Lactose Synthetase. Modification of Carboxyl Groups in α-Lactalbumin*

Tsau-Yen Lin†

ABSTRACT: The carboxyl groups in α-lactalbumin, one of the two component proteins of bovine lactose synthetase, were modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and glycinamide. At pH 4.75 the treatment caused rapid inactivation of lactose synthetase activity and an average of 20 residues of carboxyl groups were converted into C(=O)NH-CH_2C(=O)NH_2 derivative within 400 min. An homologous reaction on hen egg-white lysozyme, which resembles α-lactalbumin in amino acid composition and sequence, led to modification of 8 out of 11 carboxyl groups with a loss of the cell-wall lytic activity. Further treatment of these modified proteins with the carbodiimide and [^{14}C]glycinamide in 4 M guanidine hydrochloride to attain a maximal extent of modification brought about incorporation of 0.6 and 2.1 residues of the nucleophile, respectively. The major ^{14}C labels were located in residues 63–79 (specific radioactivity 0.3 mole of [^{14}C]glycinamide/molecule) in α-lactalbumin and in residues 34–45 (Glu-35; specific radioactivity 0.92 mole of [^{14}C]glycinamide/molecule), residues 6–13 (Glu-7; specific radioactivity 0.44), and residues 62–68 (Asp-66; specific radioactivity 0.54) in lysozyme. These results suggest that (1) no carboxyl group in α-lactalbumin has the unique property comparable with Glu-35 of lysozyme; (2) even if similarities exist in gross conformations of these two proteins, the environments of all carboxyl side chains in α-lactalbumin appear to be more accessible to the modifying reagent than those in lysozyme; (3) free carboxyl groups in α-lactalbumin may be essential for maintaining its biological activity. The modified α-lactalbumin does not catalyze lactose synthesis with A protein, nor inhibit uridine diphosphate galactose-*N*-acetyl-D-glucosamine galactosyl transferase activity and lactose formation catalyzed by the native α-lactalbumin. The activity could not be protected from carboxyl modification by including in the reaction mixture either uridine diphosphate D-galactose, D-glucose, mono- and oligo-*N*-acetyl-D-glucosamine, or lactose. Tentative results suggest that partial protection could be achieved by binding with A protein. The possible mode of carboxyl participation in lactose synthesis is discussed.

The lactose synthetase from bovine milk which catalyzes the formation of lactose from UDP-D-galactose and D-glucose (Babad and Hassid, 1964, 1966) consists of two proteins, A and B, both of which are required for the enzyme activity (Brodbeck and Ebner, 1966). The lower molecular weight component, B protein, was shown to be α-lactalbumin (α-LA),[1] which is a milk protein of mol wt 14,500 with 123 amino acid residues (Brodbeck *et al.*, 1967). Brew *et al.* (1967) have recently demonstrated that chicken egg-white lysozyme and bovine α-LA have closely similar amino acid sequences. The similarities in the primary structures also suggest that α-LA may have a conformation analogous to that established for lysozyme (Blake *et al.*, 1967; Browne *et al.*, 1969). If the two sequences are aligned so that maximum homology can be obtained, it was noted that the residue at 52 in the primary structure of chicken lysozyme may be preserved in α-LA, whereas the amino acid corresponding to the Glu-35 in the lysozyme is His-32 or Thr-33 in α-LA (Hill *et al.*, 1969). The side chains of Glu-35 and Asp-52 have been inferred as the essential groups for general acid–base catalysis of the bond cleavage exerted by lysozyme (Phillips, 1967). Thus, the histidine residue in α-LA may be assumed to exercise the function of Glu-35 in lysozyme if any functional similarity exists in these two proteins. However, evidence indicates that chicken lysozyme does not participate in lactose synthesis, nor does α-LA act on lysozyme substrates. In fact, Brew, *et al.* (1968) demonstrated that the separated A protein catalyzes UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase activity, and α-LA (B protein) modifies the acceptor specificity of the enzyme from *N*-acetyl-D-glucosamine to D-glucose.

In order to clarify the relationship between the structure of α-LA to its function, advantage was taken of the carbodiimide method of Hoare and Koshland (1967) to modify carboxyl groups in this protein. Since this procedure which converts the carboxyl group into C(=O)NHR derivative proved to be useful for exploring the state of carboxyls in chicken lysozyme (Lin and Koshland, 1969), it seems likely that equally significant information about the role and nature of carboxyl groups in α-LA could be obtained by examining the effect of modification on the functional activity, and by comparing the result with that obtained on the similar treatment of chicken egg-white lysozyme. The data indicate that the integrity of carboxyl side chains of α-LA is essential for maintaining the B protein activity. Furthermore, it appears that the state of carboxyl groups in α-LA differs from that of the carboxyl side chains in chicken lysozyme with regard to their accessibility to the modifying reagent.

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† Present address: Department of Biochemistry, Merck Sharp & Dohme Research Laboratories, Rahway, N. J. 07065.

[1] Abbreviations used are: α-LA, α-lactalbumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; AMS, aminomethanesulfonic acid.

Material and Methods

Preparation of α-LA (B Protein) and A Protein Fraction from Bovine Milk. Unpasteurized whole milk (3 l.) was first treated with ammonium sulfate (264 g of ammonium sulfate/l.) and centrifuged (9000 rpm for 30 min, Sorvall GSA rotor). The purification procedure of Brodbeck *et al.* (1967) was then followed on the supernatant solution except that 300 g/l. of ammonium sulfate was added to obtain the lactose synthetase activity, and the second ammonium sulfate precipitation was omitted. The α-LA fraction obtained from Bio-Gel P-30 column (5 × 150 cm) was further purified on a microgranular DEAE-cellulose column (2 × 35 cm) with a linear gradient prepared from 20 mM Tris-HCl buffer with 5 mM $MgCl_2$ (pH 7.4) (500 ml) and 400 mM Tris-HCl buffer with 5 mM $MgCl_2$ (pH 7.4) (500 ml). The dialyzed and lyophilized material gave a single Nigrosin-stained band that migrated toward the cathode on starch gel electrophoresis in pH 3.3 aluminum lactate buffer.

The A fraction obtained from the Bio-Gel chromatography was further separated by gel filtration on Sephadex G-100 column (2.5 × 150 cm) in 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$. The protein fraction under the peak of UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase activity was stored at −10° with 2 mM dithiothreitol before use (Figure 1). This fraction was not homogeneous since two additional protein components devoid of A protein activity could be separated on a DEAE-cellulose column, with a gradient of KCl (0–0.4 M) in the Tris-HCl buffer. The gel filtration behavior of A protein activity indicated a molecular weight of approximately 65,000 when the following proteins of known molecular weight were used as standards: chymotrypsinogen, ovalbumin, inorganic pyrophosphatase, bovine serum albumin, and alcohol dehydrogenase.

Activity Assay. Activity of α-LA and its derivatives to promote lactose snythesis in the presence of A protein (the B protein activity) was assayed with a constant amount of A fraction which contained an excess of A protein activity (the activity to catalyze lactose synthesis in the presence of α-LA) to saturate the B protein activity. For each preparation of A protein fraction the lactose synthetase activity was determined with various amounts on a constant amount of α-LA and the quantity of A fraction which gave no increase in lactose synthesis with further increase of its amount was taken to be the saturating quantity for the specific amount of α-LA. The activities of the native and the modified α-LA were compared under the condition of assay in which a linear response could be obtained in respect to the concentration of the protein.

