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ARTICLE *in* BIOCHEMISTRY · JULY 1988

Impact Factor: 3.02 · DOI: 10.1021/bi00415a031 · Source: PubMed

Published in Biochemistry, July 26, 1988, pp. 5601-5610, by the American Chemical Society

Accessibility and Mobility of Lysine Residues in β-Lactoglobulin

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Received July 16, 1987; Revised Manuscript Received February 18, 1988

ABSTRACT: N^{ϵ}-[$^2H_6$]Isopropyllysyl-β-lactoglobulin was prepared by reductive alkylation of β-lactoglobulin with [$^2H_6$]acetone and $NaBH_4$ to provide a ^2H (NMR) probe for the study of lysine involvement in lipid–protein interactions. Amino acid analysis showed 80% of the protein's 15 lysine residues to be labeled. Unmodified lysine residues were located through peptide maps produced from CNBr, tryptic, and chymotryptic digests of the labeled protein. Lys_{47} was not modified; $Lys_{135,138,141}$, located along an amphipathic helical rod, were each partially unmodified. All other lysine residues were at least 90% modified. Average correlation times calculated from ^2H NMR spectra were 20 and 320 ps for 8.7 and 3.3 residues, respectively, in 6 M guanidine hydrochloride; in nondenaturing solution, values of 70 and 320 ps were obtained for 6.5 and 3.2 residues, respectively, with the remaining 2.3 modified residues not observed, suggesting that side chains of lysine residues in unordered or flexible regions were more mobile than those in stable periodic structures. ^2H NMR spectra of the protein complexed with dipalmitoylphosphatidylcholine confirmed the extrinsic membrane protein type behavior of β-lactoglobulin previously reported from ^{31}P NMR studies of the phospholipids complexed with β-lactoglobulin. Although no physiological function has yet been identified, comparison of these results with the X-ray structure [Papiz et al. (1986) *Nature (London) 324*, 383–385] supports the hypothesis that residues not accessible for modification may help to stabilize the cone-shaped β-barrel thought to contain binding sites for small lipid-soluble molecules.

β-Lactoglobulin (β-lg),[1] the major whey protein of bovine milk, has been used extensively as a model for the study of the interactions of globular proteins. Its interactions in vitro include complex formation with retinol (Hemley et al., 1979; Fugate & Song, 1980) and other aromatics (Farrell et al., 1987) and specific binding of sodium dodecyl sulfate (Jones & Wilkinson, 1976; Hillquist-Damon & Kresheck, 1982), alkanes (Wishnia & Pinder, 1966), and guanidinium ions (Pace & Marshall, 1980). Binding of β-lg to triglycerides (Smith et al., 1983) and phospholipids (Brown et al., 1983) in a manner similar to that of the peripheral membrane protein myelin (Fraser & Deber, 1984) has also been reported. Recent comparisons which show partial sequence identity (Pervaiz & Brew, 1985; Godovac-Zimmermann et al., 1985) and similar crystal structure (Papiz et al., 1986; Sawyer et al., 1985; Newcomer et al., 1984) with human retinol binding protein suggest that β-lg may have had a role in ruminant nutrition as a carrier of small, lipid-soluble molecules such as vitamin

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[1] Abbreviations: β-lg, β-lactoglobulin; RCM-β-lg, disulfide-reduced, S-carboxymethylated β-lg; Ip-β-lg, isopropyllysyl-β-lg; RCM-Ip-β-lg, reduced, S-carboxymethylated Ip-β-lg; Ip-RCM-β-lg, reductively alkylated after reduction and S-carboxymethylation; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; TPCK-trypsin, trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; CNBr I, cyanogen bromide fragment of β-lg which eluted first on gel permeation chromatography; Ip CNBr I, cyanogen bromide fragment of Ip-β-lg which eluted first on gel permeation chromatography.

A; however, the actual physiological function is still not clear.

Specifically modified amino acid residues can function as probes in studies of protein structure and function. Lysine residues are frequently found in catalytic and allosteric sites as well as on the protein surface where they may enhance solubility or provide a site for the electrostatic attachment of peripheral membrane proteins to phospholipids. Reductive alkylation allows an isotopic label to be covalently bound to a lysine residue where it may be expected to act as a probe of protein structure. Means and Feeney (1968) developed a general alkylation technique using aliphatic aldehydes or ketones with sodium borohydride as the reducing agent. Jentoft and Dearborn (1979) developed an alternative method using [^{13}C]formaldehyde and cyanoborohydride to reductively methylate proteins for ^{13}C NMR studies. In either case, the reaction is specific, only ϵ- and α-amino groups are modified, and there is no change in the charge distribution on the amino group. Reaction conditions are mild, and extensive modification occurs with minimal disturbance to native conformation or biological activity. The level of modification may be determined by monitoring a radioactive label (Jentoft & Dearborn, 1979), by titrating the remaining amino groups with TNBS (Habeeb, 1966), or from an amino acid analysis. However, when the modification is less than stoichiometric, these methods provide little information about the locations of fully or partially modified residues. While varying levels of modification have been reported, the only assignment of specific modified residues has been based on ^{13}C NMR spectra (Jentoft et al., 1981; Gerken et al., 1982; Sherry & Teherani, 1983).

