Selective Reduction of Cystine I–VIII in α-Lactalbumin of Bovine Milk†

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ABSTRACT: Bovine α-lactalbumin was reduced by dithioerythritol in aqueous solutions at pH 7.0 in two steps: a fast step followed by a much slower one. At the end of the fast step the partially reduced protein was carboxymethylated with 2-iodoacetic acid, and a product containing two carboxymethylcysteine residues per α-lactalbumin (2CM-αLA) was isolated in about 95% yield. By fragmentation of 2CM-αLA and characterization of the fragments, it has been established unequivocally that cystine I–VIII was the only reduced and carboxymethylated disulfide bond in 2CM-αLA. No other residues have been effected. 2CM-αLA is a homogeneous product, as was judged from its electrophoretic mobility on ion-exchange columns and on polyacrylamide disc electrophoresis in several different pH systems. Although the ability of 2CM-αLA to bind anti-α-lactalbumin antibodies cannot be distinguished from that of the native protein, 2CM-αLA has only about 50% of the biological activity of α-lactalbumin in the lactose synthesis reaction, and about 35% of the inhibitory capacity of α-lactalbumin in the inhibition of *N*-acetyllactosamine synthesis.

α-Lactalbumin is one of the two proteins which take part in the biosynthesis of lactose (Babad and Hassid, 1964, 1966; Brodbeck and Ebner, 1966; Ebner *et al.*, 1966; Brodbeck *et al.*, 1967). Although enzymic activity of α-lactalbumin has never been detected, it was suggested that α-lactalbumin modified the enzyme galactosyltransferase in a substrate-dependent interaction to promote lactose synthesis (Morrison and Ebner, 1971).

Bovine α-lactalbumin is a single polypeptide chain composed of 123 amino acids. Its eight half-cystine residues form four disulfide cross-links between cysteines 6–120 (I–VIII), 28–111 (II–VII), 61–77 (III–V), and 73–91 (IV–VI) (Brew *et al.*, 1970). No definite information is available concerning the actual three-dimensional structure of α-lactalbumin, although a molecular model has been proposed by Browne *et al.* (1969).

The disulfide bonds of α-lactalbumin, lysozyme, and many other proteins, play a key role in stabilization of the native three-dimensional structure of the protein. Disruption of all the disulfide bonds in proteins leads to complete loss of biological activity (for a review, see Anfinsen, 1964, 1965–1966). While the importance of the disulfide bonds of proteins has been well established, one may ask whether all of the disulfide bonds in a protein are equally important for its biological activity and three dimensional structure. There is evidence that partial disruption of these bonds need not significantly effect the biological activity of a protein, as was shown in the cases of bovine pancreatic ribonuclease (Sperling *et al.*, 1969; Neuman *et al.*, 1967), trypsin and trypsinogen (Light and Sinha, 1967), bovine trypsin inhibitor (Kress and Laskowski, 1967), papain (Shapira and Arnon, 1969), and immunoglobulin G (Fleischman *et al.*, 1962).

Studies with hen egg-white lysozyme have shown that disruption of one (Caputo and Zito, 1961) or two (Azari, 1966) disulfide bonds resulted in significant loss of biological activity. In the present study we show that one of the disulfide bonds of bovine α-lactalbumin, *i.e.*, that of cystine I–VIII, is important for biological activity or for maintaining the pertinent features of the macromolecularc onformation of this protein.

Materials

Proteins. Bovine α-lactalbumin was isolated from raw skimmed milk by a modification of the method of Aschaffenberg and Drewry (1957) as described by Castellino and Hill (1970). α-Lactalbumin thus obtained was a pure protein, as judged by its amino acid composition and by disc gel electrophoresis at pH 8.9 (Davis, 1964). Partially purified galactosyltransferase (A-protein) was isolated by a combination of the methods used by Brodbeck and Ebner (1966) and Brew *et al.* (1968). L-(1-Tosylamido-2-phenyl)ether chloromethyl ketone treated trypsin was prepared by treating trypsin with L-(1-tosylamido-2-phenyl)ether chloromethyl ketone (both purchased from Worthington Biochemical Corp.) according to Carpenter (1967). Pyruvate kinase type 1 from rabbit skeletal muscles (Sigma Chemical Co.) contained nucleotide diphosphokinase and lactate dehydrogenase necessary for the spectrophotometric assay of UDP. Bovine α-chymotrypsin was purchased from Worthington Biochemical Corp. Rabbit antiserum to α-lactalbumin was a gift of Dr. E. Maron. ^{125}I-Labeled lactalbumin (1.2×10^7 cpm/μmol) was prepared according to Hunter and Greenwood (1962). 8CM-αLA[1] was prepared essentially as described by Shechter *et al.* (1972) for 8CM-lysozyme.

Reagents. UDP-galactose, ATP, and Tris were products of Sigma Chemical Co. Dithioerythritol was a product of Cyclo Chemicals. NADH was purchased from P-L Biochemicals, Inc. D-Glucose and analytical grade urea were products of BDH Ltd.; urea was recrystallized from 95% ethanol. Phosphoenolpyruvate was obtained from Böhringer GmBH.

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[1] Abbreviations used are: 8CM-αLA, α-lactalbumin derivative in which all four disulfide bonds were reduced and carboxymethylated; 2CM-αLA, α-lactalbumin derivative in which cystine I–VIII was reduced and carboxymethylated; 2CM-6PE-αLA, 2CM-αLA derivative in which all three disulfide bonds were reduced and pyridinethylated; CM-cysteine, *S*-carboxymethylcysteine; PE-cysteine, *S*-β-(4-pyridinethyl)cysteine.