In addition to the A protein fraction and α-LA or its derivatives, the reaction mixture contained 15 μmoles of Tris-HCl (pH 7.4), 10 μmoles of $MnCl_2$, 6.0 μmoles of D-glucose, and 75 mμmoles, 15,000 cpm, of UDP-D-[^{14}C]-galactose (New England Nuclear) in a total volume of 0.2 ml. After incubation at 37° for 10 min, the mixture was passed through a Dowex 1 column made in a Pasteur pipet, which was previously washed with 0.1% lactose and water. The column was washed with 1.5 ml of water and the effluent was dried on a planchet and counted in a Nuclear-Chicago gas-flow counter or, alternatively, aliquots of the effluents were counted in Bray's scintillation solvent (Bray, 1960) with a Nuclear-Chicago Mark I scintillation spectrometer. For measurement

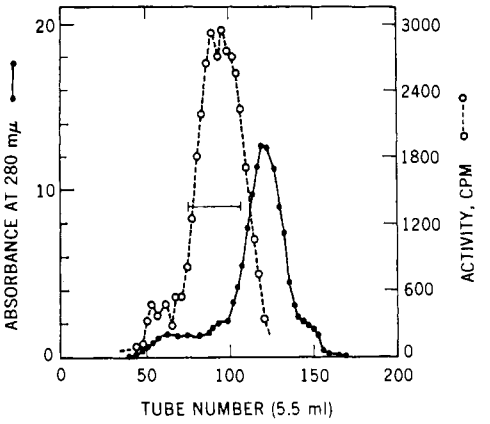

FIGURE 1: Gel filtration on Sephadex G-100 (2.5 × 150 cm) of the UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase fraction previously obtained from the Bio-Gel P-30 chromatography of an $(NH_4)_2SO_4$-fractionated bovine milk. The column was equilibrated and eluted with 20 mM Tris-HCl buffer containing 5 mM $MgCl_2$ (pH 7.4). The galactosyl transferase activity was determined with 0.1-ml aliquots. The fractions marked were pooled for further studies.

of UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase in the A protein fraction the same procedure was used, except that D-glucose was replaced by *N*-acetyl-D-glucosamine. Suitable controls such as determination without A protein or α-LA were included. The A protein preparations used in this study could catalyze the formation of 5–9 mμmoles of *N*-acetyllactosamine from UDP-D-galactose per min per mg of protein; 1 mg of protein of these preparations formed 8–16 mμmoles of lactose/min under the condition where the presence of excess α-LA permitted a maximal lactose formation. In all cases the activities were expressed as counts per minute of ^{14}C or millimicromoles of lactose and/or *N*-acetyllactosamine formed in 1 min.

Concentration of α-LA was determined from the absorbance at 280 mμ using the value $E^{1\%}_{280}$ 20.1 (Ebner *et al.*, 1966). The amount of the modified α-LA was also estimated by amino acid analysis on the acid hydrolysate assuming three alanyl residues per molecule of the protein. Protein content in A protein preparations was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standards.

The activities of the native and the carboxyl-modified lysozyme were measured by *Micrococcus lysodeikticus* cell lysis at pH 7.0 in 0.1 M potassium phosphate buffer (Shugar, 1952). The decrease in absorbance at 450 mμ of the cell suspension was followed with the Beckman DB spectrophotometer equipped with a recorder and a scale expander. Normally about 5 μg of the native lysozyme was employed.

Modification of Carboxyl Groups in Proteins. The procedure for modification has been described previously in the studies on chicken egg-white lysozyme (Lin and Koshland, 1969), which is essentially that of Hoare and Koshland (1967). α-LA (10 mg/ml), glycinamide hydrochloride (Aldrich: initial concentration, 1 M), and NaCl (0.2 M) in water at pH 4.75 or 7.0 were treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Ott Chemical; initial concentration, 0.1 M) at 25° for the desired intervals. For preparative reactions in which longer than 300 min were required, an additional

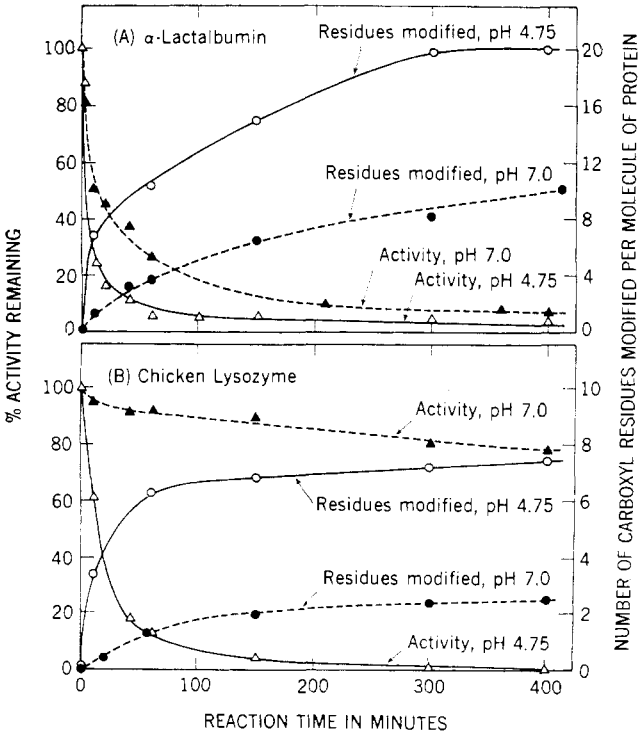

FIGURE 2: Time dependence of reaction of α-lactalbumin and chicken egg-white lysozyme with EDC–glycinamide at pH 4.75 and 7.0. The reaction mixture contained protein (10 mg/ml; about 0.7 mM), glycinamide (initial concentration 1 M), NaCl (0.2 M), and EDC (initial concentration 0.1 M). The reaction was initiated by addition of EDC and proceeded at the desired pH, 25°, by controlling with a pH-Stat. At each time interval, aliquots were taken and quenched with ten volumes of 1 M sodium acetate buffer (pH 4.75). The materials were exhaustively dialyzed against water, lyophilized, and treated with a known quantity of water. Aliquots were taken for determinations of enzyme activity and extent of carboxyl modification. The specific enzyme activity before and after dialysis was found to be identical. For α-LA, lactose synthetase activity is shown, which was determined with an A-protein fraction which could saturate a specific amount (30 μg) of α-LA. The cell wall lytic activity is indicated for lysozyme.

amount of EDC (10–15% of the initial quantity) was applied after the 200th min. The pH of the reaction mixtures was controlled by a Radiometer pH-stat assembly. The reaction was terminated by adding five volumes of 1 M sodium acetate (pH 4.75) and the mixture was subjected to exhaustive dialysis against water for 2 days at 4°. An identical preparation through the same treatment but without EDC served as control.

For protection experiments, A fraction which contains at least twice the amount of the saturation level of A activity of the lactose synthetase relative to a constant amount of α-LA was first incubated with the latter protein at 25° for 10 min in the presence of $MnCl_2$ (0.1 M) and glucose (0.1 M) and the modification reaction followed (350 min). A control was run in which the A protein fraction was first modified with EDC reagent for 350 min, followed by further reaction in the presence of added α-LA for 350 min with an additional amount of the reagent.

For determination of the extent of modification, aliquots (about 1 mg) of protein were hydrolyzed in 6 N HCl at 105°

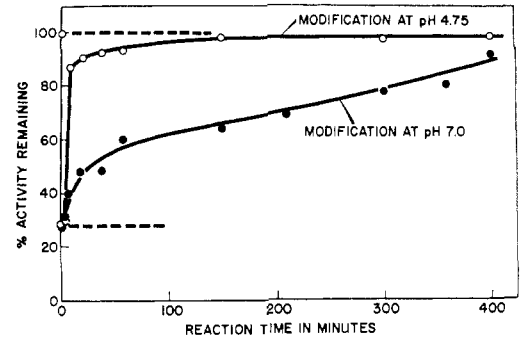

FIGURE 3: Effect of modification of carboxyl groups in α-lactalbumin on its inhibitory action on UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase activity of A protein. Time dependence of the modification is shown. The conditions of the reaction were the same as described in the legend for Figure 2. The assay for galactosyl transferase was conducted with 30 mM *N*-acetyl-D-glucosamine. The level of transferase activity without influence of α-LA was taken as 100%. With 30 μg of α-LA 72% of the transferase (A protein) activity was inhibited under the condition of the assay. The amounts of the modified protein equivalent to 30 μg of the unmodified α-LA were taken for assay comparison.

for 22 hr in a vacuum-sealed glass tube and analyzed with a Beckman-Spinco Model 120C amino acid analyzer. The increase in glycine content was regarded as the glycinamide residues incorporated into carboxyl groups of protein by carbodiimide (Hoare and Koshland, 1967; Lin and Koshland, 1969).

The tyrosine residues which were partially modified by EDC were regenerated to free tyrosine by treatment of the modified protein with 0.75 M NH_2OH at pH 6.5 (Carraway and Koshland, 1968).

