Involvement of Histidine-32 in the Biological Activity of α-Lactalbumin†

Jean-Paul Prieels,‡ John E. Bell,§ Melvin Schindler,¶ Francis J. Castellino,‖ and Robert L. Hill*

ABSTRACT: Reaction of either native or carboxymethylated bovine and human α-lactalbumins with diethyl pyrocarbonate at pH 6.1 results in the ethoxyformylation of up to two histidine residues in the bovine and one histidine residue in the human protein. At low concentrations (1 mg/mL) of bovine α-lactalbumin, modification of up to one histidine residue per molecule did not affect biological activity; however, modification of a second residue resulted in complete loss of activity. At higher concentrations (46 mg/mL), modification of a single histidine residue resulted in complete loss of activity. Carboxymethylated bovine α-lactalbumin, containing primarily 1,3-dicarboxymethylhistidine-68 and 3-carboxymethylhistidine-32, was found to incorporate one ethoxyformyl group per molecule on reaction with diethyl pyrocarbonate, and the modified derivative was totally inactivated. Proton magnetic resonance spectroscopy indicated that only histidine-32 was ethoxyformylated under conditions in which the biological activity of either native or carboxymethylated bovine α-lactalbumin was lost. Ultraviolet and circular dichroic difference spectra of either native or carboxymethylated bovine α-lactalbumin indicated that ethoxyformylation produced no gross conformational changes. Specificity studies with model compounds including histidine and its *N*-methyl and *N*-carboxymethyl derivatives indicated that the N(1) nitrogen of the imidazole group is specifically ethoxyformylated by diethyl pyrocarbonate. These studies, in conjunction with those on α-lactalbumin and its carboxymethyl derivatives, suggest that the N(1) nitrogen of the imidazole group of histidine-32 in bovine α-lactalbumin is intimately involved in the interaction of α-lactalbumin with UDP-galactose *N*-acetylglucosamine β1→4-galactosyltransferase in the formation of lactose synthase.

It is well established that α-lactalbumin forms a unique complex with UDP-galactose *N*-acetylglucosamine β1→4-galactosyltransferase (EC 2.4.1.22) to form lactose synthase during lactation in higher mammals (Brodbeck & Ebner, 1966; Brew et al., 1968; Hill & Brew, 1975; Brew & Hill, 1975). In the absence of exact knowledge of the three-dimensional structures of either α-lactalbumin, the transferase or the lactose synthase complex, little is known of the precise nature of the interaction of α-lactalbumin with galactosyltransferase. Previous chemical modification studies of α-lactalbumin have helped identify individual residues that may be involved in the interaction of α-lactalbumin with galactosyltransferase. Thus, modification of bovine α-lactalbumin with *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide has implicated tryptophan-26 and tryptophan-118 in this association (Bell et al., 1975). Similarly, histidine-32 has been shown to be essential for the association of human α-lactalbumin with galactosyltransferase by modification studies with diethyl pyrocarbonate (Schindler et al., 1976). In contrast carboxymethylation of bovine α-lactalbumin suggests that introduction of a carboxymethyl group on histidine-32 does not abolish the activity (Castellino & Hill, 1970).

Earlier studies (Pradel & Kassab, 1968; Melchior & Fahrney, 1970; Thomé-Beau et al., 1971; Tudball et al., 1972; Blumberg et al., 1973; Burstein et al., 1974; Lee et al., 1976) have shown that diethyl pyrocarbonate reacts with the side chains of cysteine, tyrosine, lysine, and histidine; however, this reagent can be particularly useful in modification studies of histidine since the formation of the ethoxyformylhistidine derivative can be monitored at 242 nm (Ovadi et al., 1967) and is deethoxyformylated by treatment with hydroxylamine (Mühlrâd et al., 1967). In addition, it has been suggested that ethoxyformylation takes place specifically at the N(1) nitrogen of the imidazole ring of histidine (Mühlrâd et al., 1967).

Since histidine-32 is present in all of the α-lactalbumins whose complete amino acid sequences have been established to date (Vanaman et al., 1970; Brew, 1972; Findlay & Brew, 1972), studies on the modification of bovine α-lactalbumin with diethyl pyrocarbonate have been performed in an attempt to reconcile the observation that histidine-32 in human α-lactalbumin is essential for its biological activity (Schindler et al., 1976), whereas carboxymethylation of histidine-32 in bovine α-lactalbumin did not totally abolish its ability to form lactose synthase (Castellino & Hill, 1970). In addition, to confirm the suggested specificity of diethyl pyrocarbonate for the N(1) nitrogen of histidine residues (Mühlrâd et al., 1967), the ethoxyformylation of a series of histidine derivatives has been studied. Analysis of the properties of α-lactalbumins modified by either diethyl pyrocarbonate or iodoacetate or both reagents suggests that ethoxyformylation of N-1 of the imidazole ring of histidine-32 destroys the activity of α-lactalbumin but that carboxymethylation of the N-3 ring nitrogen of the same histidine residue does not. These studies support the view that histidine-32 is involved in a specific interaction with the galactosyltransferase in lactose synthase.