^{14}C-Labeled 2-iodoacetic acid was purchased from New England Nuclear, and diluted with nonradioactive 2-iodoacetic acid (Eastman Organic Chemicals). 4-Vinylpyridine was a product of Baker Chemical Co.

Methods

Analytical Procedures

Spectrophotometric measurements were performed with a Beckman Acta V or a Cary 14 Spectrophotometers, Quartz cells with 1-cm light paths were used.

Amino acid analyses were performed with a Beckman-Spinco Model 120C automatic amino acid analyzer after hydrolysis in 6 N HCl for 22 hr (Spackman *et al.*, 1958; Spackman, 1963; Friedman *et al.*, 1970).

Amino-terminal sequence analysis was performed according to a modification (Hermodson *et al.*, 1972) of the method of Edman and Begg (1967) using a Beckman sequencer (Model 890A). The products of degradation were identified by gas–liquid chromatography.

α-Lactalbumin concentrations were determined spectrophotometrically at 280 nm using $E_{280\ nm}$ = 29,000 l. mol^{-1} cm^{-1}, or by amino acid analysis assuming three residues of alanine and one residue of arginine (Brew *et al.*, 1970).

Radioactivity measurements were performed with a Packard Model 3003 TriCarb liquid scintillation spectrometer, and a Packard Model 320E TriCarb flow monitor, attached to the amino acid analyzer.

Acrylamide disc electrophoresis was performed with a Canalco apparatus at pH 8.9 and 4.3 using 7% gels (Davis, 1964; Reisfeld *et al.*, 1962).

Assay Procedure. Activity of α-lactalbumin and its derivatives to promote lactose synthesis in the presence of galactosyltransferase and glucose, was assayed according to the method of Brodbeck and Ebner (1966). By this method, UDP formation in the lactose synthesis reaction was followed spectrophotometrically at 340 nm according to Davidson (1959). A standard assay mixture contained, in a final volume of 2 ml: Tris-HCl buffer (pH 7.4), 100 μmol; phosphoenolpyruvate, 3 μmol; ATP, 0.25 μmol; $MnCl_2$, 80 μmol; NADH, 0.5 μmol; UDP-D-galactose, 60 μmol; pyruvate kinase (a crude preparation, containing lactate dehydrogenase), 1 mg; partially purified galactosyltransferase and α-lactalbumin. Activities of native and modified α-lactalbumin derivatives were compared under assay conditions in which a linear response could be obtained with respect to the concentration of the protein (10–80 μg/assay mixture).

Inhibition of *N*-acetyllactosamine synthesis by α-lactalbumin and its derivatives was assayed under similar conditions. The reaction mixture contained: $MnCl_2$, 10 μmol; UDP-galactose, 0.8 μmol; *N*-acetylglucosamine, 50 μmol; no glucose. All other ingredients were the same as above.

Immunological Procedure. To 0.2 ml of rabbit antiserum to α-lactalbumin, increasing amounts of antigen (5–50 mmol) were added, in a final volume of 0.6 ml of 0.1 M phosphate buffer–saline (pH 7.2). The mixture was incubated for 2 hr at 37°, ^{125}I-labeled α-lactalbumin (25 μl of a solution of 10^6 cpm/0.9 mg per ml) was added to each sample and the samples were incubated for 2 more hr at 37°. A saturated solution of ammonium sulfate (0.4 ml) was then added, and the mixture was left overnight at 4°. The precipitate was collected by centrifugation, washed extensively with 40% ammonium sulfate, and its radioactive content was determined with a liquid scintillation spectrometer.

Experimental Section

Partial Reduction of α-Lactalbumin. α-Lactalbumin was dissolved in 0.5 M Tris-hydrochloride buffer (pH 7.0; previously deaerated by a stream of nitrogen; 1 mM EDTA), to a final concentration of 0.5 μmol/ml, and placed in a stirred Radiometer titration vessel under a slow stream of nitrogen gas. Measured amounts of dithioerythritol solutions were then added, and reaction was allowed to proceed at room temperature. At different times, samples were removed and carboxymethylate with 2-iodoacetic acid (threefold excess over dithioerythritol) in 0.5 M Tris-hydrochloride buffer (pH 7.0) having the same volume as the protein solution. After alkylation for 30 min the mixture was dialyzed extensively against 0.05 M ammonium bicarbonate, and lyophilized.

Complete Reduction and Alkylation of α-Lactalbumin. The protein (50 mg of α-lactalbumin or 2CM-αLA) was dissolved in 5 ml of 1 M Tris-hydrochloride buffer–8 M urea (pH 8.0) containing 0.01% EDTA. Dithioerythritol (30 mg) was added and the reduction proceeded for 1 hr at room temperature.

CARBOXYMETHYLATION. Iodoacetic acid (150 mg) was added to the stirred reaction mixture, and after 20 min, the mixture was subjected to gel filtration on a Sephadex G-25 column (2 × 45 cm) and eluted with 0.05 M ammonium bicarbonate. The fractions corresponding to the protein peak were pooled and lyophilized.

PYRIDINETHYLATION. 4-Vinylpyridine (75 μl) was added to the stirred reaction mixture, under nitrogen and pyridinethylation was performed for 45 min. The reduced and alkylated protein was isolated by gel filtration as above.

