and Morris (1960). The pH 4.7 buffer was the acetic acid–pyridine system of Schwartz (1963).

Peptides were stained with ninhydrin (0.1 g/dl in acetone) and were found to lie on a diagonal passing through the origin, except for those peptides containing cystine or cysteine. These peptides moved from the diagonal toward the positive electrode due to the extra charge produced by the oxidation to cysteic acid.

The maps were washed free of excess ninhydrin with acetone, and the peptides that did not lie on the diagonal were cut from the sheet, eluted from the paper with 2–3 ml of 6 M HCl and hydrolyzed for 22 hr at 110°.

[^{14}C]Iodoacetamide Treatment. The free sulfhydryl group of β-lactoglobulin variants was reacted with [^{14}C]iodoacetamide. A solution of the protein (100–200 mg, in 0.1 M Tris-HCl–0.027 M Na_2EDTA, pH 7.5 buffer (1.9 ml)) was mixed

with [^{14}C]iodoacetamide (4-fold molar excess for one -SH group per monomer, in 0.1 ml; 52.3 Ci/mole diluted about ten times with nonradioactive reagent) followed by 10 M urea (8 ml in 0.1 M Tris-HCl–0.027 M Na_2EDTA), to produce a final solution 8 M in urea and with an apparent pH of 7.8–7.9. After the required reaction time in urea at 25°, the mixture was diluted with an equal volume of 0.5 M sodium acetate buffer (pH 5.0) and dialyzed against 0.01 M acetic acid and then water (3 × 2 l.) for a total of at least 14 hr. The material was lyophilized and used directly for the initial work in which radioactive peptides were isolated from a subtilisin digest, while for the subsequent sequence determination, and the ratio of labeling, if more than one site for the sulfhydryl exists, the dilsulfide bridges were reduced and the cysteines produced reacted with nonradioactive iodoacetamide.

In order to obtain a measure of the extent of -SH/-SS- interchange which occurs in 8 M urea at pH 7.9, in the absence of iodoacetamide, additional samples of bovine β-lactoglobulins A and B were dissolved and mixed with urea solution under the same conditions as above, except that the [^{14}C]iodoacetamide was not added until the protein had been exposed to 8 M urea for 1.5 hr (1 hr for B); the final mixture was diluted with 0.5 M sodium acetate buffer (pH 5.0), 10 min after the addition of the ^{14}C reagent. Subsequent treatments were as for the above samples.

Composition of Subtilisin Peptides. The ^{14}C-labeled carboxymethylated protein was digested with subtilisin (1:50, w/w) in ammonium bicarbonate solution (0.5 g/dl) for 4 hr at 37° after which 2-mercaptoethanol (0.1/4 ml) was added for 30 min before the mixture was lyophilized. During the enzymic digestion at pH 8 there would be ready interchange to give all -SS- forms and so the mercaptoethanol was added to reduce these to cysteine residues and so simplify the mixture of peptides. Two samples (2–3 mg) were subjected to electrophoresis at pH 4.7 (40 V/cm, 1 hr). The strip containing the peptides from one sample was exposed to performic acid vapor for 2 hr, dried *in vacuo* over KOH pellets, and then subjected to electrophoresis at pH 1.9 (40 V/cm, 1 hr) at right angles to the first electrophoresis. The other sample was not oxidized but subjected directly to the electrophoresis at pH 1.9. The ^{14}C-labeled peptides in each were detected by autoradiography and further separated by ascending chromatography in pyridine–isoamyl alchohol–water (7:7:6, v/v; Bell *et al.*, 1968) and again detected by autoradiography. The major radioactive spots were eluted with 6 M HCl and hydrolyzed in evacuated tubes at 110° for 22 hr, prior to amino acid analysis.

Reduction and Carboxymethylation (Nonradioactive). The procedure used for reduction and carboxymethylation of the ^{14}C-labeled β-lactoglobulins was similar to that described by Crestfield *et al.* (1963). The sample (100 mg) was dissolved in buffer (1.9 ml, 0.2 M Tris-HCl–0.027 M Na_2EDTA, pH 8.5); 10 M urea (8 ml in 0.2 M Tris-HCl–0.027 M Na_2-EDTA, pH 8.5) through which nitrogen had been bubbled was added followed by 0.1 ml of 2-mercaptoethanol. The mixture was left in a stoppered tube under nitrogen for 4 hr at 25° after which 270 mg of iodoacetamide in 2 ml of water was added. After a further 20 min the mixture was diluted with sodium acetate (5 ml, 0.5 M, pH 5.0) and dialyzed against 0.001 M acetic acid and water, then lyophilized.