Experimental Procedure

Materials. Bovine (Castellino & Hill, 1970) and human (Schindler et al., 1976) α-lactalbumins and the bovine galactosyltransferase (Bell et al., 1976) were prepared from fresh,

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, and the Chemistry Department, University of Notre Dame, Notre Dame, Indiana. *Received August 17, 1978; revised manuscript received January 31, 1979.* The studies reported here were supported by research grants from the National Heart and Lung Institute, National Institutes of Health (HL-06400), and the National Science Foundation. Dr. Jean-Paul Prieels was the recipient of a NATO postdoctoral fellowship, 1976, 1977.

* Department of Biochemistry, Duke University Medical Center, Durham, NC 27710.

‡ Laboratoire de Chimie Générale I, Faculté des Sciences, Université Libre de Bruxelles, B1050, Brussels, Belgium.

§ Department of Biochemistry, The University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642.

¶ Department of Microbiology, University of Connecticut, Health Center, Farmington, CT 06032.

‖ Chemistry Department, University of Notre Dame, Notre Dame, IN 46556.

0006-2960/79/0418-1771$01.00/0

raw, skim milk by published methods. Diethyl pyrocarbonate was obtained from Aldrich Chemical Corp. Iodoacetic acid was recrystallized from petroleum ether (bp 66–70 °C) and stored in the dark. [^{14}C]Iodoacetic acid (specific activity 12 mCi/mmol) was obtained from New England Nuclear Corp. 1-Methyl-L-histidine and 3-methyl-L-histidine were from Sigma Chemical Co. All other reagents were of the highest grade commercially available.

Carboxymethylation of α-Lactalbumin. Reaction of bovine (100 mg) and human α-lactalbumin (50 mg) with iodoacetate was performed as described previously (Castellino & Hill, 1970). After reaction at room temperature for 3 days in the dark at pH 6.5, excess reagents were removed by gel filtration on a column (2 × 20 cm) of Sephadex G-25 eluted with 2 mM ammonium bicarbonate. The protein fractions were pooled and, after lyophilization, dissolved in 5 mL of 20 mM sodium phosphate, pH 7.8. Separation of the reaction products was achieved by ion-exchange chromatography on a column (2 × 60 cm) of DEAE-Sephadex A-25 equilibrated with 20 mM sodium phosphate, pH 7.8. The column was developed with a linear gradient formed with the equilibration buffer and 0.4 M NaCl, which allowed the separation of the different reaction products. For both proteins the last eluting peak (respectively 58% and 50% of the total protein for human and bovine α-lactalbumin) was used for reaction with diethyl pyrocarbonate.

Synthesis of Carboxymethylated Derivatives of Histidine. 1-Carboxymethyl-, 3-carboxymethyl-, and 1-3-dicarboxymethylhistidine were obtained after reaction of iodoacetate with *N*-acetyl-L-histidine by published methods (Crestfield et al., 1963). Separation of the three derivatives was achieved by ion-exchange chromatography on a column (2.5 × 18 cm) of Dowex AG-50-X8 (200-400 mesh) equilibrated with 0.05 M pyridineacetic acid buffer, pH 2.55, and eluted with a linear gradient formed with equal volumes (250 mL) of equilibration buffer and 0.5 M pyridineacetic acid, pH 3.75.

Ethoxyformylation of α-Lactalbumin and Histidine Derivatives. Reaction of diethyl pyrocarbonate with α-lactalbumin or the histidine derivatives was performed in 50 mM sodium phosphate, pH 6.1, at room temperature as described previously (Schindler et al., 1976). The extent of reaction was measured by recording the increase in absorbance at 242 nm due to the formation of monoethoxyformylated histidine [E_M of 3200 cm^{-1} (Mühlrâd et al., 1967)].

Amino Acid Analysis. The extent of carboxymethylation of bovine and human α-lactalbumins was determined from the amino acid composition of acid hydrolysates (6 N HCl, 24 h, in vacuo) of α-lactalbumin derivatives. Each derivative was oxidized with performic acid prior to hydrolysis in order to calculate the exact amount of 3-carboxymethylhistidine, which emerges close to the cysteine peak during amino acid analysis. Amino acid compositions were determined on a Spinco Model 120B automatic amino acid analyzer as described previously (Castellino & Hill, 1970).

Enzymic Studies. The effect of carboxymethylation and ethoxyformylation of α-lactalbumin upon the ability of α-lactalbumin to enhance lactose synthesis or to inhibit *N*-acetyllactosamine synthesis was followed by measuring the incorporation of UDP-[^{14}C]galactose into glucose or *N*-acetyllactosamine as described previously (Bell et al., 1976).