Ion-Exchange Chromatography of Reduced and Carboxymethylated α-Lactalbumin Derivatives. α-Lactalbumin, 2CM-αLA, or 8CM-αLA was dissolved in 0.05 M Tris-hydrochloride buffer, 0.03 M NaCl (pH 8.9; 10 mg/ml), and loaded on a DEAE-cellulose column (1.0 × 15 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl (0.03–0.3 M) in 0.05 M Tris-hydrochloride buffer (pH 8.9), 500 ml in each chamber, at a rate of 60 ml/hr, 10 ml/fraction, at room temperature.

CNBr Cleavage of 2CM-6PE-αLA. Cleavage with cyanogen bromide was performed by the method of Gross and Witkop (1962). Approximately 35 mg of 2CM-6PE-αLA was dissolved in 5 ml of 80% formic acid, cyanogen bromide (100 mg) was added, and the reaction was allowed to proceed for 20 hr at room temperature. Water (40 ml) was added, and the mixture was lyophilized.

Enzymatic Digest of α-Lactalbumin Derivatives. SUCCINYLATION. The protein was dissolved in a solution of 0.05 M sodium acetate (0.05 M $CaCl_2$, 30 mg/10 ml), and placed in a stirred Radiometer titration vessel at 2°. The pH was adjusted to 8.0 with a solution of 1 N NaOH. Succinic anhydride (150 mg) was added in five portions during a period of 1 hr, and the pH was maintained at 8 with 10 N NaOH. The reaction was allowed to proceed for an additional hour, at which point the temperature was raised to 37°, and the pH was adjusted to 8.4.

TRYPTIC DIGEST. L-(1-Tosylamido-2-phenyl)ether chloromethyl ketone treated tryspin was added (60 μl of a solution of 10 mg/ml) and the digestion was carried out for 6 hr. A solution of 0.1 N NaOH was used as a titrant. The completion of the digest was proven by subjecting a portion of the digestion mixture to hydrolysis and amino acid analysis, while another portion was further digested with carboxypeptidase B. One mole of carboxy-terminal arginine per α-lactalbumin or its derivative was released by this treatment; carboxy-terminal lysine was not released.

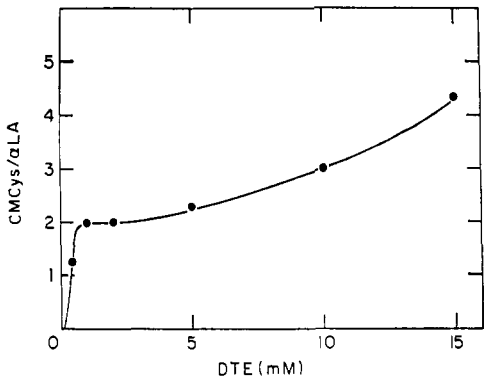

FIGURE 1: Extent of formation of CM-cysteine residues in α-lactalbumin upon reduction with increasing concentrations of dithioerythritol and subsequent treatment with iodoacetic acid. Reduction was performed at protein concentration of 0.5 μmol/ml in Tris-hydrochloride buffer (pH 7.0) for 20 min at room temperature.

CHYMOTRYPTIC DIGEST. α-Chymotrypsin was added (60 μl of a solution of 10 mg/ml) and the digestion was carried out for 1 hr. The pH was kept constant with a solution of 0.1 N NaOH as a titrant.

Results

Partial Reduction of α-Lactalbumin. The extent of reduction of α-lactalbumin in aqueous solutions was investigated as a function of the reaction time and the molar ratio of dithioerythritol to α-lactalbumin. Figure 1 describes the course of the reduction of the disulfide bonds in α-lactalbumin as a function of dithioerythritol concentration. It can be seen that at dithioerythritol concentrations of 1–4 μmol/ml, corresponding to a molar ratio of dithioerythritol over S–S bond of 0.5–2, an average of about one disulfide bond per α-lactalbumin was reduced within 20 min. A larger number of disulfide bonds could be reduced by increasing the concentration of dithioerythritol in the reaction mixture.

Preparation of 2CM-αLA and 2CM-6PE-αLA. In order to obtain a homogeneous product, we chose to reduce α-lactalbumin with a twofold excess of dithioerythritol over the protein for 10 min, and to alkylate the released sulfhydryl groups with 2-iodoacetic acid.[2] The reduced and alkylated α-lactalbumin derivative thus obtained was purified by gel filtration on a Sephadex G-25 column and by ion-exchange chromatography on a DEAE-cellulose column (Figure 2). The main protein peak from this column (fractions 34–46, Figure C) was pooled, concentrated, dialyzed against 0.05 M ammonium bicarbonate, and lyophilized. Amino acid analysis of the modified protein indicated that it contained two carboxymethylcysteine residues per α-lactalbumin molecule (Table I). This product will be denoted as 2CM-αLA. In another preparation, [1-^{14}C]-2-iodoacetic acid (29,700 cpm/μmol) was used, and the product was denoted as 2C*M-αLA. 2CM-αLA (and 2C*M-αLA) was further reduced with dithioerythritol in 8 M urea, and the released sulfhydryl groups were alkylated with 4-vinylpyridine. The product was isolated by gel filtration and will be referred to as 2CM-6PE-αLA (or 2C*M-6PE-αLA, respectively). Its amino acid composition is presented in Table I. Carboxymethylcysteine was the only radioactive amino acid in 2C*M-αLA.

[2] Other sulfhydryl agents have also been used. The characterization of these selectively modified α-lactalbumin derivatives is under investigation.