Pepsin Peptide. The reduced and fully carboxymethylated product was digested with pepsin (1:50, w/w) for 4 hr at 37°. The mixture was freeze-dried and subjected to electrophoresis at pH 4.7 (40 V/cm, 1 hr) and the radioactive band located, cut out, sewn onto a fresh sheet of Whatman No. 3MM paper, and electrophoresed at pH 1.9 (40 V/cm, 1 hr). Further purification of the radioactive material was obtained by ascending chromatography in pyridine–isoamyl alcohol–water (7:7:6, v/v). After locating the band by autoradiography the peptide was eluted with pyridine–water (1:3, v/v) or ammonium bicarbonate (0.5 g/dl). The composition and purity of a sample of the peptide was determined by hydrolysis and amino acid analysis. This peptide prepared from a sample of β-lactoglobulin B was used for sequence determination while in all the variants studied the peptide was digested with subtilisin (80 μg) in ammonium bicarbonate solution (2 ml) for 4 hr at 37°. After lyophilization the mixture was subjected to two-dimensional electrophoresis at pH 8.9 and 1.9, both 40 V/cm, 1 hr. The radioactive fragments were detected by autoradiography, eluted with 6 M HCl, then hydrolyzed in the presence of thiodiglycol, and analyzed for amino acids and specific activity of the CMCys.

Sequence Determination. The dansyl-Edman technique as described by Gray (1967) was used for determining the amino acid sequence of the pepsin peptide from β-lactoglogulin B. Thiodiglycol was added to the HCl used for hydrolysis to protect the dansyl-CMCys derivative. The dansyl derivatives of the possible amino acids were readily separated and identified by comparison to known markers on thin-layer chromatograms (silica gel, Baker-flex, J. T. Baker Chemical Co., Phillipsburg, N. J.) using two solvent systems: benzene–pyridine–acetic acid (80:20:2, v/v) and chloroform–*tert*-amyl alcohol–acetic acid (70:30:3 v/v) (Morse and Horecker, 1966). Autoradiography of the thin-layer sheets was used to determine whether the dansyl-CMCys derivative from each position in the peptide was radioactive.

Effect of Denaturation. In addition to the reaction with [^{14}C]iodoacetamide after exposure to 8 M urea for 1.5 hr, described above, the following experiment was performed. A sample of β-lactoglobulin B was denatured in 7 M urea at pH 7.5 and 25° for 24 hr. Denatured protein was precipitated by dilution with 2 M ammonium sulfate at pH 5.2. The precipitated protein was dialyzed against 0.01 M HCl and then hydrolyzed with pepsin for 4 hr at 37°. A diagonal peptide map at pH 4.7 was prepared and stained with ninhydrin. After photography, excess of ninhydrin was removed by washing with acetone. It was then examined as described under Results.

Results

Location of Disulfide Bridges. In considering the results below it is important to recall the positions in the polypeptide chain of the half-cystine (Cys) residues in bovine β-lactoglobulin A and B as determined by Frank and Braunitzer (1967). There are 162 amino acids in the chain and on their sequence the 5 Cys residues were numbered 57, 69, 70, 123, and 160. The partial sequence modified as the result of the present work is presented later as Figure 7. The principal modification relevant to the disulfide bridging is that a residue of glutamine was found to separate the two residues of Cys reported to be adjacent at 69 and 70. The composition of the larger peptide from residues 60 to 70 is the same with regard to glutamic acid as reported and therefore the numbering was altered to make the residues of Cys to be at 68 and 70, with Gln at 69.

Peptic digests of each of the variants, A, B, and C, gave similar patterns of Cya[1]-containing peptides in diagonal

[1] Abbreviation used is: Cya, cysteic acid.

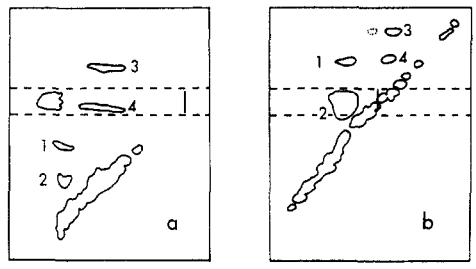

FIGURE 1: Diagonal peptide maps (two-dimensional electrophoresis) of a peptic digest of bovine β-lactoglobulin B. The strips were oxidized with performic acid vapor after the first electrophoresis: (a) pH 1.9, pH 1.9 and (b) pH 4.7, pH 4.7.

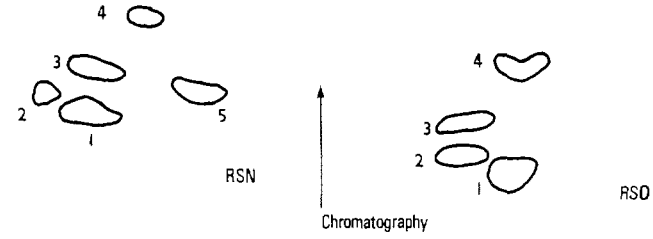

FIGURE 3: Autoradiographs of an ascending chromatographic separation of peptides cut from the electrophoretic map shown in Figure 2. Chromatography in pyridine–isoamyl alcohol–water (7:7:6, v/v).

maps at pH 1.9 and 4.7. Maps for the B variant are shown in Figure 1.

In each map peptide 1 was the oxidized C-terminal fragment Glx-Glx-Cya-His-Ile. Peptide 2 in each case contained relatively high amounts of lysine and corresponded to the sequence around Cys-123. Better resolution was obtained at pH 1.9 than at pH 4.7. The material on the central strip at pH 4.7 contained large amounts of contaminants with only a trace of Cya. However amino acid analysis of the area below the sewn strip agreed with that of peptide 2 from the pH 1.9 map, which, for the B variant, was Lys_3,Cya,Asx,Glx_5,Gly,-Ala_2,Ile_2,Leu. The analyses correspond to the sequence of residues 114–130, shown in Figure 7.