Spectroscopic Studies. Proton magnetic resonance spectra of native and ethoxyformylated α-lactalbumin were obtained using a Varian XL-100-15 NMR spectrometer, operating at 100.1 MHz, locked on ^{2}H, and operating in the pulsed Fourier transform mode. At least 128 scans and 4000 data points were analyzed. A 1000-Hz spectral width and a pulse of 20 μs was used. The operating temperature was maintained with a Varian Model V-6040 temperature regulator. Ultraviolet difference spectra were recorded on a Cary Model 15 spectrophotometer using a 1-cm light path. Cell blanks were subtracted from the difference spectra. Spectra were routinely run with modified protein in the sample beam and native protein in the reference beam. Circular dichroic spectra were recorded on a Dichrographe III spectrophotometer (CNRS, Roussel-Jouan) using a 2-cm light path. Difference spectra were calculated from the appropriate pairs of spectra.

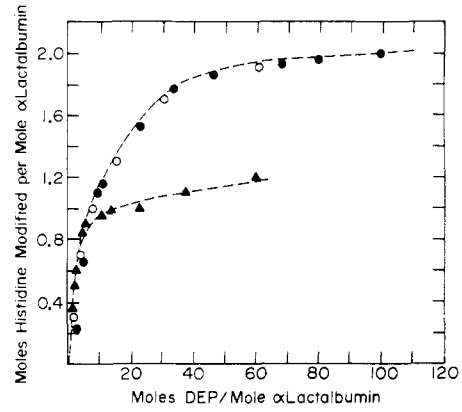

FIGURE 1: Extent of ethoxyformylation of the histidine residues in bovine and human α-lactalbumins as a function of the molar ratio of diethyl pyrocarbonate to α-lactalbumin. α-Lactalbumin (1 mL) in 0.05 M sodium phosphate buffer, pH 6.15, was mixed with diethyl pyrocarbonate in ethanol (40 μL) to give the appropriate molar ratio and incubated at 25 °C for 1 h. The absorbance change at 242 nm was monitored with time to estimate the number of histidine residues modified. Human α-lactalbumin, 1 mg/mL (▲); bovine α-lactalbumin, 1 mg/mL (●) and 46 mg/mL (○).

Results

The Extent of Modification of Human and Bovine α-Lactalbumin by Diethyl Pyrocarbonate and Identification of the Histidine Residues Modified. Figure 1 shows the extent of modification of the histidine residues in bovine and human α-lactalbumins by diethyl pyrocarbonate as judged by changes in absorption at 242 nm. Approximately one residue is modified in each α-lactalbumin at about a tenfold molar excess of diethyl pyrocarbonate to α-lactalbumin. On increasing the molar ratio of the reactants, no further modification of human α-lactalbumin was observed, although a maximum of two residues of histidine per molecule were modified in bovine α-lactalbumin.

The ethoxyformylhistidine residues in the α-lactalbumins formed on reaction with diethyl pyrocarbonate are too unstable to be easily identified by isolation and characterization of the modified histidine-containing peptides; thus, proton magnetic resonance spectroscopy was employed to identify which of the three histidine residues in bovine α-lactalbumin had reacted. Earlier studies had shown that histidine was the sole residue modified in human α-lactalbumin (Schindler et al., 1976). From previous spectral studies, it was possible to assign the proton resonances for each histidine residue (Bradbury & Norton, 1975). Figure 2 compares the NMR spectra of native, bovine α-lactalbumin, and α-lactalbumin modified at a molar ratio of diethyl pyrocarbonate to α-lactalbumin of 9:1 (46 mg of α-lactalbumin/mL), conditions that allow modification of only one histidine residue per molecule (Figure 1). These spectra indicate that histidine-32 (308 Hz) is substantially modified since it is almost completely removed from the spectrum. Histidine-107 (386–387 Hz) is essentially un-

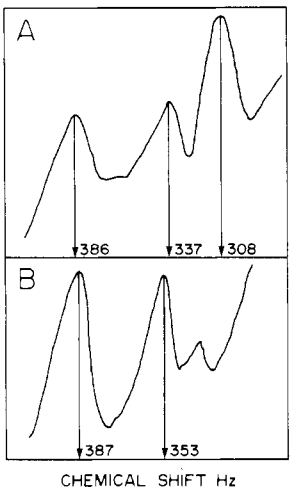

FIGURE 2: The proton NMR spectra of (A) native and (B) ethoxyformylated bovine α-lactalbumins. The ethoxyformylation was performed as described in Figure 1 with a ninefold molar ratio of diethyl pyrocarbonate; α-lactalbumin was at a final concentration of 46 mg/mL.