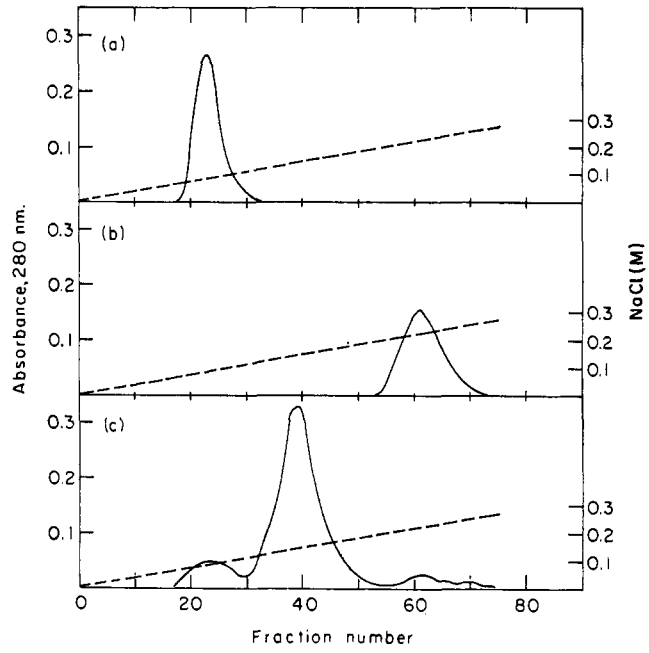

FIGURE 2: Ion-exchange chromatography of α-lactalbumin derivatives. The protein was dissolved in 0.05 M Tris-hydrochloride buffer, 0.03 M NaCl (pH 8.9), and applied to a DEAE-cellulose column (1.0 × 15 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl (0.03–0.3 M) in Tris-hydrochloride buffer (pH 8.9), 500 ml in each chamber, at a rate of 60 ml/hr, 10 ml/fraction: (a) α-lactalbumin (10 mg); (b) 8CM-αLA (12) mg); (C) 2CM-αLA (30 mg).

*Sequence Analysis of the Amino-Terminal End of 2C*M-6PE-αLA.* Half-cystine residue I in α-lactalbumin is located at

TABLE I: Amino Acid Composition of α-Lactalbumin Derivatives.

Amino Acid[a]	α-Lactalbumin Theor[b]	α-Lactalbumin Found	8CM-αLA	2CM-αLA	2C*M-6PE-αLA
Aspartic acid	21	20.7	21.2	20.3	21.1
Threonine	7	6.4	6.3	6.4	6.3
Serine	7	6.3	6.3	5.9	6.0
Glutamic acid	13	13.1	13.2	12.7	13.2
Proline	2	2.2	2.1	2.2	2.2
Glycine	6	6.2	6.2	5.4	5.6
Alanine	3	*3.0*	*3.0*	*3.0*	*3.0*
Half-cystine	8	6.8		5.1	
Valine	6	5.8	5.7	5.9	5.7
Methionine	1	0.9	0.9	0.9	1.2
Isoleucine	8	7.6	7.7	7.4	7.5
Leucine	13	12.3	12.1	12.2	12.3
Tyrosine	4	3.7	3.6	3.8	3.9
Phenylalanine	4	3.9	4.0	4.1	3.9
Lysine	12	12.2	11.8	11.6	12.3
Histidine	3	3.1	2.9	3.0	3.1
Arginine	1	1.1	1.0	1.1	1.1
Tryptophan[c]	4	3.9	3.8	3.9	3.7
CM-cysteine			7.6	1.7	2.1
PE-cysteine					5.8

[a] Not corrected. [b] According to Brew *et al.* (1970). [c] Determined spectrophotometrically.

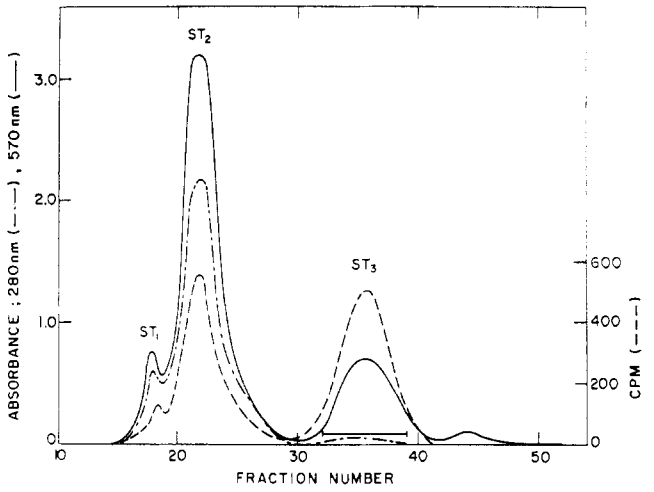

FIGURE 3: Gel filtration of the tryptic digest of 2C*M-αLA. The lyophilized digest was dissolved in approximately 3 ml of 30% aqueous acetic acid and applied to a Sephadex G-75 column (1.5 × 85 cm) developed at a rate of 15 ml/hr, 2.0 ml/fraction. Fractions 20–28 (ST_2) and 32–39 (ST_3) were pooled. The elution was monitored by absorption at 280 mn (—·—), by ninhydrin color value of 50-μl aliquots (after partial alkaline hydrolysis) (—), and by the radioactivity present in 50-μl aliquots (- - -).

sequence position 6, counting from the amino-terminal end of the protein molecule (Brew *et al.*, 1970). Therefore, in order to check the possibility that cystine I–VIII was the reduced and alkylated bond in 2C*M-αLA, 2C*M-6PE-αLA was subjected to a short automatic sequence analysis (eight turns) according to an adaptation (Hermodson *et al.*, 1972) of the method of Edman and Begg (1967). The amino acid phenylthiohydantoins were identified by gas–liquid chromatography and the radioactivity in each sample was determined by liquid scintillation. The results are presented in Table II. As can be seen from this table, about 40% of the radioactivity of 2C*M-6PE-αLA was removed in turn 6, which had been identified as carboxymethylcysteine; no pyridinethylcysteine was detected in this turn.