Labeling of the Unmodified Carboxyl Groups with [^{14}C]*-Glycinamide and Localization of the Labeled Sites.* The proteins with previously modified carboxyls were treated with EDC and [^{14}C]glycinamide in 4.5 M guanidine for 300 min at pH 4.75 and 25°. The radioactive proteins were reduced and carboxymethylated (Crestfield *et al.*, 1963). The materials were digested with *N*-tosyl-L-phenylalanylchloromethane-treated trypsin (Kostka and Carpenter, 1964) at pH 8.0 and room temperature for 20 hr. About 3-mg aliquots of the lyophilized digests were applied on S & S orange ribbon acid-washed paper (48 × 55 cm) and chromatographed in 1-butanol–pyridine–HOAc–H_2O (15:10:3:12) for 18 hr. The paper strip was cut into 1.25-cm wide segments and counted in 10 ml of diluted Liquifluor (Packard) with a liquid scintillation counter. The paper segments under radioactive peaks were combined, washed thoroughly with toluene, and then extracted with 1 M aqueous pyridine. The lyophilized [^{14}C]peptides were again successively analyzed in a similar manner by paper electrophoresis in pyridine–acetic acid–water (pH 3.6) and in pyridine–acetic acid–water (pH 6.5) (Bennett, 1967). The homogeneity of the [^{14}C]peptides was tested on cellulose thin-layer chromatography in 1-butanol–pyridine–HOAc–H_2O. Ninhydrin, hypochlorite, and Ehrlich's reagent (Easley, 1965) were used to visualize peptide spots. The peptides were identified from the amino acid composition with the known sequences elucidated by Brew *et al.* (1967) for α-LA and by Canfield (1963) and Jollès *et al.* (1963) for chicken lysozyme.

TABLE I: Comparison of Amino Acid Composition of the Purified α-Lactalbumin and Its Modification Products.[a]

	α-Lactalbumin			Derivatives		
	Found	Lit.[b]	Theory[c]	I[d]	II[e]	III[f]
Aspartic acid	21.7	21.8	21	21.0	21.5	21.2
Threonine	6.0	7.0	7	6.0	6.2	6.0
Serine	5.7	7.2	7	5.7	5.4	5.5
Glutamic acid	13.9	13.6	13	14.2	13.6	13.5
Proline	1.7	2.1	2	1.6	1.6	1.9
Glycine	5.8	6.6	6	*25.6*	*25.8*	*25.3*
Alanine	3.0	3.7	3	3.0	3.0	3.0
Half-cystine	3.7[g]	8.2[i]	8	3.6	3.9	3.7
Valine	5.8	6.2	6	5.9	5.8	5.9
Methionine	1.1	1.0	1	1.0	1.1	1.1
Isoleucine	7.6	8.1	8	7.7	7.7	7.6
Leucine	13.8	13.7	13	13.3	13.4	13.7
Tyrosine	3.9	4.6	4	*2.8*	*2.0*	*3.8*
Phenylalanine	4.0	4.2	4	4.1	4.0	4.1
Lysine	12.4	12.2	12	12.0		
Histidine	3.0	2.9	3	2.8		
Arginine	1.0	1.0	1	0.9		
Tryptophan[h]	3.8	5.3	4	3.9		

[a] Values are expressed as molar ratios normalized to a value of 3.0 for alanine. [b] Gordon and Ziegler (1955). [c] Brew *et al.* (1967). [d] α-LA was treated with EDC–glycinamide for 400 min, and the major fraction obtained from chromatography on sulfoethyl-Sephadex C-25 was analyzed. [e] α-LA was treated with EDC–glycinamide for 400 min in the presence of 4 M guanidine hydrochloride. [f] The purified major fraction of the modified α-LA was treated with 0.75 M NH_2OH for 1.5 hr at pH 6.5 and 25°, and dialyzed. [g] Cystic acid obtained from the performic acid oxidized α-LA and its modified derivative III was found to be 7.1 and 7.5 residues per molecule (uncorrected for decomposition by acid hydrolysis), respectively. [h] Tryptophan was determined by the method of Barman and Koshland (1967) with 2-hydroxy-5-nitrobenzyl bromide. [i] Estimated as cystic acid (Gordon and Ziegler, 1955).

[^{14}C]Glycinamide with a specific radioactivity of 22.4 × 10^3 cpm/μmole was prepared from ^{14}C-labeled glycine (New England Nuclear) through methyl esterification and amination (Smith and Slonim, 1948). Guanidine was obtained from Mann Research Laboratories in the form of hydrochloride.

Spectral Measurements. Ultraviolet absorption spectra and difference spectra were recorded with a Cary 14 recording spectrophotometer. Optical rotatory dispersion and circular dichroism spectra were measured with a Cary 60 recording spectropolarimeter and expressed as degrees centimeters squared per decimole.

Results

Reaction of EDC and Glycinamide with α-LA at pH 4.75 and 7.0. In Figure 2a the extent to which the carboxyl groups in α-LA were modified at pH 4.75 and 7.0 is plotted against the reaction time. The effect of the modification on the activity of α-LA is also illustrated. Comparison of the initial rates of inactivation and modification (the pseudo-first-order rate constants: 12.0 × 10^{-2} and 5.0 × 10^{-2} min^{-1}, respectively) suggests a close correlationship between the biological activity and integrity of carboxyl groups in α-LA. At both pH conditions, more than 90% of the lactose synthetase activity (B protein activity) was lost, while approximately 20 residues of carboxyl groups/molecule of α-LA were modified at pH 4.75 and 10 residues at pH 7.0, respectively, after 400-min reaction. About two to four residues of carboxyl groups were modified rapidly in the initial few minutes with concomitant loss of more than 50% activity.

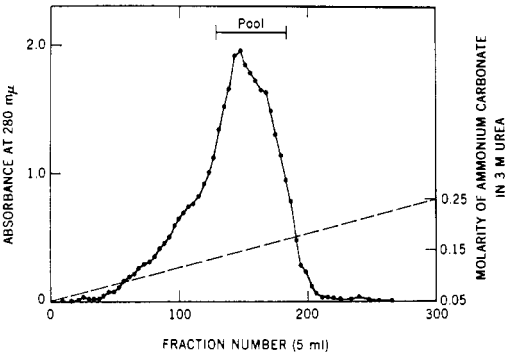

FIGURE 4: Chromatography on sulfoethyl-Sephadex C-25 (2.5 × 100 cm) of α-lactalbumin modified with EDC–glycinamide (500 mg) at 25°, pH 4.75, for 400 min. The column was equilibrated with 0.05 M ammonium carbonate buffer (pH 9.2) in 3 M urea, and eluted with a linear concentration gradient made of 750 ml of the equilibrium buffer and 750 ml of 0.45 M ammonium carbonate buffer (pH 9.2) in 3 M urea. The fractions marked were collected and dialyzed against water to eliminate urea and salts. The material was lyophilized, dissolved in the equilibrium buffer, and subjected to rechromatography.

The corresponding effect of carboxyl modification on the ability of α-LA to inhibit *N*-acetyllactosamine synthesis catalyzed by A protein is shown in Figure 3. Under the condition of assay the native α-LA inhibited about 70% of the A protein activity, while modification of carboxyl groups caused loss of the inhibitory effect. The product from a 400-min period of reaction showed no effect on the A-protein activity.

Although no information on the exact number of free carboxyl groups in α-LA molecule is available at the present time (Brew *et al.*, 1967), from the number of carboxyl groups that can be modified by carbodiimide reagent in 4 M guanidine the number appears to be approximately 19 ± 1 residues/molecule (Table I). Thus, at pH 4.75 almost all carboxyl groups in α-LA appeared to be modified within 400 min.