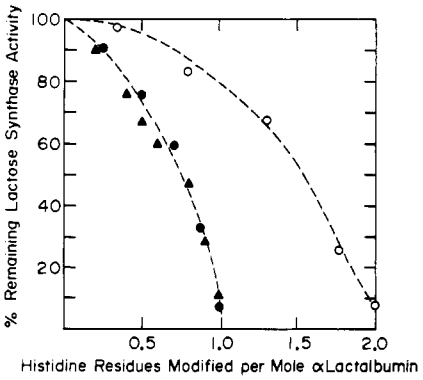

FIGURE 3: The lactose synthase activity of bovine and human α-lactalbumins as a function of the extent of ethoxyformylation. Lactose synthase activity was estimated as described earlier (Bell et al., 1976) in 0.05 M sodium cacodylate, pH 7.4, with 20 mM glucose, 40 mM $MnCl_2$, 0.2 mM UDP-galactose, and 0.25 mg/mL α-lactalbumin. Human α-lactalbumin, 1 mg/mL (▲), and bovine α-lactalbumin, 46 mg/mL (●) and 1 mg/mL (○), were reacted with diethyl pyrocarbonate as described in Figure 1 with varying ratios of diethyl pyrocarbonate:α-lactalbumin.

perturbed and histidine-68 shifts about 16 Hz (δ 337–353). This interpretation of the spectra is consistent with spectral studies on human α-lactalbumin, which does not contain histidine-68, but also shows the 308-Hz peak to be lost from the spectrum.

The Activity of Diethyl Pyrocarbonate Modified α-Lactalbumins. The ability of bovine and human α-lactalbumins to support lactose synthesis or to inhibit *N*-acetyllactosamine synthesis in the presence of UDP-galactose *N*-acetylglucosamine galactosyltransferase was examined. As shown in Figure 3, conditions that modify only one histidine residue per molecule in either α-lactalbumin lead to its inactivation. It is unclear why the extent of inactivation is not directly proportional to the extent of modification. Although no activity was found when one residue was modified, each of the modified derivatives was completely reactivated after incubation for 1 h at pH 7.5 with 200 mM hydroxylamine.

A different inactivation profile was obtained for bovine α-lactalbumin modified at 1 mg/mL, where a maximum of two residues of ethoxyformylhistidine are formed per molecule (Figure 3). Modification of one residue of histidine in bovine α-lactalbumin results in little inactivation, and only after two

Table I: The Histidine and Carboxymethylhistidine Contents of Bovine and Human α-Lactalbumin and Their Carboxymethyl Derivatives

	(residues/molecule)			
	bovine α-lactalbumin		human α-lactalbumin	
amino acid	native	CM-His derivative[a]	native	CM-His derivative[a]
histidine	3.0	0.85	2.0	1.0
3-CM-His	0	1.06	0	0.7
1,3-$(CM)_2$-His	0	0.85	0	0.1

[a] 1-Carboxymethylhistidine was not detected in either the human or bovine carboxymethyl-α-lactalbumins. The single methionine residue in bovine α-lactalbumin and the two methionine residues in human α-lactalbumin were fully carboxymethylated, but earlier studies (Castellino & Hill, 1970) showed that *S*-carboxymethylmethionyl-α-lactalbumins are fully active.

Table II: Kinetic Constants for Native and Carboxymethylated Bovine and Human α-Lactalbumins

	K_m(app) (mM)	rel V_{max} (arbitrary units)
bovine α-lactalbumin		
native	0.009	6.3
carboxymethylated	0.068	6.7
human α-lactalbumin		
native	0.0036	4.5
carboxymethylated	0.041	4.4

histidine residues are modified is the remaining activity lost.

Modification of Bovine Carboxymethyl-α-lactalbumin by Diethyl Pyrocarbonate. The foregoing results suggest that the pattern of ethoxyformylation of bovine α-lactalbumin varies with the concentration of α-lactalbumin. The spectral studies (Figure 2) clearly reveal that only histidine-32 has been modified in α-lactalbumin reacted at a 9:1 molar ratio of diethyl pyrocarbonate to protein at an α-lactalbumin concentration of 46 mg/mL. The resulting ethoxyformylhistidine-32 α-lactalbumin is inactive, whereas an active monoethoxyformyl-α-lactalbumin is formed on reaction of bovine α-lactalbumin with diethyl pyrocarbonate under the same conditions except at an α-lactalbumin concentration of 1 mg/mL. These results suggest that different histidine residues are modified by diethyl pyrocarbonate at the low (1 mg/mL) and high (46 mg/mL) concentrations of α-lactalbumin. Support for this conclusion was obtained by examination of the reaction of diethyl pyrocarbonate with carboxymethyl-α-lactalbumin derivatives. Bovine and human α-lactalbumins were carboxymethylated with iodoacetic acid, as described earlier (Castellino & Hill, 1970) and under Experimental Procedure. The histidine and carboxymethylhistidine contents of the carboxymethyl derivative obtained in greatest yield for each α-lactalbumin are listed in Table I. On the basis of earlier results, the major amounts of the 1,3-dicarboxymethylhistidine in bovine carboxymethyl-α-lactalbumin are derived from histidine-68 which reacts much faster than histidine-32. Thus, the 3-carboxymethylhistidine in this preparation is primarily histidine-32 in bovine as well as in human α-lactalbumin. Histidine-107 is not modified in either protein. In accord with earlier studies (Castellino & Hill, 1970), the carboxymethylated α-lactalbumins have lowered biological activities, which are reflected in the increased apparent K_m values listed in Table II. Reaction of bovine carboxymethyl-α-lactalbumin (0.7 mg/mL) with a 30-fold molar excess of diethyl pyrocarbonate resulted in modification of 0.8 residue of histidine, as judged by the change in ab-