Assuming an average stepwise yield of 95.5% (from the yields of glutamic acid in turns 1 and 7), the radioactivity content of CM-cysteine at turns 6 and 7 (overlap) was 53% of the total radioactivity in 2C*M-6PE-αLA.

Site of Reduction of α-Lactalbumin by Dithioerythritol. The short automatic sequence analysis of 2C*M-6PE-αLA clearly indicated that CM-cysteine at position 6 was one of the two half-cystine residues which had been reduced and carboxymethylated in 2C*M-αLA. The other half-cystine residue should be, therefore, half-cysteine-120. In order to prove it, 2C*M-αLA was succinylated with succinic anhydride (Klotz, 1967) and its arginyl peptide bond (Arg_{10}) was digested with trypsin as described under Methods.

TABLE II: Automatic Sequence Analysis of 2C*M-6PE-αLA.[a]

Turn	Sequence: Theor[b]	Sequence: Found	nmoles	cpm
1	Glu	Glu	140	11
2	Gln	Gln		0
3	Leu	Leu		0
4	Thr	Thr		0
5	Lys	*c*		14
6	Cys	CMCys		3430
7	Glu	Glu	109	154
8	Val	Val		7

[a] 2C*M-6PE-αLA (145 nmol, 8600 cpm) was subjected to automatic sequence analysis (see Methods). [b] According to Brew *et al.* (1970). [c] Poor identification. CMCys, *S*-carboxymethylcysteine.

TABLE III: Amino Acid Composition of Peptide Fragments of α-Lactalbumin Derivatives.[a]

Amino Acid[a]	ST_3 1–10 Theor	ST_3 1–10 Found	CB_3 91–123 Theor	CB_3 91–123 Found	CB_3-C 119–123 Theor	CB_3-C 119–123 Found
Aspartic acid		0.1	3	2.7		
Threonine	1	0.9		0.2		
Serine		0.1	1	1.0		
Glutamic acid	3	3.0	3	3.0	1	1.0
Glycine			1	0.8		0.1
Alanine			2	1.7		0.1
Half-cystine	1		3		1	
Valine	1	1.0	2	1.6		
Isoleucine			2	1.6		0.2
Leucine	1	0.9	6	4.9	2	1.3
Tyrosine			1	0.6		
Phenylalanine	1	0.9				
Lysine	1	1.1	6	5.2	1	1.1
Histidine			1	0.7		
Arginine	1	0.9				
CM-cysteine		0.8		0.7		0.8
PE-cysteine				1.8		
Tryptophan			2	*c*		
Per cent of isolation		85				47

[a] According to the sequence by Brew *et al.* (1970). [b] Not corrected. [c] Not determined.

Figure 3 illustrates the separation pattern of this digest on a Sephadex G-75 column. Three radioactive ninhydrin-positive peaks were isolated, and the distribution of the radioactivity among peaks ST_1, ST_2, and ST_3 (Figure 3) was 9, 44, and 47%, respectively. The fractions corresponding to peaks ST_2 and ST_3 were pooled and lyophilized. Peak ST_3 was subjected to high-voltage paper electrophoresis at pH 3.75 which proved its homogeneity. The amino acid composition of peptide ST_3, which was isolated in 84% yield, indicated that it was composed of residues 1–10 of 2C*M-αLA (Table III). and contained 47% of the CM-cysteine content of 2C*M-αLA.

Peptide ST_2 which contained the other CM-cysteine residue was further reduced by dithioerythritol in 8 M urea, and the released sulfhydryl groups were alkylated with 4-vinylpyridine. This modified peptide was cleaved with cyanogen bromide in 80% formic acid, and the fragments were separated by gel filtration on a Sephadex G-100 column. The elution pattern of the cleavage products is presented in Figure 4.

As can be seen from this Figure , peak CB_1 contained 20% of the radioactivity, peak CB_2 was nonradioactive, and peak CB_3 contained about 80% of the radioactivity of the cyanogen bromide digest of peptide ST_2 (from Figure 3). The fractions corresponding to peak CB_3 were pooled and lyophilized.

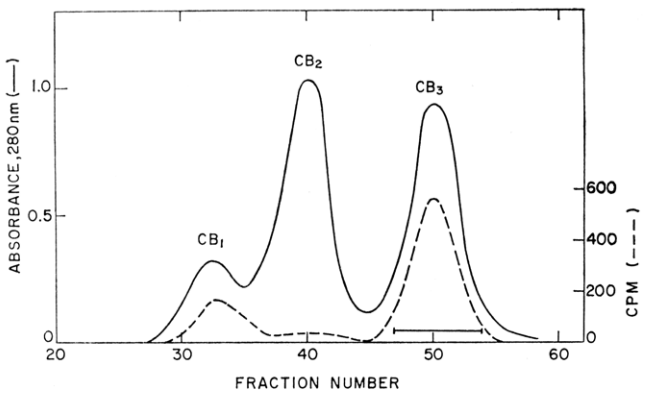

FIGURE 4: Gel filtration of the cyanogen bromide digest of peptide ST_2. The digest was applied to a Sephadex G-100 column (1.5 × 85 cm) in 50% formic acid. The column was developed at a rate of 11 ml/hr, 1.7 ml/fraction, and was monitored by the absorption at 280 nm (——) and the radioactivity content of 50-μl aliquots (- - -).