As illustrated in Figure 2b, a similar experiment conducted on chicken egg-white lysozyme confirmed the previous observation with glycine methyl ester as the modifying nucleophile (Lin and Koshland, 1969) that complete inactivation can be attained after about 200-min treatment at pH 4.75, and an average of 7.5 residues among 11 carboxyl groups present in lysozyme were modified in a 400-min period of reaction. The pseudo-first-order rate constants for the initial phases of inactivation and of carboxyl modification were 4.6 × 10^{-2} and 2.9 × 10^{-2} min^{-1}, respectively. Modification

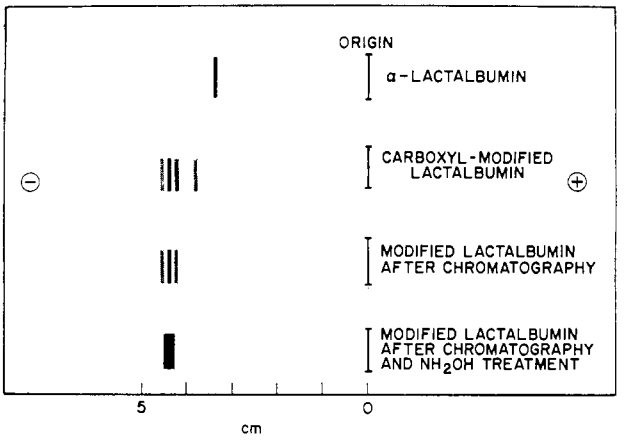

FIGURE 5: Electrophoresis of α-lactalbumin and its carboxyl-modified derivatives on starch gel. The gel was prepared from 14% Connaught starch suspension in 0.01 M aluminum lactate adjusted to pH 3.3 with lactic acid. The gel was stained with 0.1% Nigrosin in 1% HOAc in methanol for 5 min and then washed with 5% HOAc in methanol. About 40 μg of each protein preparation was analyzed at 12 V/cm, 3 hr at 23°.

at pH 7, however, produced a much less effective result; more than 80% of the native activity remained, while 2.5 residues out of 11 carboxyl groups were modified after reacting for 400 min.

Characterization of Modification Products. In order to study further the structure–function relationship relevant to carboxyl side chains, a modification reaction on an expanded scale (500 mg of α-LA) was carried out for 400 min. The reaction was arrested by addition of five volumes of sodium acetate and the mixture was dialyzed exhaustively against water at 4° and lyophilized. The modified protein was then chromatographed on a sulfoethyl-Sephadex C-25 column which was previously equilibrated with 0.05 M ammonium carbonate buffer (pH 9.2) in 3 M urea by a linear gradient made of 750 ml of the equilibrium buffer and 750 ml of 0.45 M ammonium carbonate buffer (pH 9.2) in 3 M urea (Figure 4). After rechromatography, the major component was dialyzed against water and lyophilized. This material showed a single major band on the starch gel electrophoresis in pH 3.3 aluminum lactate buffer (Figure 5).

Table I lists the amino acid compositions of the native α-LA as well as various preparations of the carboxyl-modified protein. Besides the expected increase in the amounts of glycine, other amino acids except tyrosine were shown to be unaffected by the carboxyl modification. Loss of tyrosine residues was conspicuous, but their recovery to the level of the native protein could be achieved by treatment of the modified protein with hydroxylamine as described by Carraway and Koshland (1968). Nevertheless, such treatment did not give the modified protein the activity of lactose synthesis. The glycine residues incorporated into α-LA were the same in the presence or absence of 4 M guanidine; this is an interesting contrast to the result of a similar modification on chicken lysozyme in which at least two carboxyl groups were unavailable to react with the modifier unless denaturating the protein with guanidine.

Since the modifying nucleophile, glycinamide, converts the carboxyl groups to $C(=O)NHCH_2C(=O)NH_2$ derivatives

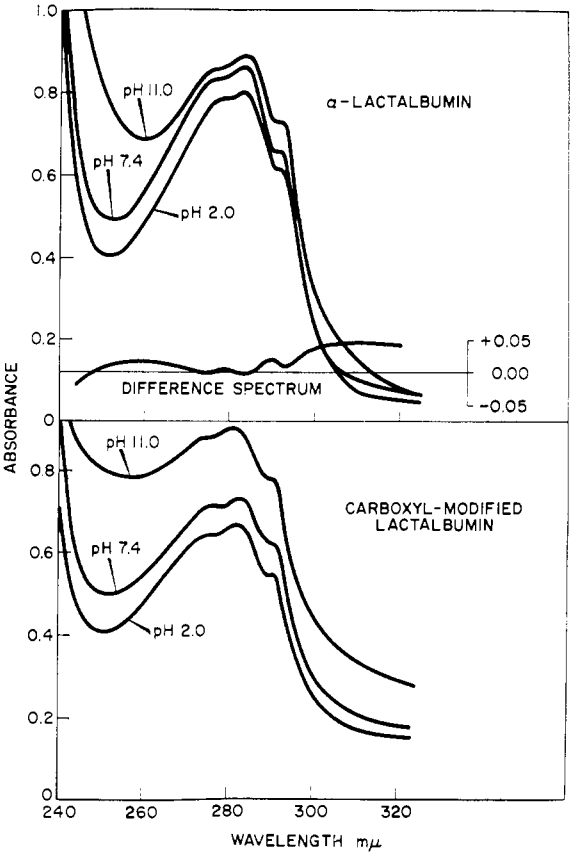

FIGURE 6: Ultraviolet spectra and difference spectra of α-lactalbumin and its carboxyl-modified derivative. The difference spectrum was obtained with the solutions of α-LA and the modified protein, both having A_{280} 0.645 (2.2×10^{-5} M for the former and 2.1×10^{-5} M for the latter) at pH 7.4 in potassium phosphate buffer (0.2 M). α-LA was in the reference cuvet. α-LA was modified with EDC–glycinamide, chromatographed on sulfoethyl-Sephadex C-25, and treated with NH_2OH. Spectra were taken with a Cary Model 14 spectrophotometer at 24°. ϵ_{280} for the native and the modified α-LA are 2.9×10^4 and 3.0×10^4, respectively. $\Delta\epsilon_{290}$ was found to be approximately +720.

with reduction of the net negative charge and an increase in size, α-LA modified with aminomethanesulfonic acid (AMS) was also prepared for comparison. AMS converts the carboxyls into $C(=O)NHCH_2SO_3^-$ form with retention of the net charge in the protein and with an increase in size. The modification was almost quantitative with 400-min reaction because further treatment of this modified protein with EDC and glycinamide in 4 M guanidine incorporated only 0.4 residue of glycine/molecule of protein. The AMS-modified α-LA was found to be functionally inactive. In contrast to using glycinamide, in this case the hydroxylamine treatment could not give satisfactory recovery in tyrosine (2.1 residues/molecule).

The lysine residues detected upon acid hydrolysis of the purified α-LA derivative and the native protein after deamination by nitrous acid (Anfinsen *et al.*, 1962) were 0.82 and 0.2 residue/molecule, respectively. Since α-LA contains 12 residues of lysine/molecule, the possible formation of inter- or intrachain cross-linkage during the carboxyl modification reaction through lysine ε-amino groups must be insignificant.

TABLE II: Modification of Chicken Egg-White Lysozyme with EDC–Glycinamide.

Protein	Glycine Recovd after Acid Hydrolysis[a]	Carboxyl Groups Modified[a]
Native lysozyme	12.0	
Lysozyme modified for 400 min at pH 4.75	20.3	8.3
Lysozyme–glycinamide modified for 300 min at pH 4.75 in 4 M guanidine	23.2	11.2
Lysozyme modified for 400 min at pH 4.75 in 4 M guanidine	23.1	11.1

[a] In residues per molecule.

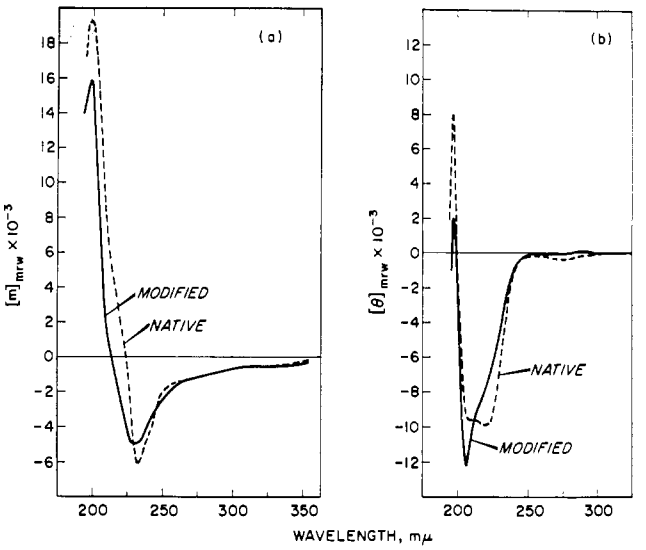

FIGURE 7: The optical rotatory dispersion (a) and the circular dichroism (b) spectra of α-lactalbumin and its carboxyl-modified (NH_2OH treated) derivative in potassium phosphate buffer (0.05 M pH 7.4) at 23°. Measurements were conducted in a Cary Model 60 spectropolarimeter with circular dichroic accessory using 1-, 10-, and 50-mm cells. Concentrations of both proteins were chosen to yield absorbance no greater than 1.4 at any wavelengths. Optical rotations and ellipticieties in degrees centimeters squared per decimole are plotted against wavelength. The mean residue weights of the native and the carboxyl-modified (with 19 residues of bound glycinamide) α-LA were taken to be 118 and 109, respectively.