Table III: The Nature of the Reaction of Diethyl Pyrocarbonate with Native and Carboxymethylated α-Lactalbumins and Histidine Derivatives

compound[a]	concn of compd (μM)	molar excess of diethyl pyrocarbonate	absorbance change at 242 nm		no. of His residues modified (residues/molecule)	fraction reacted (%)	half-life at 25 °C, pH 6.1 (h)
			theor	obsd			
bovine CM-α-lactalbumin	4.7	30	0.15	0.12	0.81		1.5
bovine α-lactalbumin (native)	5.6	34	0.36	0.35	1.9		>168
human CM-α-lactalbumin	4.4	30	0.14	0.13	0.9		16
human α-lactalbumin (native)	5.4	40	0.17	0.16	0.95		>168
His	2.8	24	0.90	1.1		120	>168
1-Me-His	2.1	31	0.67	0.41		60	0.25
3-Me-His	2.2	30	0.70	0.45		64	0.3
1-CM-His	2.8	24	0.90	0		0	
3-CM-His	2.4	28	0.77	0.56		73	0.4
1,3-$(CM)_2$-His	2.6	50	0.83	0		0	

[a] Histidine and the histidine derivatives were the L isomers.

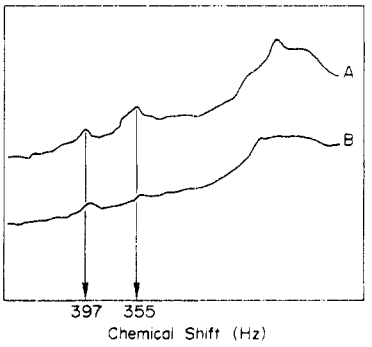

FIGURE 4: Proton NMR spectra of (A) carboxymethylated bovine α-lactalbumin and (B) ethoxyformylated, carboxymethylated α-lactalbumin. Ethoxyformylation was performed with a ninefold molar ratio of diethyl pyrocarbonate, with α-lactalbumin at a concentration of 37 mg/mL.

sorption at 242 nm (Table III) and complete loss of its residual biological activity. Nuclear magnetic resonance spectra of carboxymethylated bovine α-lactalbumin before and after ethoxyformylation are shown in Figure 4. The H_2 protons of histidines-107 and -32 are clearly seen in the carboxymethylated α-lactalbumin which contains 1 mol of 1,3-dicarboxymethylhistidine-68 and 1 mol of 3-carboxymethylhistidine-32 (curve A). The H_2 proton of histidine-68 is not observed due to the rapid exchange of the proton in the disubstituted histidine, as has been noted before (Olofson et al., 1964; Browne et al., 1969). After ethoxyformylation with a ninefold molar excess of diethyl pyrocarbonate, which by absorbance measurements at 242 nm contained 0.85 mol of modified histidine/mol of protein, the H_2 proton of histidine-32 is no longer observed in the spectrum (curve B). The resonance due to histidine-107, though broadened somewhat, is still clearly present in the spectrum, indicating that histidine-107 is not ethoxyformylated under these conditions. Since histidine-68 is unessential for the biological activity of bovine α-lactalbumin, this result suggests that inactivation of the carboxymethyl derivative by diethyl pyrocarbonate is due to ethoxyformylation of histidine-32 or 3-carboxymethylhistidine-32. Carboxymethylated human α-lactalbumin also incorporated about 0.9 residue of ethoxyformyl groups and resulted in a totally inactive derivative (Table III), in accord with earlier reports (Schindler et al., 1976).

Conformational Studies on Modified α-Lactalbumins. To monitor possible conformational changes in bovine and human α-lactalbumins on modification with either iodoacetic acid or diethyl pyrocarbonate, or both, ultraviolet and circular dichroic difference spectra were recorded in the 250–310-nm region.

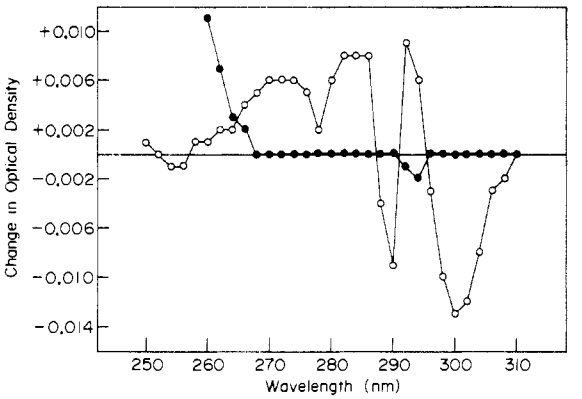

FIGURE 5: Ultraviolet difference spectra of carboxymethylated α-lactalbumin vs. native bovine α-lactalbumin (○—○); ethoxyformylated α-lactalbumin vs. native α-lactalbumin (●–●); and ethoxyformylated, carboxymethylated α-lactalbumin vs. carboxymethylated α-lactalbumin (●–●). All spectra at α-lactalbumin concentrations of 23 μM in 50 mM phosphate buffer, pH 6.1.