Although peptides 3 and 4 of Figure 1 appear to lie on a vertical line, they did not represent a pair of disulfide bridged peptides. Amino acid analyses showed them to be due to variable cleavage by pepsin about the region 54–72; smaller peptides were not observed and so it was not possible to determine which residues were bridged within that part of the sequence.

Location of ^{14}C-Labeled Carboxymethylated Cysteine—Assuming a Single Position. Since the disulfide bonding in the region of the protein chain from residue 57 to 70 was not resolved by simple diagonal peptide mapping, attempts were made to solve the problem using peptide maps of digests of [^{14}C]iodoacetamide treated β-lactoglobulin B. In this work it was assumed that the one cysteine residue per monomer was located at a single position. Initially pepsin was used for hydrolysis and although this was followed by digestion with subtilisin the peptides were larger and did not purify easily. However a very plausible argument was made (Ralston, 1969) that Cys-69 (in Frank and Braunitzer's sequence) was the free cysteine residue and that there was a disulfide bridge between Cys-57 and Cys-70.

The difficulties inherent in the investigation are the need to obtain an enzymic cleavage between the two residues of Cys close to one another, the variable recovery of CMCys and Cys on acid hydrolysis after elution from peptide maps (including apparent oxidation to Cya or complete loss) and the separation and purification of a family of peptides produced by a nonspecific enzyme. A fresh attempt was made using a longer time of digestion with subtilisin, followed by reduction with mercaptoethanol of the various –SS– bridged peptides to their cysteine forms. Peptide maps, one of which had included an oxidation with performic acid before the second electrophoresis, were compared and the major radioactive peptides were further separated and purified by chromatography. Bovine β-lactoglobulin B was used so that the alanine substitution would help define the sequence from the composition of peptides when the order of residues 71 and 72 (Val and Leu) was not known. Comparison of the peptide maps shown in Figure 2 reveals that only peptide 5 was strongly affected by the performic acid oxidation, such that it must include a residue of Cys (in addition to the labeled CMCys) which has been oxidized to cysteic acid and so be more acidic after oxidation. The other peptides 1–4 can be taken to be the result of cleavage between the CMCys and any other residue of Cys, and so their composition could indicate which residue of Cys had been labeled and hence had occurred as cysteine in the protein. Peptide 2 eluted from the chromatogram shown in Figure 3 (RSN) had one residue of alanine with an apparent contamination of glutamic acid. The other peptides were either not pure or were larger fragments but none contained leucine without alanine present. Peptide 5 from the oxidized map RSO gave the composition Cya,CMCys,Glu. Examination of the partial sequence of Frank and Braunitzer would suggest that the residue of alanine defines the free cysteine which was labeled with [^{14}C]-iodoacetamide as Cys-69; however the glutamic acid seen in peptides 2 and 5 was not consistent with the published sequence.

Sequence Determination. To clarify this discrepancy it was necessary to check the sequence in the vicinity of residues 69–70. A sample of fully carboxymethylated β-lactoglobulin B was prepared for the sequence determination by first reacting the one residue of cysteine with [^{14}C]iodoacetamide and then reducing the disulfide bridges and carboxymethylating the four cysteines produced with nonradioactive iodoacetamide. A pepsin digest of the product was found to contain a single major radioactive peptide and this was purified by electro-

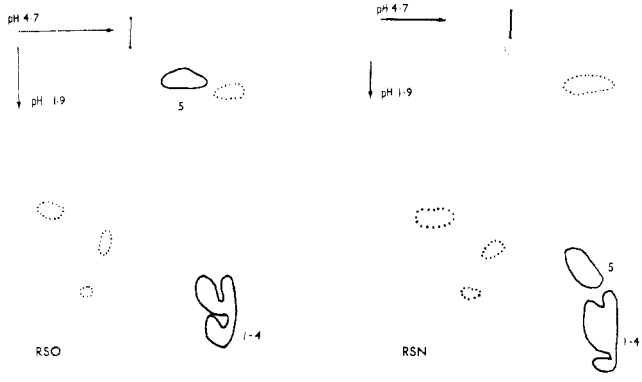

FIGURE 2: Autoradiographs of the two-dimensional electrophoretic separations of a subtilisin digest of ^{14}C-labeled carboxymethylated bovine β-lactoglobulin B. First electorphoresis at pH 4.7, second at pH 1.9. In RSO the strip was oxidized with performic acid vapor after the first electrophoresis; in RSN the oxidation was omitted.