A comparison of ultraviolet absorption spectra of the modified and the unmodified α-LA was made as shown in Figure 6. The small difference in the two spectra may suggest that the overall conformation of the protein and the relative configuration of the aromatic amino acid residues were not significantly affected by introducing glycinamide into the carboxyl groups. However, this supposition was not supported by an analysis of the optical rotatory dispersion and the circular dichroism spectra of the two proteins (Figure 7). The two optical rotatory dispersion spectra are quite similar except that some differences are noticed in the wavelength ranges 235–270 and 205–220 mμ, the region associated with rotational properties of the polypeptide backbone structure. The most significant difference was observed in the circular dichroism spectra: the negative band near 275 mμ was markedly diminished in the modified protein, and the flat region from 220 to 205 mμ exhibited by the native protein became steeper with a minimum at 207 mμ. While it is difficult at present to make an assignment of the chromophores or conformations responsible for either of the changes, the possibility of moderate change in the conformation of α-LA by chemical modification cannot be excluded.

Although as indicated in Table I, carboxyl groups in α-LA were easily modified with EDC–glycinamide, carboxyls in chicken lysozyme could not be quantitatively modified under the same conditions, unless the protein was denatured or the reaction carried out in the presence of guanidine (a concentration higher than 3.5 M). This particular behavior of lysozyme toward carbodiimide–AMS or carbodiimide–glycine methyl ester reagents has been described (Lin and Koshland, 1969). A comparable experiment with EDC–glycinamide reagent gave a similar result as shown in Table II.

Treatment of the Carboxyl-Modified Proteins with EDC–[^{14}C]Glycinamide in the Presence of 4 M Guanidine. The modified α-LA with 19 altered carboxyl groups and the modified lysozyme with 8.3 residues of carboxyl groups modified with glycinamide were further treated with EDC and ^{14}C-labeled glycinamide in the presence of 4 M guanidine at pH 4.75 for 300 min, and the amounts of [^{14}C]glycine introduced into each protein by the reagent were analyzed after dialysis and lyophilization. The results are summarized in Table III. The fact that only a small amount of ^{14}C was incorporated into the premodified α-LA substantiates the previous evidence that all carboxyl groups in α-LA can easily react with the modifier. On the contrary, more than two residues of carboxyls in lysozyme were not susceptible to the reagent.

In Tables IV and V the amino acid compositions and the radioactive specific activities of the ^{14}C-labeled peptides which were derived from the [^{14}C]glycinamide incorporated α-LA and lysozyme are shown, respectively. It is evident that the γ-carboxyl group of the glutamic acid residue 35 in

TABLE III: Complete Modification of Carboxyl Groups of α-Lactalbumin and Chicken Lysozyme in Two Stages.

Protein	Carboxyls Modified in 400 min; Purified and Treated with NH_2OH[a]	Carboxyls Modified in a Second Treatment with [^{14}C]Glycinamide–EDC and 4 M Guanidine[a]	
		By Amino Acid Anal.	By ^{14}C-Measurement
α-Lactalbumin	19.4	0.6	0.7
Chicken lysozyme	8.3	2.2	2.1

[a] In residues per molecule.

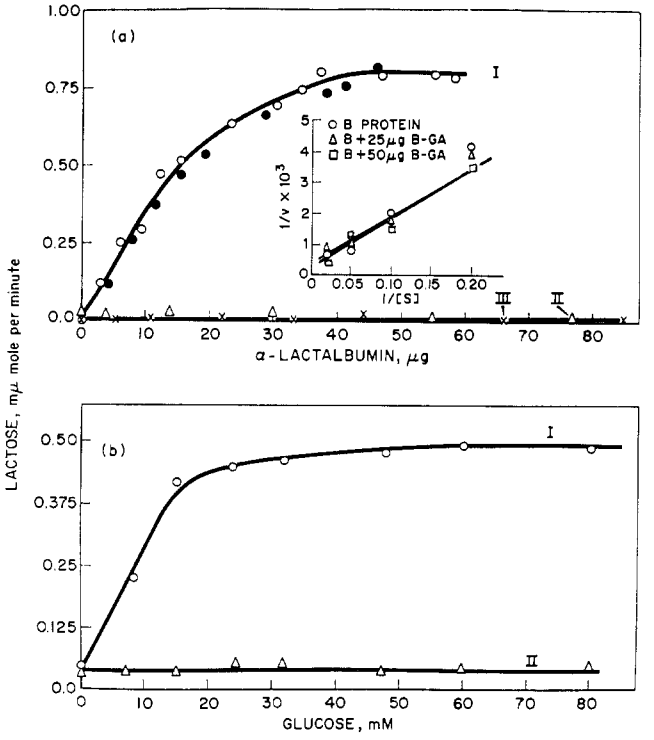

FIGURE 8: Effect of the amounts of modified and unmodified α-lactalbumin (a) and D-glucose concentrations (b) on the activity of lactose synthesis. The assays were carried out under the conditions described in Material and Methods except that the amount of α-LA or the concentration of D-glucose was varied. Before measurements, various amounts of an A protein fraction were tested on a specific amount of α-LA to ascertain the quantity of A fraction which can saturate α-LA for lactose synthesis. (I) Unmodified α-LA (B protein, —○— and —●— different experiments); (II) carboxyl-modified α-LA, chromatographed (B-GA, —△—); (III) carboxyl-modified α-LA after chromatography and NH_2OH treatment, —x—

TABLE IV: Amino Acid Compositions of [^{14}C]Glycinamide-Labeled Peptides Derived from Modified α-Lactalbumin and Chicken Lysozyme.[a]

	α-Lactalbumin		Chicken Lysozyme		
Amino Acid	L-1	L-2[b]	H-1	H-2	H-3
Aspartic acid	6.1 (6)	4.3 (5)	2.9 (3)	0.4[c]	2.1 (2)
Threonine	0.1	1.0 (1)	1.8 (2)	0.1	0.1
Serine	2.9 (3)	0.2	1.0 (1)	0.2	0.2
Glutamic acid	1.0 (1)	0.2	1.9 (2)	0.9 (1)	0.2
Proline	1.1 (1)				
Glycine	3.8 (4)	3.2 (3)	1.3 (1)	1.3 (1)	2.2 (2)
Alanine	0.1	0.1	1.0 (1)	2.8 (3)	0.2
Valine	0.1	1.1 (1)		0.1	
Methionine	0.1	0.9 (1)		0.9 (1)	
Isoleucine	1.9 (2)	0.8 (1)			0.1
Leucine	0.3	1.9 (2)		1.0 (1)	
Tyrosine					
Phenylalanine	0.1	1.0 (1)	1.9 (2)		
Lysine	1.1 (1)	1.1 (1)		1.0 (1)	
Histidine	0.9 (1)				
Arginine			1.0 (1)		1.0 (1)
Tryptophan					1.6[d] (2)
Carboxymethyl-cysteine	1.8 (2)	0.8 (1)		1.2 (1)	0.6[e] (1)

[a] Each column represents the results of a single analysis without correction for decomposition during hydrolysis. The molar ratios were calculated by normalizing to a value of 1.0 for glutamic acid (L-1), for alanine (H-1), for leucine (H-2), for arginine (H-3), and for phenylalanine (L-2), respectively. The figures in the parentheses are the theoretical values (nearest integer) for the assigned tryptic peptides (*cf.* Table V). [b] Another radioactive peptides with amino acid composition similar to that of residues 16–58 in α-LA was detected but not fully characterized. [c] Further purification of this peptide fraction removed a nonradioactive aspartic containing peptide. [d] The values were obtained from alkaline hydrolysate; the peptide showed strong positive reaction to Erhlich reagent. [e] The low value was probably due to loss of carboxymethylcysteinyl residues in the course of purification of peptides by paper electrophoresis on papers (Harris, 1967).

lysozyme can not react with the modifier; about 90% of this carboxyl group is still unmodified even after reaction for 400 min at pH 4.75. Moreover, partial shielding of the side chains of Asp-66 and Glu-7, which are all closely situated to the disulfide linkages, seems to be associated with the unique environment of the Glu-35 γ-carboxyl group. This observation confirms again the earlier result of carboxyl modification of lysozyme with glycine methyl ester or AMS as the nucleophiles (Lin and Koshland, 1969).