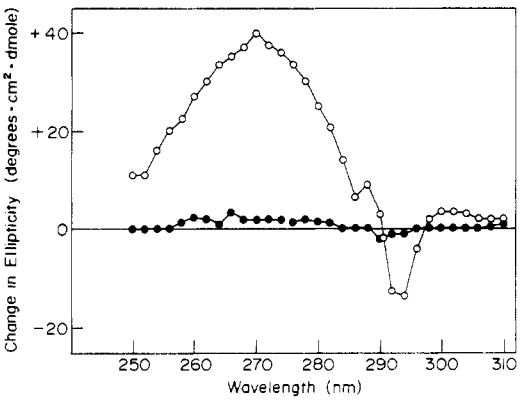

FIGURE 6: Circular dichroism difference spectra of carboxymethylated α-lactalbumin (○—○); ethoxyformylated α-lactalbumin vs. native α-lactalbumin (●—●); and ethoxyformylated, carboxymethylated α-lactalbumin vs. carboxymethylated α-lactalbumin (●—●). All spectra at α-lactalbumin concentrations of 23 μM in 50 mM phosphate buffer, pH 6.1.

The ultraviolet difference spectra (Figure 5) for carboxymethylated α-lactalbumin vs. native bovine α-lactalbumin indicated major changes in absorption at 272, 283, 290, 292, and 300 nm upon carboxymethylation. The ultraviolet difference spectra for ethoxyformylated α-lactalbumin vs. native α-lactalbumin, and for the ethoxyformylated, carboxymethyl-α-lactalbumin vs. carboxymethyl-α-lactalbumin, showed only minor absorbance changes in this region upon

ethoxyformylation. Similar results (not shown) were obtained with human α-lactalbumin.

Circular dichroic difference spectra indicated (Figure 6) major ellipticity changes upon carboxymethylation of native bovine α-lactalbumin at 270 nm (positive) and 294 nm (negative). Ethoxyformylation of either native or carboxymethyl-α-lactalbumin resulted in no significant changes in ellipticity in the 250–310-nm region. As with the ultraviolet difference spectra, similar results were obtained with human α-lactalbumin.

Reaction of Diethyl Pyrocarbonate with Histidine and Its N-Methyl and N-Carboxymethyl Derivatives. The foregoing studies on the reaction of carboxymethyl-α-lactalbumins with diethyl pyrocarbonate suggest that inactivation of α-lactalbumin by this reagent results from ethoxyformylation of the N-1 atom of the imidazole ring. Thus, it was of interest to examine the reactivity of histidine and its methylated and carboxymethylated derivatives with diethyl pyrocarbonate. The results obtained are listed in Table III. The extent of ethoxyformylation was estimated by measuring the increase in absorbance at 242 nm by assuming the extinction coefficient for monoethoxyformylhistidine [3200 cm^{-1} (Ovadi et al., 1967)]. Histidine, 1-methylhistidine, 3-methylhistidine, and 3-carboxymethylhistidine reacted to give from 60 to 120% of the theoretically expected monoethoxyformyl derivatives, which showed varying degrees of stability under the conditions used. These results indicate that diethyl pyrocarbonate can react with either the 1 or 3 N of the imidazole ring. However, the complete lack of reaction of diethyl pyrocarbonate with 1-carboxymethylhistidine and 1,3-dicarboxymethylhistidine implies that the N-1 nitrogen of the imidazole ring is preferentially modified in the carboxymethylhistidines, although perhaps not in the methylhistidines. This reaction specificity is in accord with the conclusion that the N-1 atom of the imidazole ring of histidine-32 is preferentially ethoxyformylated in both human and bovine α-lactalbumin.

Discussion

Although earlier studies (Pradel & Kassab, 1968; Melchior & Fahrney, 1970; Thomê-Beau et al., 1971; Tudball et al., 1972; Blumberg et al., 1973; Burstein et al., 1974; Lee et al., 1976) have shown that diethyl pyrocarbonate reacts with the side chains of cysteine, tyrosine, and lysine in addition to that of histidine, it is unlikely that reaction with side chains in α-lactalbumin other than histidine has occurred to any significant extent in the studies reported here. The protein is devoid of thiol groups and, since there was no change in the ultraviolet absorption spectrum of α-lactalbumin (from 260 to 290 nm) after reaction with diethyl pyrocarbonate, modification of tyrosine phenolic hydroxyl groups is unlikely. Ethoxyformylation of the ε-amino groups of lysine could have occurred, but, if so, such modification is of no significance because treatment of ethoxyformylated α-lactalbumin with hydroxylamine restores its activity, and, ethoxyformyl histidine, but not ethoxyformyllysine, is deethoxyformylated by this treatment (Mühlrâd et al., 1967). The specificity of diethyl pyrocarbonate for ethoxyformylation of the N-1 imidazole nitrogen atom of histidine has been noted earlier (Mühlrâd et al., 1967) and confirmed by the present studies. 1-Carboxymethylhistidine and 1,3-dicarboxymethylhistidine were unreactive with diethyl pyrocarbonate, whereas 3-carboxymethylhistidine, 1-methylhistidine, and 3-methylhistidine were reactive.