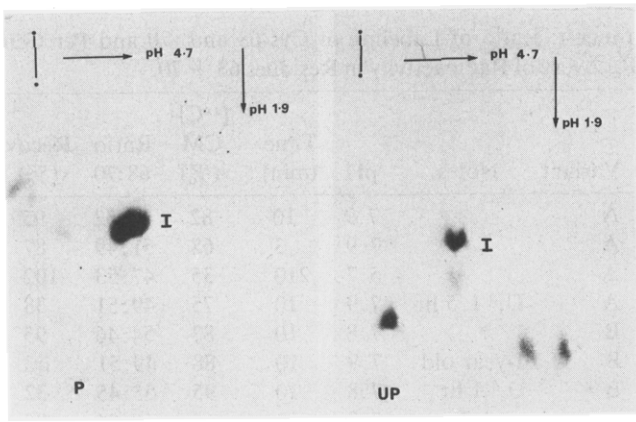

FIGURE 4: Autoradiographs of a two-dimensional electrophoretic separation of peptic digests of reduced and fully carboxymethylated bovine β-lactoglobulin A. First electrophoresis at pH 4.7, second at pH 1.9. P indicates [^{14}C]iodoacetamide added to the β-lactoglobulin *prior* to standing in 8 M urea at pH 7.9 for 10 min. UP indicates [^{14}C]iodoacetamide added *after* the protein had been in 8 M urea for 1.5 hr. Peptide I is Val-CMCys-Gln-CMCys-Leu.

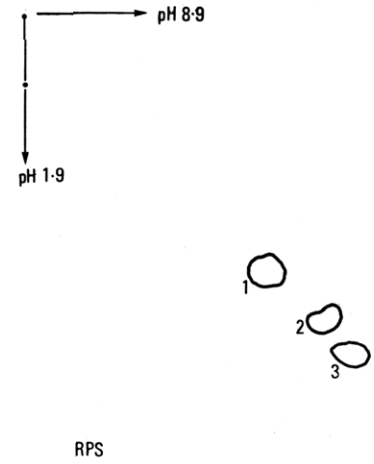

FIGURE 5: Autoradiograph of a two-dimensional electrophoretic separation of a subtilisin digest of peptide I in Figure 4. First electrophoresis at pH 8.9, second at pH 1.9.

phoresis and chromatography. Amino acid analysis showed that it was a pentapeptide with the composition $CMCys_2$,Glu,-Ala,Leu. Application of the dansyl-Edman technique clearly showed the sequence to Ala-CMCys-Gln-CMCys-Leu. The amide on glutamine was assigned on the basis of mobilities of peptides and the recovery of glutamine (identified on analyzer) from an Edman degradation of the dipeptide CMCys-Gln derived from a subtilisin digest of the carboxymethylated protein. Autoradiography of the thin-layer sheets used to identify the dansyl derivatives showed that the CMCys in both positions in the chain was apparently radioactive. That is, a proportion of each Cys residue had had a free sulfhydryl group available for reaction with the [^{14}C]iodoacetamide, although a total of only one sulfhydryl group per monomer occurs.

The β-lactoglobulin B used for the sequence determination had been stored as moist crystals at 4° for about 1 year and so it was thought that -SH/-SS- interchange may have occurred in that time. Therefore fresh crystallized samples of the A and B variants were prepared from freshly collected milk of typed cows.

Ratio of Labeling If Multiple Sites for the Sulfhydryl Group. The following procedure for the determination of the ratio of labeling if the free sulfhydryl group occurs at each residue of Cys was used. The fresh sample of β-lactoglobulin variant was dissolved in buffer, [^{14}C]iodoacetamide was added followed by urea in buffer to give a final solution 8 M in urea with an apparent pH of 7.8–7.9. Preliminary experiments had indicated that under these conditions about 90% of the single sulfhydryl group per monomer was carboxymethylated in 10 min and so routinely the reaction in urea was stopped by dilution with pH 5 buffer at 10 min. After dialysis and lyophilization the radioactive product was reduced and carboxymethylated with nonradioactive iodoacetamide in urea to yield material in which all five residues of Cys were converted to CMCys, to be chemically homogeneous but heterogeneous with regard to ^{14}C isotope, only those residues of Cys which had originally had a free sulfhydryl group being labeled. An autoradiograph of the two-dimensional electrophoretic separation of a pepsin digest of this fully carboxymethylated protein is shown in Figure 4 (P). For all variants there was a single major radioactive peptide, the composition of which, after further chromatographic purification, showed that it was the pentapeptide containing the two CMCys residues, the A ↔ B:Val ↔ Ala substitution being evident. By comparison, Figure 4 (UP) shows the autoradiograph of the A variant which had been allowed to stand in 8 M urea at 25° for 1.5 hr prior to the addition of [^{14}C]iodoacetamide for 10 min, the subsequent handling being identical. Several more radiopeptides are clearly evident. The pentapeptide I was less intense while the peptide below and to the left on the map analyzed for the C-terminal peptide (Glx-Glx-CMCys-His-Ile; specific activity of CMCys indicated 28% ^{14}C carboxymethylation). Extensive interchange had obviously taken place during the period in urea in the absence of iodoacetamide. This sample served to show that under the standard conditions when iodoacetamide was present while the protein was in urea and the exposure limited to 10 min, such interchange was negligible. The equivalent radioactive peptides were barely detectable on the autoradiographs of the standard treatment (Figure 4 (P)). Amino acid analysis of these showed that while the peptides were present in good yield the specific activity of the CMCys indicated that less than 3% of the total radioactivity was involved.

The purified pepsin peptide I was digested with subtilisin, which was found to cleave after the glutamine residue and the fragments Val-CMCys-Gln (peptide 2) and CMCys-Leu (peptide 3) were separated from one another and from undigested material (peptide 1) by two-dimensional electrophoresis at pH 8.9, pH 1.9 (Figure 5).