The amino acid composition of peptide CB_3, which corresponded to residues 91–123 of 2C*M-αLA is presented in Table III. Peptide CB_1 corresponded to some uncleaved peptide ST_2 (20%).

Peptide CB_3 (residues 91–123 of α-lactalbumin, which contained *one* CM-cysteine residue and two PE-cysteine residues, was digested by α-chymotrypsin and the digest was separated on a Sephadex G-50 column. The elution pattern of this digest is presented in Figure 5. Only one radioactive peak was present. The fractions corresponding to this peak, peak CB_3-C (Figure 5), were pooled, lyophilized, and rechromatographed on a Sephadex G-15 column. Amino acid composition analysis of this radioactive peptide indicated that it corresponded to residues 119–123 (Table III, peptide CB_3-C). Peptide CB_3-C which was isolated in an overall yield of 47%, contained CM-cysteine-120, which participated in disulfide bond I-VIII of α-lactalbumin.

Properties of 2CM-αLA. ELECTROPHORETIC MOBILITY. 2CM-αLA migrated on acrylamide gels (pH 8.9) as a single band. For comparison the mobility of native α-lactalbumin and 8CM-αLA is also included (Figure 6).

THE BIOLOGICAL ACTIVITY of 2CM-αLA to promote lactose synthesis in the presence of galactosyltransferase (A protein) and glucose, was measured and compared with that of the native protein. 2CM-αLA had about 50% of the biological activity of the native protein.

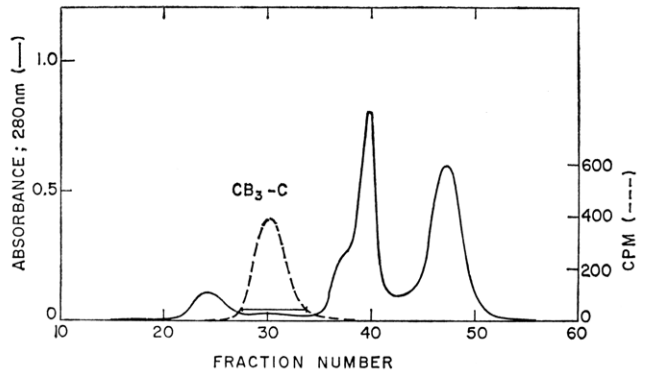

FIGURE 5: Gel filtration of the chymotryptic digest of peptide CB_3. The digest was applied to a Sephadex G-50 superfine column (1.5 × 85)in 0.05 M ammonium bicarbonate. The column was developed at a rate of 25 ml/hr, 5 ml/fraction, and was monitored by the absorption at 280 nm (——) and by the radioactivity content of 100-μl aliquots (- - -).

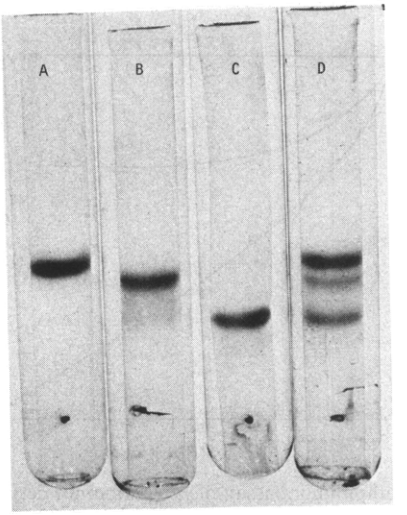

FIGURE 6: Disc gel electrophoresis of reduced and carboxymethylated derivatives of α-lactalbumin at pH 8.9 in 7% gel according to Davis (1964): (A) α-lactalbumin; (B) 8CM-αLA; (C) 2CM-αLA; (D) α-lactalbumin + 2CM-αLA + 8CM-αLA.

INHIBITION OF THE BIOSYNTHESIS OF *N*-ACETYLLACTOSAMINE BY 2CM-αLA was compared with that of native α-lactalbumin at pH 7.5. As can be seen from Figure 7, 2CM-αLA has about 35% of the inhibitory capacity of α-lactalbumin in this assay. 8CM-αLA does not inhibit this reaction.

IMMUNOLOGICAL PROPERTIES OF 2CM-αLA. The capacity of 2CM-αLA to bind rabbit antibodies to α-lactalbumin is presented in Figure 8. As can be seen from this figure, α-lactalbumin and 2CM-αLA bind rabbit anti α-lactalbumin antibodies to the same extent. In 8CM-αLA, about 30% of the antigenic determinants were still preserved, while hen egg-white lysozyme does not cross-react with rabbit anti-α-lactalbumin antibodies.