The studies reported here support the view (Schindler et al., 1974) that histidine-32 of human and bovine α-lactalbumin may play an important role in the interaction of α-lactalbumin and galactosyltransferase that leads to formation of lactose synthase (Brodbeck & Ebner, 1966; Brew et al., 1968; Hill & Brew, 1975; Brew & Hill, 1975). Moreover, they suggest that the N-1 atom of the imidazole ring of histidine-32 cannot be substituted without inactivation of the α-lactalbumin. Substitution of the N-3 ring atom of histidine-32 by a carboxymethyl group does not appear to lead to complete inactivation of α-lactalbumin since the carboxymethylhistidine derivatives of α-lactalbumin examined here and in earlier studies (Castellino & Hill, 1970) retained partial activity, and complete inactivation occurred only on ethoxyformylation of the unsubstituted N-1 ring atom. The partial inactivation observed on carboxymethylation of the N-3 nitrogen of histidine-32 may result from the apparent conformational changes resulting from carboxymethylation, as detected by ultraviolet and circular dichroic difference spectra studies. Alternatively, it may be that carboxymethylation of N-3 alters the properties of the N-1 nitrogen atom. The remarkably different effects on the activity of α-lactalbumin by substitution of the N(1) and N(3) atoms of histidine-32 suggest a high degree of specificity in the interaction between α-lactalbumin and the galactosyltransferase.

The total loss of function of α-lactalbumin on ethoxyformylation of the N-1 nitrogen of histidine-32 is a direct result of modification and not due to conformational changes induced in the protein by modification. Ethoxyformylation of neither native nor carboxymethylated α-lactalbumin produces any significant change in the optical properties of the aromatic residues in the protein as detected by difference spectroscopy, suggesting that the environment of these residues is not altered by ethoxyformylation. Both techniques used, however, show changes resulting from carboxymethylation, indicating that these techniques are sensitive to the environment of the aromatic residues and that carboxymethylation produces some conformational changes in α-lactalbumin.

Much has been written concerning the homology between α-lactalbumin and lysozyme (Warme et al., 1974; Browne et al., 1969), and it has been suggested that the cleft region of lysozyme, which could be partly conserved in α-lactalbumin, may provide part of the binding site for the sugar acceptor substrates of the galactosyltransferase. The studies reported here and some previous studies (Bell et al., 1975) indicate that this "cleft" region in α-lactalbumin may be intimately associated with the interaction between α-lactalbumin and galactosyltransferase. Histidine-32 should be located in this "cleft" region. While these two studies provide strong evidence that a "cleft" region of a α-lactalbumin is involved in its association with galactosyltransferase, they lend no support to the notion that α-lactalbumin may provide additional binding interactions with the sugar acceptor substrate. Currently there is no evidence from any type of chemical modification studies that this might be the case. It seems, therefore, far more likely that α-lactalbumin plays its unique role by perturbing enzyme–substrate equilibria in favor of increased substrate binding, as has been previously suggested on the basis of kinetic studies of the lactose synthase system (Bell et al., 1976).

Particularly noteworthy in the present studies is the difference in the reactivity of diethyl pyrocarbonate with the two reactive histidine residues in bovine α-lactalbumin. At low concentrations (1 mg/mL) of α-lactalbumin, the first residue ethoxyformylated is probably histidine-68, and its modification results in little loss of biological activity. Inactivation of α-lactalbumin occurs only when it is ethoxyformylated after modification of a second histidine residue, most likely histi-

dine-32. In contrast, only histidine-32 is ethoxyformylated on reaction of α-lactalbumin at high concentrations (37 mg/mL) with diethylpyrocarbonate. These differences in modification as a function of α-lactalbumin concentration probably reflect the fact that histidine-68, which is not essential for supporting the activity of α-lactalbumin and is, indeed, absent in human α-lactalbumin, cannot react because of the aggregation of α-lactalbumin at high protein concentrations (Kronman et al., 1967).