The similar mobility of the peptides 2 and 3 at pH 8.9 is a confirmation that the glutamic acid seen on hydrolysis occurs as the amide glutamine in the peptides and protein; the three peptides are separated at pH 1.9 (toward cathode) and 8.9 (toward anode) solely in order of their size. The radioactive peptides were eluted and the specific activity of the CMCys in each was determined. Table I lists the ratio of labeling in the two positions (Cys-68 and -70), together with analytical data to the extent of carboxymethylation with ^{14}C-labeled reagent and the percentage of radioactivity accounted for in the two residues. It was found that in the A variant treated for 10 min at pH 7.9, 82% labeling was achieved with the [^{14}C]iodoacetamide and 95% of the radioactivity was accounted for in the two residues 68 and 70 in a ratio of 48:52.

The initial determinations were made on A and B variants isolated from homozygous animals, using a 10-min treatment at pH 7.8–7.9. In order to try to answer the several questions

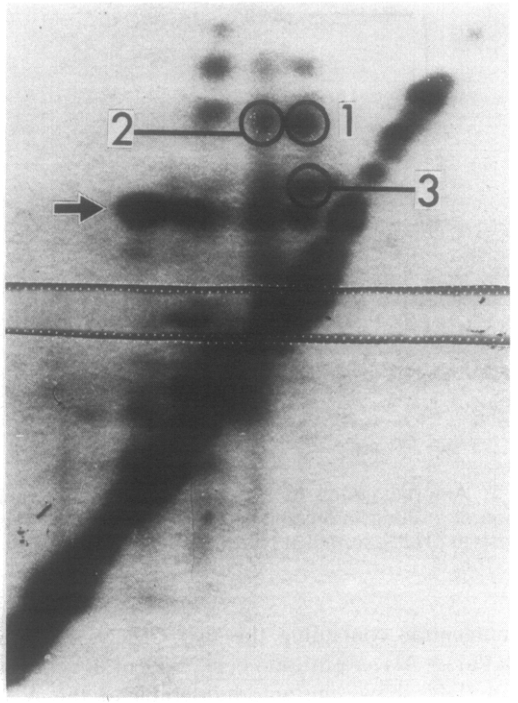

FIGURE 6: Diagonal peptide map of a pepsin digest of bovine β-lactoglobulin B after denaturation in 7 M urea at pH 7.5 for 24 hr. Electrophoresis conducted at pH 4.7, pH 4.7 with performic oxidation after the first electrophoresis. The arrow indicates peptides staining red with Pauly's reagent. The circled peptides were eluted and analyzed.

TABLE I: Ratio of Labeling of Cys-68 and -70 and Per Cent Recovery of Radioactivity in Residues 68 + 70.

Variant	Notes	pH	Time (min)	[^{14}C]-CM (%)	Ratio 68:70	Recov (%)
A		7.9	10	82	48:52	95
A		7.9	3	68	51:49	87
A		5.7	210	35	47:53	102
A	U,[a] 1.5 hr	7.9	10	75	49:51	38
B		7.8	10	83	54:46	95
B	1-year old	7.9	10	88	49:51	nd
B	U,[a] 1 hr	7.8	10	95	55:45	32
A	ex A Dr	7.8	10	66	46:54	95
Dr[b]	ex A Dr	7.8	10	66	47:53	87
A	ex AB				48:52	
		7.9	10	69		89
B	ex AB				47:53	
C		7.9	20	76	51:49	80
Caprid		7.9	10	61	50:50	92

[a] Pretreatment with urea for the period shown, in the absence of iodoacetamide. [b] Dr indicates β-lactoglobulin$_{\text{Droughtmaster}}$.

raised by the unexpected result, the study was extended to include the other samples and conditions shown in the table. The lowest pH treatment considered practicable was at pH 5.7 for 210 min because at pH 5.0 only about 20% carboxymethylation was achieved in 14 hr in the 8 M urea. In all the variants and treatments performed similar results, within the accuracy of the determinations, were obtained, with the exception that only 80% of the total radioactivity was accounted for in residues 68 and 70 in the case of bovine β-lactoglobulin C, although the ratio of labeling of 68:70 was maintained at 50:50 (within ±5%). The sample of the C variant had been stored in lyophilized form at 4° for over 1 year. There were not any obvious extra peptides evident on the two-dimensional electrophoretogram of the pepsin digest of this treated sample of C.

Effect of Urea Denaturation. It has already been noted that the bottom left-hand peptide in Figure 4 (UP) was the C-terminal fragment of β-lactoglobulin A. Analysis of two of the other spots evident in Figure 4 (UP) showed them to be the result of variable cleavage by pepsin and to both contain Cys-123. None of the spots analyzed was recognizably due to cleavage about Cys-57. In Figure 4 (P) there were some faint spots below the origin and about level with peptide I; the intensity of these did not increase in the denatured sample (Figure 4 (UP)) and analysis showed them to be due to larger peptic peptides which included the residues 68 and 70.

A diagonal peptide map of bovine β-lactoglobulin B, that had been subjected to the action of 7 M urea at 25° for 24 hr, was prepared and is shown in Figure 6.