Discussion

The present study indicates that the disulfide bond I-VIII in bovine α-lactalbumin is more susceptible to reduction by dithioerythritol than the other disulfide bonds of this pro-

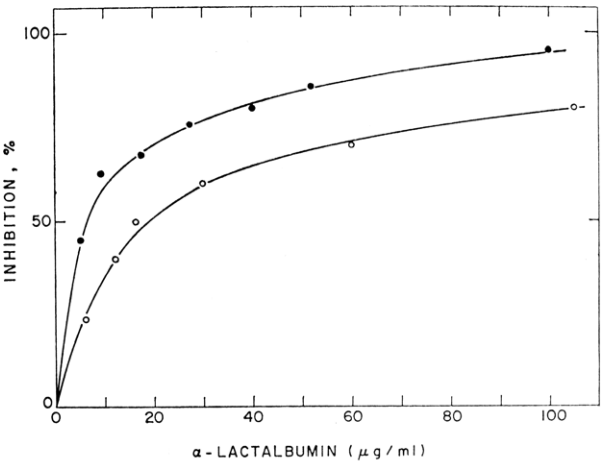

FIGURE 7: Inhibition of *N*-acetyllactosamin synthesis by α-lactalbumin and 2CM-αLA. The assay was performed in Tris-hydrochloride, 50 mM (pH 7.4); $MnCl_2$, 5 mM; UDP-galactose, 0.4 mM; *N*-acetylglucosamine, 25 mM, essentially as described by Fitzgerald *et al.* (1970).

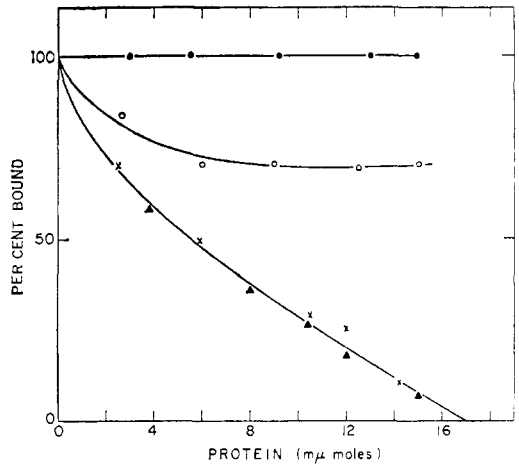

FIGURE 8: Radioimmunoassay of α-lactalbumin derivatives. Ordinates shows percentage of ^{125}I precipitated from 22 μg of ^{125}I-labeled α-lactalbumin (2.5×10^4 cpm) by 0.2 ml of a solution of rabbit antiserum to α-lactalbumin, plotted against the unlabeled protein added on the abscissa: α-Lactalbumin (▲); 2CM-αLA (×); 8CM-αLA (○); hen egg-white lysozyme (●).

tein molecule. Studies of the course of the reduction of the disulfide bonds of α-lactalbumin by dithioerythritol indicate that there is an initial fast reduction step followed by a much slower one. By choosing the right reduction conditions, we were able to trap and isolate an α-lactalbumin derivative in which only one disulfide bond was reduced, and the liberated sulfhydryl groups were alkylated with 2-iodoacetic acid. This modified α-lactalbumin derivative contained two carboxymethylcysteine residues (2CM-αLA).

The proof that cystine I–VIII was the reduced disulfide bond was obtained by sequence analysis of 2C*M-αLA or its pyridinethylated derivative 2C*M-6PE-αLA. A relatively short automatic sequence analysis (eight turns) indicated that half-cystine I (residue 6) of bovine α-lactalbumin was a reduced and carboxymethylated residue in 2CM-αLA. This residue contained about 50% of the total radioactivity (owing to the carboxymethyl moiety) of 2CM-αLA. The other 50% of the radioactivity most probably is associated with the other half-cystine residue of this bond, namely, half-cystine VIII (residue 120) of α-lactalbumin (Brew *et al.*, 1970).

2C*M-αLA was fragmented by enzymatic (trypsin and chymotrypsin) and nonenzymatic (CNBr) cleavages, and the two peptides containing half-cystine I (residues 1–10 of α-lactalbumin) and half-cystine VIII (residues 119–123 of α-lactalbumin) were isolated. Those were the only radioactive peptides of 2C*M-αLA. It may thus be concluded that cystine I–VIII is more susceptible to reduction by dithioerythritol than any other disulfide bond in bovine α-lactalbumin.

A comparison of these studies with those of cleavage of the disulfide bonds in α-lactalbumin by mercaptoethanol (Attassi *et al.*, 1970) reveals differences in the reactivity of α-lactalbumin toward dithioerythritol and mercaptoethanol. With the latter reagent, about 2.5 disulfide bonds were split within 1 hr, and 2 M guanidine-HCl was required for complete reduction of all four disulfide bonds in α-lactalbumin. With dithioerythritol, bond I–VIII splits readily, and the remaining disulfide bonds may subsequently react, although at much slower rates.

Although dithioerythritol has a much lower reduction potential than mercaptoethanol, one would expect that with somewhat higher concentrations of mercaptoethanol, a complete reduction of *all* four disulfide bonds of α-lactalbumin could be obtained. Preliminary experiments with purified α-lactalbumin indicated that mercaptoethanol, at a concentration of 10 mM, is indeed capable to cause complete reduction of the disulfide bonds of α-lactalbumin at pH 7.5 (with no denaturant added).