The assignment of the labeled peptides from α-LA on its primary structure depended entirely on the composition of peptides derived from the reduced and carboxymethylated α-LA through tryptic digestion. The sequences of these radioactive peptides were not determined due to the limited amount of peptides isolated. The enzymic cleavage product was not completely soluble in 1 M aqueous pyridine. However, when the whole material was applied on paper and subjected to paper electrophoresis, the major radioactivity resided in the mobile and pyridine-soluble fractions. Since none of the peptide fractions separated had a specific activity greater than 0.5, it appears that few carboxyls in this protein at pH 4.75 are buried and inaccessible to the modifying reagent.

Effect of Carboxyl-Modified α-LA on A Protein Activity. The effect of the carboxyl modification on the biological activity of α-LA was examined with the preparation of 400-min reaction. The chromatographically purified product did not catalyze the formation of lactose from D-glucose in the presence of UDP-D-galactose and A protein when tested with various amounts of the modified α-LA and glucose (Figure 8). The modified protein also lost the capacity to inhibit *N*-acetyl-D-glucosamine-UDP-D-galactose galactosyl transferase activity of A protein (Figure 9).

Treatment of the modified α-LA with hydroxylamine did not restore the functional activity. Thus, the partial modification of tyrosine residues by EDC did not contribute to the loss in the activity.

It is apparent that maximal capability of A protein to

TABLE V: Specific Radioactivity and Possible Incorporated Sites in the Primary Structures of α-Lactalbumin and Chicken Lysozyme.

	Modified α-Lactalbumin (0.7 [^{14}C]Glycinamide/Molecule)		Modified Chicken Lysozyme (2.1 [^{14}C]Glycinamide/Molecule)		
	L-1	L-2[a]	H-2		
Possible radioactive site[a]	63–79	80–93	34–45	6–13	62–68
Possible carboxyl residue modified with [^{14}C]glycinamide[b]	Asp-66 Asp-64 Asp-78	Asx-82 Asx-83 Asx-84 Asx-87 Asx-88	Glu-35	Glu-7	Asp-66
Specific radioactivity of [^{14}C]glycinamide residue per peptide	0.3	0.1	0.92	0.44	0.54

[a] Another radioactive peptide which was not fully identified possessed an amino acid composition close to that of residues 16–58 in α-LA. The specific radioactivity was about 0.1 mole of [^{14}C]glycine/molecule of peptide as residues 16–58. [b] Identification was made by comparison of amino acid composition with the known sequences of lysozyme and α-lactalbumin. The residues which have not been completely characterized as aspartyls are indicated as Asx (Brew *et al.*, 1967). The indicated regions in the native lysozyme sequence contain a single carboxyl side chain, respectively.

utilize D-glucose as the galactosyl transferase acceptor could not be attained other than by its interaction with α-LA. For example, acetamide (up to 45 mM) together with D-glucose (18 mM) could not exert the function of α-LA. Since α-LA modified with EDC–AMS did not exhibit activity, the change in the overall charges on α-LA due to carboxyl modification with EDC–glycinamide might not be the major cause for the loss of lactose synthetase activity. However, despite the carboxyl-modified chicken lysozyme competing with the native lysozyme to bind the cell wall substrate (see also Frieden, 1956), the carboxyl-modified α-LA did not affect the reaction of lactose formation catalyzed by A protein and α-LA (Figure 8).

Effect of Sugars and A Protein Fraction on Carboxyl Modification of α-LA. The activity of α-LA to promote synthesis of lactose could not be protected from the loss of activity due to carboxyl modification by including in the reaction systems the following sugars: D-glucose, lactose, D-galactose, *N*-acetyl-D-glucosamine, or cellobiose. Concentrations of these sugars were tested up to 1 M. With 0.05 M UDP-D-galactose in the reaction system, the modified protein was as inactive as the carboxyl-modified α-LA obtained by the modifying reagent alone.

In their study of milk lactose synthetase complex without separation of A and B components, Babad and Hassid (1966) reported the optimal pH for lactose synthesis to be 7.4. At this pH it would be expected that A and B (α-LA) proteins would have the best productive association for the synthesis of lactose in the presence of UDP-D-galactose, D-glucose, and $MnCl_2$. When the effect of pH on the activity of α-LA to promote lactose synthesis in the presence of A protein was examined, the optimal activity was also found to be in the range of pH 7.2–7.4. At pH 5.5, 35% of the optimal activity was found; 54% at pH 6.5; 68% at pH 8.0; and 35% at pH 8.5.

Hence, attempts were made to modify α-LA with EDC–glycinamide at pH 7.0 in the presence of an A protein fraction containing A protein activity twice that of the saturation level for the amount of α-LA. The modified α-LA retained some B protein activity, but evaluation of the result of protection was ambiguous, since as mentioned before the rate of carboxyl modification at pH 7 was sluggish and the reaction incomplete within 400 min.

Therefore, despite the lactose synthetase activity at pH 5 being less than 30% of that at pH 7.4, α-LA was then subjected to reaction with EDC–glycinamide in the presence of the A protein fraction, D-glucose (0.1 M), and $MnCl_2$ at pH 5.0 with an appropriate control run as described in Methods. The materials were treated with the normal procedure and chromatographed on a Bio-Gel P-30 column with 20 mM Tris-HCl–5 mM $MgCl_2$ and pH 7.4. Some residual activity was detected in the α-LA modified in the presence of A protein fraction (Figure 10). Each modified α-LA preparation was further subjected to chromatography on a Bio-Gel column until a single component was obtained on the starch gel electrophoresis in aluminum lactate buffer (pH 3.3). The two preparations were practically identical in their electrophoretic behavior. However, when these proteins were measured for the activity of lactose synthesis, approximately 15–20% of the original native α-LA activity was detected in the A-protein-treated modified α-LA in comparison to less than 5% for the modified protein of the control experiment (Figure 11). Acid hydrolysis of these proteins indicated that 20.3 ± 1.0 residues of glycine were present in the A-protein-treated modified α-LA, whereas 24.6 ± 0.9 residues were in the α-LA modified along with the denatured A protein fraction. Inasmuch as 6 residues of glycine are present in the native α-LA, and approximately 25.5 residues of glycinamide could be incorporated into this protein by EDC in the presence of 4 M guanidine, it seems likely that by interaction with A protein during the reaction roughly 4 residues of carboxyl groups in α-LA became less susceptible to the modifying reagent with

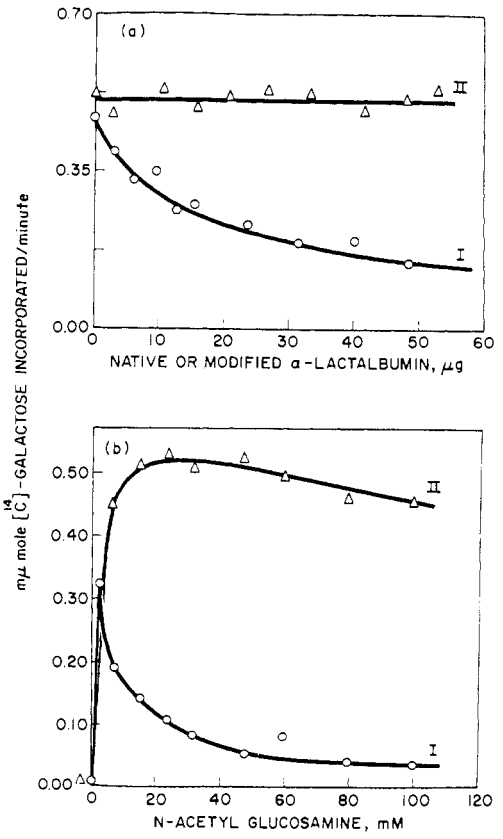

FIGURE 9: Effects of the native and the carboxyl-modified α-lactalbumin on the UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase activity. (a) The activity in the presence of various quantities of the native and the modified α-LA; (b) the effect of concentration of *N*-acetyl-D-glucosamine. The conditions of the assay were as described in Material and Methods. (I) Native α-LA, —○—; in (b), 78 μg; (II) carboxyl-modified α-LA, —△—; in (b), 79 μg.