After removal of excess ninhydrin, the peptides labeled 1–3 were cut from the map and subjected to amino acid analysis. The rest of the map was sprayed with Pauly's diazo reagent (Stepka, 1957). The peptide lying on a horizontal line, indicated by an arrow in Figure 6, stained red (indicative of histidine containing peptides) which would identify it as the C-terminal fragment containing Cys-160. The fact that a line rather than a single spot was obtained by the diagonal mapping would suggest that Cys-160 was bridged to a variety of other peptides (not just Cys-123).

Peptides 1 and 2 had the same composition and corresponded to all of the sequence between Cys-57 and Cys-70. Peptide 3 contained only one Cya and appeared to be a small fragment of the chain between Cys-57 and -70. The analyses and histidine staining served to show that the peptides off the diagonal did represent Cya-containing fragments. Thus it is evident that the number and pattern of the "off-diagonal" peptides are markedly altered following the action of urea.

Discussion

The peptides containing Cys-123 and Cys-160 from bovine A, B, and C variants were found to lie on a vertical line in diagonal peptide maps and therefore are considered to be disulfide bridged. Although the region of the protein chain containing Cys-57, Cys-68 (formerly designated 69), and Cys-70 did not result in observable pairs of disulfide bridged peptic peptides on diagonal maps, it was possible to isolate radioactive peptides from [^{14}C]iodoacetamide-treated β-lactoglobulin B which, with the modifications we have made for the sequence of residues in that region, suggested that Cys-68 is the residue which occurs as cysteine in the protein and hence that Cys-57 is bridged to Cys-70. However it became evident that both Cys-68 and -70 in all samples studied were labeled by the ^{14}C-labeled reagent and that the ratio of labeling was 1:1, with 90 ± 5% of the radioactivity accounted for on those two residues in the A and B variants, but with 80% accounted for in the C variant. The results are summarized in Figure 7, on the partial sequence reported by Frank and Braunitzer (1967, 1968) and modified as the result of the present work. In earlier stages of the investigation it was

reported that the sulfhydryl group occurred at only position 69 (now 68) (Ralston, 1969; McKenzie and Ralston, 1971; McKenzie, 1970, 1971). In reaching this conclusion it was assumed that the sulfhydryl would be at one position only and the interpretation depended on the composition of isolated peptide fragments. Recently a Belgian group (Mainferme *et al.* 1971; Martial *et al.*, 1971; Pérez Gómez *et al.*, 1971) have concluded that the sulfhydryl is located at position 70 with disulfide bridges from 57 to 69 and 123 to 160. This conclusion would have been made on the assumption of a single position and was based on the composition of peptides isolated after cyanogen bromide cleavage and digestion with pepsin and thermolysin. They noted finding an excess of 0.8 residue of glutamic acid in a peptide containing Cys-70 but did not investigate the sequence. The shortest time which they used for reaction of the sulfhydryl group with alkylating reagents was 6 hr at pH 8.3. The difference between their results and ours could arise from the different methods of cleavage and separation resulting in preferential isolation of different sets of peptides. Assuming a single position the composition of one purified labeled fragment would be considered to be sufficient to define the system. The long time, for reaction of the sulfhydryl group, used by Préaux and coworkers could result in a specific -SH/-SS- interchange, while there could be preferential labeling of a more exposed or reactive form of the sulfhydryl group in the absence of considerable unfolding such as occurs in urea.

The present result showing the sulfhydryl group in alternate positions at Cys-68 and -70 in equal proportion has been obtained from both fresh and stored samples of the purified protein. The methods we have used for the isolation of the β-lactoglobulins were originally developed to minimize dissociation, conformational change and -SH/-SS- interchange during the fractionation. Thus, unless there is a very facile and complete interchange between Cys-68 and Cys-70, the present result should be valid for the protein as it occurs in milk and in the mammary gland.

One possible explanation considered for this result, when first obtained with the B variant from a homozygous animal, was that there were two genetically determined forms of the polypeptide chain (B and B′) and that the folding and different disulfide bridging reflected this difference. If that were the case then if the relevant peptides were separated after treatment of β-lactoglobulin AB from a single heterozygote either the B or B′ form would be expected to be found, along with either an A or A′ form. Likewise our previous observation that carbohydrate was added to the A variant polypeptide chain in the $A_{Droughtmaster}$ and $B_{Droughtmaster}$ heterozygotes (Bell *et al.*, 1970) could have been due to the existence of an A′ form of protein which would have the same amino acid composition but different sequence, disulfide bridging and position of the cysteine compared to the A form. These differences could be recognized within the mammary gland, such that carbohydrate would be added to only the A′ form and therefore if A and Droughtmaster variants were separated and treated, the peptides would be expected to show the cysteine in different positions. In the AB and A and Droughtmaster peptides, the cysteine was found still to occur in alternate positions, all with the same 50:50 (within ±5%) ratio, and so the results cannot be explained on the basis of a genetic difference.

Alkylation of the sulfhydryl group in β-lactoglobulin with iodoacetamide is slow at pH 7.9 in the absence of urea, but rapid once urea is added. The use of iodoacetamide in urea results in the total time of exposure to pH 7.9 being significantly reduced so that -SH/-SS- interchange should also be reduced and the unfolding in urea should prevent preferential labeling of a more exposed or reactive form of the sulfhydryl group (*cf.* "competitive labeling"; Kaplan *et al.*, 1971). The autoradiographs and specific activity measurements of the two-dimensional separation of the pepsin digests of the fully carboxymethylated β-lactoglobulins showed that very little interchange to Cys-123 or -160 had occurred during the treatment.