At present, no definite information is available concerning the actual three-dimensional structure of α-lactalbumin, although a molecular model, which is based on the striking similarity between the amino acid sequences of bovine α-lactalbumin and hen egg-white lysozyme has been proposed by Browne *et al.* (1969). A number of chemical and physical studies of these proteins support this proposal, although some of these studies do suggest a more expanded structure for α-lactalbumin. Recent low-angle X-ray scattering data for lysozyme and α-lactalbumin suggest similar shapes in solution (Krigbaum and Kugler, 1970; Pessen *et al.*, 1971). The susceptibility of the disulfide bonds of lysozyme to reduction was studied some years ago. Caputo and Zito (1961) and Azari (1966) have reported that disruption of one or two disulfide bonds in lysozyme did not result in a parallel loss of enzymatic activity. Lysozyme was quite resistant to reduction or sulfitolysis in the absence of urea. Only in the presence of 4–6 M urea, could one to two disulfide bonds be disrupted with loss of 10–30% of the biological activity. In our study, reduction of only one disulfide bond resulted in a loss of about 50% (in the lactose synthesis) and 65% (in the inhibition of *N*-acetyllactosamine synthesis) of the biological activity of α-lactalbumin. These results support the suggestion of a more compact structure for hen egg-white lysozyme and a more expanded structure around the disulfide bonds of α-lactalbumin.

The inefficiency of 2CM-αLA in serving as a "specifier" for galactosyltransferase in the biosynthesis of lactose (about 50%) and in the inhibition of *N*-acetyllactosamine synthesis (about 35%) suggests that the interaction site(s) of α-lactalbumin with galactosyltransferase has been altered. It might also indicate that these two biological activities of α-lactalbumin are associated with the same site(s) on the protein molecule.

Native α-lactalbumin and 2CM-αLA bind anti-α-lactalbumin antibodies to the same extent, indicating that there are no gross conformational differences between the two proteins. However, the fact that 2CM-αLA is more susceptible to digestion with trypsin than native α-lactalbumin[3] indicates that some minor conformational differences between the two proteins do exist.

Studies of the optical properties of 2CM-αLA as well as some chemical modifications of this and other partially reduced and alkylated α-lactalbumin derivatives are under investigation.

Preliminary attempts to introduce a mercury atom into the reduced disulfide bond of α-lactalbumin have been successful, and a detailed study of this modification of α-lactalbumin is in progress.

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[3] Y. Shechter, unpublished results.

References

Anfinsen, C. B. (1964), *in* New Perspectives in Biology, Sela, M. Ed., Amsterdam, Elsevier, p 42.

Anfinsen, C. B. (1965–1966), *Harvey Lect. 61*, 91.

Aschaffenberg, R., and Drewry, J. (1957), *Biochem. J. 65*, 273.

Attassi, M. Z., Habeeb, A. F. S. A., and Rystedt, L. (1970), *Biochim. Biophys. Acta 200*, 184.

Azari, P. (1966), *Arch. Biochem. Biophys. 115*, 230.

Babad, H., and Hassid, W. Z. (1964), *J. Biol. Chem. 239*, 946.

Babad, H., and Hassid, W. Z. (1966), *J. Biol. Chem. 241*, 2672.

Brew, K., Castellino, F. J., Vanaman, T. C., and Hill, R. L. (1970), *J. Biol. Chem. 245*, 4570.

Brew, K., Vanaman, T. C., and Hill, R. L. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 491.

Brodbeck, U., Denton, W. L., Tanahashi, N., and Ebner, K. E. (1967), *J. Biol. Chem. 242*, 1391.

Brodbeck, U., and Ebner, K. E. (1966), *J. Biol. Chem. 241*, 762.

Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C., and Hill, R. L. (1969), *J. Mol. Biol. 42*, 65.

Caputo, A., and Zito, R. (1961), *2nd Symp. Lysozyme, Milan, 1*, 29.

Carpenter, F. H. (1967), *Methods Enzymol. 11*, 237.

Castellino, F. J., and Hill, R. L. (1970), *J. Biol. Chem. 245*, 417.

Davidson, E. P. (1959), *Biochim. Biophys. Acta 33*, 238.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Ebner, K. E., Denton, W. L., and Brodbeck, U. (1966), *Biochem. Biophys. Res. Commun. 24*, 232.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.

Fitzgerald, D. K., Brodbeck, U., Kiyosawa, I., Mawal, R., Colvin, B., and Ebner, K. E. (1970), *J. Biol. Chem. 245*, 2103.

Fleischman, J. B., Pain, R., and Porter, R. R. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 174.

Friedman, M. Kull, L. H., and Canvis, J. F. (1970), *J. Biol. Chem. 245*, 3868.

Gross, E., and Witkop, B. (1962), *J. Biol. Chem. 237*, 1856.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11*, 4493.

Hunter, W. M., and Greenwood, F. C. (1962), *Nature (London), 194*, 495.

Klotz, I. M. (1967), *Methods Enzymol. 11*, 576.

Kress, L. F., and Laskowski, M., Sr. (1967), *J. Biol. Chem. 242*, 4925.

Krigbaum, W. R., and Kugler, F. R. (1970), *Biochemistry 9*, 1216.

Light, A., and Sinha, N. K. (1967), *J. Biol. Chem. 242*, 1358.

Morrison, J. F., and Ebner, K. E. (1971), *J. Biol. Chem. 246*, 3992.

Neuman, H., Steinberg, I. Z., Brown, J. R., Goldberger, R. F., and Sela, M. (1967), *Eur. J. Biochem. 3*, 171.

Pessen, H., Kumosinski, T. F., and Timasheff, S. N. (1971), *J. Agr. Food Chem. 19*, 698.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.

Shapira, E., and Arnon, R. (1969), *J. Biol. Chem. 244*, 1026.

Shechter, Y., Burstein, Y., and Patchornik, A. (1972), *Biochemistry 11*, 653.

Spackman, D. H. (1963), *Fed Proc , Fed. Amer. Chem. Soc. 22*, 244.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Sperling, R., Burstein, Y., and Steinberg, I. Z. (1969), *Biochemistry 8*, 3810.