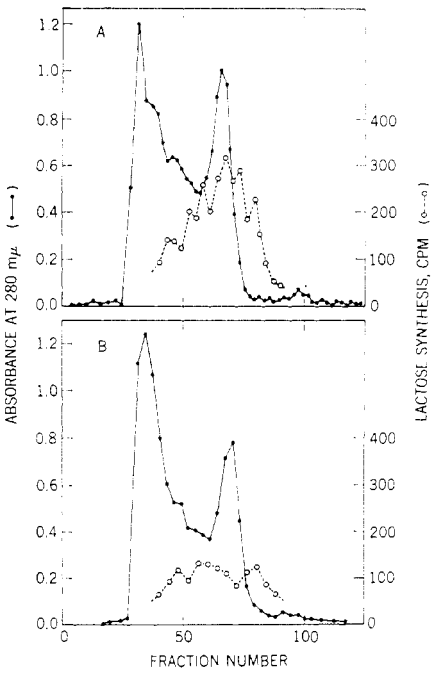

FIGURE 10: Gel filtration on Bio-Gel P-30 (2.5 × 100 cm) of the α-lactalbumin treated with EDC–glycinamide in the presence of A protein fraction, D-glucose (0.2 M) and $MnCl_2$ (0.1 M), at pH 4.75 (A), and in the presence of A protein fraction previously inactivated with EDC–glycinamide (B). The reaction conditions were described in Material and Methods; 20 mg each of α-LA and 7.5 ml of A protein fraction were used; 25 μl of the A protein fraction saturated at least 50 μg of α-LA when lactose synthetase activity was tested. After the reaction the mixtures were first dialyzed exhaustively against water, then lyophilized, dissolved in 2.5 ml of 20 mM Tris-HCl–5 mM $MgCl_2$ buffer (pH 7.4), and applied on the column. The gels were equilibrated and chromatographed with the same buffer. Fractions of 5.5 ml were collected. For measurement of lactose synthetase activity 0.2-ml aliquots were taken. In the figure the background countings were not subtracted. The fractions underlined were pooled, dialyzed, lyophilized, and rechromatographed.

concomitant retention of 15% of the native activity. Further treatment of this partially active modified protein with EDC–glycinamide caused complete loss of the activity.

Discussion

Although considerable progress in the knowledge regarding the biosynthesis of lactose has been made within the past few years (Watkins and Hassid, 1962; Babad and Hassid, 1966; Brodbeck *et al.*, 1967; Brew *et al.*, 1968), the detailed mechanism of the synthesis through interaction of two protein parts, A and B (α-LA) proteins, is still unknown. This process may be analogous to the well-defined two-component enzymes such as *Escherichia coli* tryptophan synthetase (Creighton and Yanofsky, 1966; Goldberg *et al.*, 1966), and these two component proteins may separately catalyze the parts of the overall reaction

$$\text{UDP-D-galactose} + X \rightleftharpoons \text{D-galactosyl-X} + \text{UDP} \quad (1)$$

$$\text{galactosyl-X} + \text{D-glucose} \rightleftharpoons \text{lactose} + X \quad (2)$$

Since A protein is a UDP-D-galactose-*N*-acetyl-D-glucosamine galactosyl transferase (Brew *et al.*, 1968; McGuire *et al.*, 1965), it is assumed to catalyze reaction 1 with X as *N*-acetyl-D-glucosamine residues or with an equivalent potential acceptor in the enzyme protein. Reaction 2 should be catalyzed by α-LA. This requires an assumption of two separate active sites in the two protein parts, which share a mutual intermediate. In view of the resemblance in the primary and the tertiary structures of α-LA and hen's egg-white lysozyme, this scheme appears to be attractive. Indeed, evidence has been advanced that chicken lysozyme can utilize D-glucose as the acceptor for transglycosylation reaction on *N*-acetyl-D-glucosamine oligosaccharide (Pollock and Sharon, 1969). Thus, if the conformation of α-LA retains the characteristics of the active-site cleft in lysozyme, the amino acid residues in α-LA which correspond to those in lysozyme, known to be essential to the catalysis or the substrate binding, such as Glu-35, Asp-52, and Trp-62, are likely involved in the function of lactose synthetase.

Assuming that His-32 in α-LA which is considered to be equivalent to Glu-35 of the homologous lysozyme sequence participates in the catalytic event concertedly with the possible Asp-52, it is expected that chemical modification of

these amino acid residues would destroy the activity. The present results show that carboxyl modification of α-LA produces a completely inert protein. However, carboxymethylation of α-LA at pH 5.5 or 7.5 does not cause total loss of the enzyme activity (T.-Y. Lin, P. L. Cecotti, and I. Bikel, unpublished data). Recently, Castellino and Hill (1969) reported similar results, concluding that the three histidine and the methionine residues are not completely essential for the function of α-LA. On the other hand, treatment of α-LA with hydroxynitrobenzyl bromide (Barman and Koshland, 1967) to modify tryptophanyl residues yields an inactive protein (T.-Y. Lin, unpublished data). Whether these residues are involved in binding of the substrates of A protein remains to be studied.

An alternative mechanism would be to assume that A protein itself may be capable of executing all the steps necessary for lactose synthesis, and that α-LA reacts with the enzyme as an allosteric effector either to modify the configuration of the active site to fit D-glucose or to create a new site for this sugar. The possibility of direct interaction of α-LA with the active site in A protein to provide a necessary functional group(s) for the D-glucose acceptor cannot be excluded. Brew *et al.* (1968) has already proposed that α-LA is a "specifier" protein controlling the specificity of the galactosyl acceptor for A protein.

Without available information regarding the structure and properties of the purified A protein, further consideration of the mechanism of lactose synthetase is limited. Acetamide which may form with D-glucose a configuration similar to that of *N*-acetyl-D-glucosamine did not affect or trigger the galactosyl transfer from UDP-D-galactose to D-glucose by A protein. Preliminary binding data obtained by equilibrium dialysis indicated that α-LA alone did not bind well for D-glucose and UDP-D-galactose (K_{assocn} in the order of 10^2 M^{-1} at pH 7.4, 4°, with 0.05 M Tris-HCl buffer). *N*-Acetyl-D-glucosamine and its oligosaccharide, lactose, D-galactose, and D-glucose with or without *N*-acetyl-D-glucosamine could not protect against the loss of activity due to chemical modification of carboxyl groups. These observations together with the evidence of partial protection of the α-LA function from modification by A protein suggests that some types of interaction between A and B protein components are essential for lactose synthesis. Nevertheless, Ebner *et al.* (1969) could not detect any stable complex between α-LA and the purified A protein. High levels of glucose were reported to stimulate lactose synthesis of A protein suggesting that glucose and α-LA may have similar effects on A protein. In fact, the protection experiment with A protein in the present study was of a preliminary nature; up to date, in no instance was the protection of the activity attained beyond 25% of the native lactose synthetic activity. Furthermore, the specificity of the protection by A protein has been difficult to establish because of the presence of contaminating proteins in the A protein fraction used. Before attempting to identify the site of protection in α-LA, further efforts are required to establish the conditions for maximum protection with the purified A protein.

The loss of lactose synthetase activity of α-LA as the consequence of carboxyl modification may be attributed to alteration of the functionally essential carboxyl residue(s) or to change caused in the conformation required for the activity. With present techniques, it is difficult to settle this

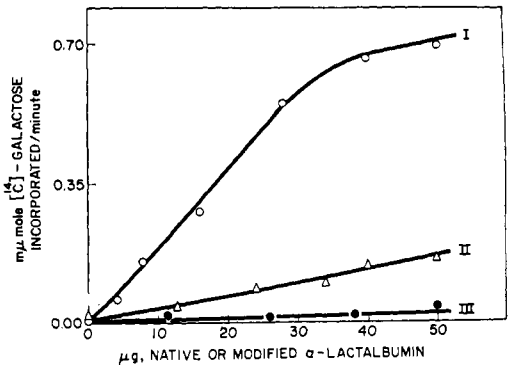

FIGURE 11: The residual lactose synthetase activity in the α-lactalbumin which was modified with EDC–glycinamide in the presence of A protein fraction. The conditions of assay for lactose synthetase were those described in Material and Methods. (I) α-LA, —○—; (II) carboxyl-modified α-LA protected by A protein fraction, —△—; (III) α-LA modified in the presence of A protein fraction previously treated with EDC–glycinamide, —●—

argument. The bulky modifying groups may also block the substrate from contact with the catalytic residue(s). Since partial protection of the activity by A protein prevented less than four carboxyl groups from modification with formation of a modified α-LA nearly indistinguishable from the unprotected modified protein, the overall conformational factor seems less important. However, the difference observed in the circular dichroic spectra of the native and the carboxyl-modified α-LA cannot be ignored. In fact, the small absorption spectral change occurring in the 290-mμ region due to modification may well be attributed to more "exposed" tryptophan residues and to change in conformation around disulfide linkages (Kronman *et al.*, 1965). In addition to the effect of modification on the chromophoric side chains, the optical rotatory dispersion and circular dichroism spectral changes suggest some possible alteration in the peptide backbone conformation (Kronman, 1968).