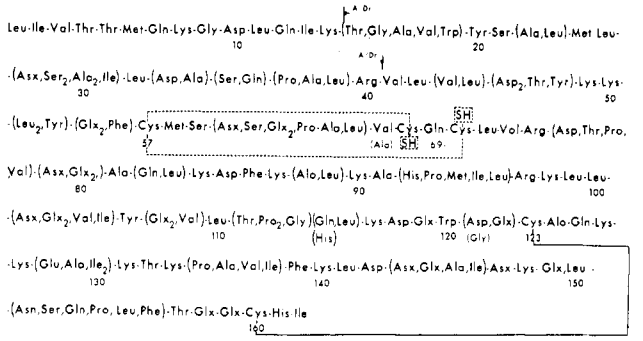

FIGURE 7: Partial amino acid sequence of bovine β-lactoglobulin A. The following substitutions for other bovine variants are shown: A ↔ B, Val ↔ Ala at residue 67 and Asp ↔ Gly at 121 or 122; B ↔ C, Gln ↔ His at 115 or 116. The Droughtmaster variant has the same amino acid composition as A, but has a carbohydrate moiety attached to the tryptic difference peptide, from residues 15 to 40. The sequence is based on that of Frank and Braunitzer (1967) for the A and B variants with the following modifications: residues 60–72, (Asx,Ser,Glx_2,Pro-Ala,Leu),Val-Cys-Gln-Cys-Leu-Val replaces (Asx,Ser,Glx_3,Pro-Ala,Leu)-Val-Cys-Cys-(Val,Leu); residues 110–117, Leu-(Thr,Pro_2,Gly),(Gln,Leu)-Lys replaces Leu-(Thr,-Glx,Pro_2,Gly,Leu)-Lys; residues 120–122, Trp-(Asp,Glx) replaces (Asp,Glx,Trp). The location of the -SH group in alternate positions at residue 68 or 70 with an -SS- bridge from 57 to 70 or 57 to 68, respectively, and the location of an -SS- bridge from 123 to 160 are those determined in the present work and are shown with broken and full lines respectively.

It is possible that the result is due to a rapid specific interchange between Cys-68 and -70; however, this would need to involve position 57, as that Cys is bridged to one or other position, and so Cys-57 should also become available for labeling, unless there were steric restrictions to the formation of intra- and/or intermolecular bridges between Cys-68 and -70. The constant ratio of labeling of Cys-68 and -70 and the high recovery of radioactivity on these positions in the 3-min treatment in urea at pH 7.9 and in the 210-min treatment at pH 5.7 would argue against any interchange in the presence of iodoacetamide being significant during the labeling period. It therefore appears that the alternate positions occur in the isolated protein and future experiments will be aimed at trying to separate the two forms and to try to answer the thermodynamic and kinetic questions raised. The folding of the polypeptide chain following synthesis appears to yield equal proportions of forms differing in their disulfide bridging and hence in their conformation. It is normally assumed that a native protein will be homogeneous with regard to its disulfide bridging even when, as in insulin, residues of Cys are adjacent in the primary sequence and the error in alternate bridging might be considered to be small.

β-Lactoglobulin A after standing in 8 M urea in the *absence* of iodoacetamide at pH 7.9 for 1.5 hr had obviously undergone extensive -SH/-SS- interchange such that residues 68 and 70 accounted for only 38% of the sulfhydryl present (*cf.* 92% when iodoacetamide was present). The Cys at posi-

tion 160 in the C-terminal peptide was labeled to about 28% after 1.5-hr exposure to urea, whereas in the native protein Cys-160 is fully bridged to Cys-123 (less than 3% labeling of Cys-160 observed). There are five residues of Cys in the protein. If the sulfhydryl were completely randomized, 20% of each position would be labeled. Therefore it appears that in the sample exposed to urea for 1.5 hr, interchange had resulted in nearly a random mixture.

We shall now discuss some more general aspects of the chemistry of β-lactoglobulins arising out of the present conclusions on the location of the –SH and –SS– groups. In the pH range 2–6.8 the bovine, ovine, and caprid β-lactoglobulins react only very slowly with –SH reagents. As the pH is increased above pH 6.8 there is a large increase in reactivity, *e.g.*, Dunnill and Green (1965) found the second-order rate constant (k' in l. mole^{-1} sec^{-1}) for the reaction of bovine β-lactoglobulin A (*ca.* 3×10^{-5} M) with sodium *p*-hydroxymercuribenzoate (*ca.* 0.6×10^{-5} M) to vary as follows: pH 6.8, $k' = 6$; pH 7.1, $k' = 91$; pH 7.8, $k' = 1030$; pH 8.5, $k' > 2.7 \times 10^4$. Pantaloni (1962) observed a marked increase in levorotation when the –SH group was blocked compared to the rotation observed in the umodified protein. Lontie, Préaux and their collaborators (see, *e.g.*, Lontie and Préaux, 1966, and Préaux and Lontie, 1966) confirmed this and showed that blocking had a profound effect on the conformational transition that bovine β-lactoglobulins undergo near pH 7. Roels *et al.* (1968a) compared the molecular size at pH 5.2, 5.6, and 8.1 of bovine β-lactoglobulins A and B with and without blocking the –SH group with *p*-hydroxymercuribenzoate, chlormerodin, and *N*-ethylmaleimide. They found the modified and unmodified protein occurred primarily as the dimer unit at pH 5.2 and 5.6. However at pH 8.1 there was enhanced dissociation of the derivatives compared with the unmodified protein, the *N*-ethylmaleimide derivative being almost completely dissociated. In an independent investigation Zimmerman *et al.* (1969) found that blocking the –SH group of the bovine A variant with 4-(*p*-dimethylaminobenzeneazo)phenylmercuric acetate, at pH above 6.9, resulted in complete dissociation to the monomer.