References

Bell, J. E., Castellino, F. J., Trayer, I. P., & Hill, R. L. (1975) *J. Biol. Chem. 250*, 7579–7585.
Bell, J. E., Beyer, T. A., & Hill, R. L. (1976) *J. Biol. Chem.* 251, 3003–3013.
Blumberg, S., Holmquist, B., & Vallee, B. L. (1973) *Biochem. Biophys. Res. Commun. 51*, 987–992.
Bradbury, J. H., & Norton, R. S. (1975) *Eur. J. Biochem. 53*, 387–396.
Brew, K. (1972) *Eur. J. Biochem. 27*, 341–353.
Brew, K., & Hill, R. L. (1975) *Rev. Physiol. Biochem. Pharmacol. 72*, 105–158.
Brew, K., Vanaman, T. C., & Hill, R. L. (1968) *Proc. Natl. Acad. Sci. U.S.A. 50*, 491–497.
Brew, K., Steinman, H. M., & Hill, R. L. (1973) *J. Biol. Chem.* 248, 4739–4742.
Brodbeck, U., & Ebner, K. E. (1966) *J. Biol. Chem. 241*, 762–764.
Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C., & Hill, R. L. (1969) *J. Mol. Biol.* 42, 65–86.
Burstein, Y., Walsh, K. A., & Neurath, H. (1974) *Biochemistry* 13, 205–210.
Castellino, F. J., & Hill, R. L. (1970) *J. Biol. Chem.* 245, 417–424.
Crestfield, A. M., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 2413–2420.
Findlay, J. B. C., & Brew, K. (1972) *Eur. J. Biochem. 27*, 65–86.
Hill, R. L., & Brew, K. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 411–490.
Kronman, J. J., Holmes, L. G., & Robbins, F. M. (1967) *Biochim. Biophys. Acta 133*, 46–55.
Lee, C. Y., Houston, L. L., & Himes, R. H. (1976) *Biochem. Biophys. Res. Commun. 70*, 50–57.
Melchior, W. B., & Fahrney, D. (1970) *Biochemistry 9*, 251–258.
Mühlrád, A., Hegui, G., & Tôth, G. (1967) *Acta Biochim. Biphys. Acad. Sci. Hung. 2*, 19–29.
Olofson, R. A., Thompson, W. R., & Michelman, J. S. (1964) *J. S.* (1964) *J. Am. Chem. Soc. 86*, 1865–1866.
Ovadi, J., Libor, S., & Elöd, P. (1967) *Acta. Biochim. Biophys. Acad. Sci. Hung. 2*, 455–458.
Pradel, L., & Kassab, R. (1968) *Biochim. Biophys. Acta 167*, 317–325.
Schindler, M., Sharon, N., & Prieels, J. P. (1976) *Biochem. Biophys. Res. Commun. 69*, 167–173.
Thomé-Beau, F., Lê-Thi-Lan, Olomucki, A., & Thoai, N. V. (1971) *Eur. J. Biochem. 19*, 270–275.
Tudball, N., Bailey-Wood, R., & Thomas, P. (1972) *Biochem. J. 129*, 419–425.
Vanaman, T. C., Brew, K., & Hill, R. L. (1970) *J. Biol. Chem. 245*, 4583–4590.
Warme, P. K., Momany, F. A., Romball, S. V., Tuttle, R. W., & Scheraga, H. A. (1974) *Biochemistry 13*, 768–781.

Synthesis of Herpes Simplex Virus DNA in Isolated Chromatin†

Karl-Werner Knopf‡

ABSTRACT: Herpes simplex virus DNA synthesis was studied in isolated chromatin (HSV chromatin) of African green monkey kidney (RC-37) cells after HSV type 1 infection. After optimizing the in vitro system, HSV chromatin was shown to synthesize both viral and cellular DNA at ratios identical with those seen in vivo. After 30 min of DNA synthesis in vitro, the DNA products were identical in size to the prelabeled parental DNA. More than 60% of the newly synthesized single-stranded DNA fragments sedimented with a sedimentation constant of greater than 10 S. HSV DNA polymerase was found to be responsible for the synthesis of 80% of all in vitro made viral and most likely also cellular DNA sequences.

In a previous report (Knopf & Weissbach, 1977), we have demonstrated that the properties and requirements of the chromatin system for DNA synthesis generally resemble those of isolated nuclei systems. Besides the fact that isolated chromatin synthesizes a larger amount of DNA, it furthermore seems to be attractive in that it functions as a natural template for exogenous DNA polymerase and in that no compartments hinder the free passage of macromolecules to the site of DNA synthesis. Therefore, it may provide a possible tool for examining the role of the known DNA polymerases and it also can lead to the discovery of proteins and factors required in DNA replication. In this report we tried to further characterize the system with the intention to show if some of the previously reported parameters for DNA synthesis can be extended to chromatin isolated from infected cells. As an experimental system, we chose the herpes virus infected cell, primarily because herpes simplex virus DNA replication occurs in the nucleus of the cells (Morgan et al., 1954) and also because HSV[1] induces its own DNA polymerase (Keir et al.,

† From the Institute for Virus Research, German Cancer Research Center, Heidelberg, West Germany. *Received March 28, 1978; revised manuscript received October 5, 1978.*

‡ Deutsches Krebsforschungszentrum, Institut für Virusforschung, Im Neuenheimer Feld 280, D-6900 Heidelberg, West Germany.

0006-2960/79/0418-1776$01.00/0