The result obtained in this work clearly indicates that nearly all carboxyl groups in α-LA can be accessible to the modifying reagent. The two regions which are not completely modified with the reagent within 400 min are apparently close to disulfide bonds. Previous studies of hen's lysozyme have shown that carboxyls situated near disulfide linkages react relatively slowly with the modifying reagent. The most intriguing observations are as follows. (1) Glu-35 in lysozyme is almost quantitatively protected from the attack of EDC–glycinamide; the two carboxyls, Glu-7 and Asp-66, are partially blocked to the modifier. The interaction between Trp-108 and Glu-35 is supposedly attributed to the unique environment around residue 35 (Blake, 1967) which also possibly confines the conformation near -Cys_6-Cys_{127}- and -Cys_{64}-Cys_{80}- to a tight arrangement, shielding Glu-7 and Asp-66 from EDC–glycinamide. (2) One of the two major regions in α-LA structure where the carboxyl group(s) was incompletely modified contains the disulfide bridge -Cys_{61}-Cys_{77}- which corresponds to -Cys_{64}-Cys_{80}- in lysozyme (Hill *et al.*, 1969). In this tryptic peptide, residues 63–79, three aspartyl residues, Asp-78, -66, and -64, are present. However, the carboxyl side chains around the disulfide linkage -Cys_6-Cys_{127}- seemed to be converted easily by the modifying reagent and only low

specific radioactivity was found in the peptide residues 6–10 and in the tryptic peptide residues 115–122 derived from [^{14}C]glycinamide-labeled α-LA. Even the highest radioactive tryptic peptide, residues 63–79, had a specific activity of 0.3 mole of [^{14}C]glycinamide/molecule of peptide, which is lower than those found in the corresponding radioactive tryptic peptides obtained from the labeled modified lysozyme. Although the environment near the two disulfide bonds mentioned above in α-LA may be similar to that of the corresponding region in the lysozyme, it seems likely that the spatial orientation in α-LA is relatively without restraint.

One of the several factors which limit the study on protection by A protein of carboxyl modification of α-LA was the sluggish reaction of modification at pH 7.0. Since no evidence was found to indicate formation of *N*-acylurea or other products of side reactions from the activated carboxyl by EDC, the possible cause of the slow reaction may be due to instability of the carboxyl–EDC adduct at pH 7 with a consequent inefficient attack by the nucleophile, glycinamide. One approach to avert this disadvantage would be to use another modification reagent, such as isoxazolium salt (Bodlander *et al.*, 1968), or to find a condition which stabilizes A protein during a longer reaction period. However, the instability of the carboxy–EDC intermediate at pH 7 itself cannot fully account for the slow loss of lysozyme activity in the modification performed at this pH. In contrast to α-LA which does not associate appreciably in the alkaline side of the isoelectric point (5.1) (Kronman *et al.*, 1964), the intermolecular association of lysozyme at pH 7 may be significant (Sophianopoulos and Van Holde, 1964; Bruzzesi *et al.*, 1965). Furthermore, in view of the work by Imoto *et al.* (1969) on the effect of pH on the formation of lysozyme–glycolchitin complex, a possible fine conformation change of the lysozyme molecule induced by a shift of pH from about 5 to 7 cannot be excluded. These two factors may form the environment around the catalytically important carboxy groups less accessible to the modifying reagent.

Browne *et al.* (1969) have recently constructed a model of α-LA on the basis of the main chain conformation established for lysozyme, and made detailed examination of the side-chain interactions in accordance with the side-chain replacements from lysozyme. The environments of the side chain were found to be such that the surface cleft is shorter in α-LA; the charged side chains are all located on the surface as found in lysozyme. While the result of this model study is consistent with the hypothesis that α-LA and lysozyme may have closely similar conformations, the model alone seems to be insufficient to provide an unequivocal explanation for the remarkable difference in the susceptibility of the carboxyl groups in these two proteins toward the carbodiimide reagent.

Acknowledgments

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Effect of 4,4-Dideuteration of Reduced Nicotinamide–Adenine Dinucleotide Phosphate on the Mixed Function Oxidases of Hepatic Microsomes*

Jordan L. Holtzman

ABSTRACT: It has been postulated for hepatic microsomal mixed-function oxidases that the electrons are transferred along an electron chain containing a flavoprotein, reduced nicotinamide–adenine dinucleotide phosphate–cytochrome *c* reductase, and the iron of cytochrome P-450. Sih, G. J., Tsong, Y. Y., and Stein, B. [(1968), *J. Amer. Chem. Soc. 90*, 5300] suggested, on the basis of kinetic evidence, that in the mitochondrial 11β-hydroxylase, reduced nicotinamide–adenine dinucleotide phosphate directly reduces the oxygen–oxygen bond. If such is the case, then the ratio of rates of hydroxylation for normal reduced nicotinamide–adenine dinucleotide phosphate to that of 4,4-dideuterio reduced nicotinamide–adenine dinucleotide phosphate (H:D) should be 2–3 due to the isotope effect of the deuterium. In hepatic microsomes the H:D is only 1.22 for ethylmorphine *N*-demethylase, 1.07 for aniline hydroxylase, and 1.12 for reduced nicotinamide–adenine dinucleotide phosphate–cytochrome P-450 reductase. The H:D of reduced nicotinamide–adenine dinucleotide phosphate–cytochrome *c* reductase is 2.00 when the deuterium is in the α position, but is 1.00 when it is in the β position. The H:D for endogenous and ethylmorphine-stimulated reduced nicotinamide–adenine dinucleotide phosphate oxidation were 0.98 and 1.18, respectively. Similarly for reduced nicotinamide–adenine dinucleotide phosphate stimulated oxygen uptake the H:D were 0.87 and 1.06, respectively. The failure to demonstrate a marked isotope effect clearly indicates that in the hepatic microsomes the proximal reducing agent of the oxygen–oxygen bond is an electron chain rather than a direct reduction by reduced nicotinamide–adenine dinucleotide phosphate.

In hepatic mixed function oxidase reactions, a molecule of oxygen, which is bound to both a substrate and cytochrome P-450, receives two electrons from a reducing agent, NADPH, to give a hydroxylated substrate and OH^-. At the present time the most widely accepted mechanism for this reaction postulates that the reduction occurs along an electron chain consisting of a flavoprotein, NADPH–cytochrome *c* reductase, and the cytochrome P-450 (Figure 1). This scheme for microsomes is supported by an extensive body of indirect evidence (Cooper *et al.*, 1965; Holtzman *et al.*, 1968). Further, Omura *et al.* (1966) have purified an analogous system from beef adrenal mitochondria which is capable of hydroxylating deoxycorticosterone in the 11β position but which differs from the microsomal system in requiring both a flavoprotein and a nonheme iron protein, in order to observe a NADPH–cytochrome *c* reductase activity.

Sih *et al.* (1968) have presented evidence for the 11β-hydroxylase system that the NADPH acts to donate a hydrogen to form a hydroperoxide rather than through an electron chain. This then rearranges to reduce the oxygen–oxygen bond. The modification of this proposal to the microsomal system is represented in Figure 2. Their arguments rest on three sets of observations. The first is that although it is possible to increase the concentration of the flavoprotein and adrenodoxin so that NADH will reduce the cytochrome P-450, it is not possible to observe hydroxylation with this cofactor. In order to achieve the same rate of reduction with NADH, they found it necessary to greatly increase the levels of the reductase and adrenodoxin. Yet, the limiting factor in the reduction is the low affinity of NADH for the flavoprotein, and if the concentration of NADH is increased, normal activity results. Secondly (D. Y. Cooper, personnel communication) they suggest that the scheme originally

* From the Laboratory of Pharmacology, Baltimore Cancer Research Center, National Cancer Institute, National Institutes of Health, Baltimore, Maryland 21211. *Received October 13, 1969.*