Considering the location of the sulfhydryl group in the peptide chain at position 68 or 70 in relation to the effect blocking it with organic mercurials and *N*-ethylmaleimide has on the behavior of the protein, we are able to make the following inferences. The conformation of the protein is such that, when the sulfhydryl group is blocked at pH 7 and above, it is sterically difficult for the monomer units to form a dimer. This effect may result from positions 68 and 70 being reasonably close to the site of contact between the monomer units or it could arise solely from a conformational change. However they are not so close that dimerization is prevented at pH values (<6) where there is no appreciable conformational change in the presence of blocking agent. The conclusion of Dunnill and Green (1965) that the large increase in k' over the small pH change near pH 7 involves both dissociation and conformational change is in agreement with our conclusions. Likewise the studies of Phillips *et al.* (1967) on comparative reactivity of the sulfhydryl groups of the bovine A, B, and C variants are in accord with our findings. On the other hand, Townend *et al.* (1969), on the basis of the molecular size of carboxymethyl, 2-hydroxyethyl, and 2-aminoethyl derivatives of bovine β-lactoglobulins A and B, concluded that the –SH group is "not located at the sites of subunit contact" and is possibly "relatively near the sites of intermolecular contact when the dimer associates to form the cyclic octamer" (*i.e.*, residues 117–126).

It is of interest to note that Kessler and Brew (1970) have isolated a porcine "β-lactoglobulin" and Bell *et al.* (1970) have isolated two variants (A and B) of this protein. It has a fairly similar amino acid composition to the ruminant β-lactoglobulins, but has *no* sulfhydryl group and two cystine residues. Under all pH conditions it exists as monomer and does not undergo the conformation transition near pH 7, and its behavior in urea is quite different from that of the ruminant β-lactoglobulins.

The finding that Cys-160 is linked to Cys-123 may be considered in relation the the studies of Kalan *et al.* (1965), Greenberg and Kalan (1965), and Phillips *et al.* (1967) on the action of carboxypeptidase on the C-terminal end of the peptide chain and the reactivity of the –SH group. The C-terminal Ile and penultimate His residues appear to be readily removed from the bovine A and B proteins, but the action becomes complex thereafter, no doubt being affected by the fact that residue 160 is cystine and not cysteine. It should also be noted that residue 123 is located in the COOH-rich region involved in the octamerization reaction of the A variant.

We have considered in detail elsewhere (McKenzie, 1967, 1971) the observations of several groups of workers that the –SH and –SS– groups of the ruminant β-lactoglobulins undergo exchange reactions in alkaline solution in the absence of urea. In this section we have already discussed the evidence from peptide-mapping and ^{14}C carboxymethylation studies for rearrangement of the disulfide groups in urea solution. Interchange is appreciable, not only at alkaline pH, but also at pH 5.2 and 3.5 (on prolonged reaction), and appears to occur *via* the monomer leading to intra- and intermolecular rearrangement (monomers, dimers, trimers, etc.) (McKenzie and Ralston, 1971, 1972[2]).

Although there is a marked increase in reactivity of the –SH group with monofunctional mercurials and *N*-ethylmaleimide when urea is present, there is a much smaller increase (compared to the native protein) in the presence of detergents as has been shown by Franklin and Leslie (1968). This is further evidence for the different states of a protein brought about by different denaturants.

It is possible that the proximity of the sulfhydryl group in position 68 or 70 to the cystine residue in position 70 or 68, respectively, is an important factor in the behavior of the protein. One also wonders whether the –SH group is hydrogen bonded (–SH···O, or –SH···N, or –SH···S), and, when the abnormal –COOH group is titrated and the conformational transition occurs near pH 7, whether such a bond is broken, both leading to increased reactivity of the group.

In a recent short communication Braunitzer *et al.* (1972) have presented a revised sequence for bovine β-lactoglobulin AB. Although they have not determined the position of the cysteine residue and disulfide bridges, their work does confirm our modifications to their previous partial structure. However, two sections of the protein chain have been interchanged after examination of cyanogen bromide cleavage fragments with an automatic sequencer. It would appear that the residues they previously numbered 98–148 become 41–91, while 41–97 become 92–148. If this revised numbering proves to be correct the alternate positions for the sulfhydryl group would then be 119 or 121 with a disulfide bridge from 106 to 121 or 106 to 119, respectively, both forms having a disulfide bridge from 66 to 160.

[2] In preparation.

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