Using [$^2H_6$]acetone, we have prepared Ip-β-lg, in which 80% of the lysine residues were converted to the N^{ϵ}-isopropyllysine form. Nonreactive lysine residues were located with the aid of peptide maps, and ^{2}H NMR was used to evaluate the dynamics of labeled lysine residues under varying conditions, as well as to examine the effects of alkylation of lysyl side chains on the interaction of β-lg with lipids. The results reported here suggest that the lysine residues of β-lg are found in several distinct environments.

Experimental Procedures

Protein Modification. β-lg (3× crystallized) was prepared from the milk of homozygous A/A cows by the method of Aschaffenburg and Drewry (1957). Preparation of Ip-β-lg by the cyanoborohydride method of Jentoft and Dearborn (1979), which is very effective for reductive methylation, was attempted. However, despite rigorous attention to reagent purity, and with variation of pH and molar ratios, only 25–45% of the lysines of β-lg could be labeled with isopropyl groups by this method; therefore, the procedure was abandoned. Ip-β-lg was then prepared following the method of Means and Feeney (1968), including the modifications of Fretheim et al. (1979). Specifically, a solution which was 1.5 mM in lysine residues [prepared by dissolving 100 mg of β-lg in 50 mL of 0.2 M sodium borate (pH 9.0, containing 20% *p*-dioxane)] was made 400 mM in acetone by the addition of 1.45 mL of [$^2H_6$]acetone (99.7 atom %). The solution was stirred at room temperature while $NaBH_4$ was added in 10 increments over 15 min to a final concentration of 530 mM. After an additional 15 min of stirring, the solution was titrated to pH 4.5 with 0.5 M HCl, dialyzed against water, and lyophilized. Disulfide bonds were reduced and S-carboxymethylated to form RCM-β-lg or RCM-Ip-β-lg by the method of Schechter et al. (1973). The isopropyllysyl derivative of RCM-β-lg (Ip-RCM-β-lg) was similarly prepared both in borate buffer and in buffer containing 6 M guanidine hydrochloride.

Characterization of Ip-β-lg. To determine the extent of lysine modification, samples of native and modified β-lg were hydrolyzed at 105 °C in 5.7 M HCl (containing 0.05% phenol) and examined by amino acid analysis (Beckman 119 CL amino acid analyzer,[2] 90-min single column, W3H cation-exchange resin) as previously described (Brown & Greenberg, 1984). The dansylation method of Weiner et al. (1972) was used to check for modification of the α-amino group. Molar concentrations of β-lg in its native and modified forms were determined spectrophotometrically from an absorption coefficient of 17 600 M^{-1} cm^{-1} at 278 nm (Townend et al., 1967; Braunitzer et al., 1973). The effects of reductive isopropylation on protein conformation and self-association were evaluated by comparing several properties of β-lg and Ip-β-lg. These included sedimentation coefficient (determined at 52 000 rpm and 20 °C), circular dichroism spectra (0.1 mg/mL, 0.05-cm cell, 260–200 nm), and electrophoretic mobility (PAGE, pH 8.9).

Peptide Maps. Disulfide-reduced, S-carboxymethylated protein was used for peptide studies. RCM-β-lg or RCM-Ip-β-lg (7 mg/mL) at pH 8.2 was digested with TPCK-trypsin (Worthington) at an enzyme:protein ratio of 1:100. Proteolysis was carried out under N_2 at 37 °C, while maintaining the pH at 8.2 with a Radiometer RTS-822 pH stat. After 1.5 h, a second aliquot of trypsin was added, and the solution was incubated for an additional 3 h and then lyophilized. Digestion of RCM-β-lg or RCM-Ip-β-lg with α-chymotrypsin (Worthington) was similar except that the pH was maintained at 8.0. RCM-β-lg or RCM-Ip-β-lg (20 mg in 1.5 mL of 70% formic acid) was treated with 100 mg of CNBr for 20 h under N_2 at room temperature in the dark, as previously described (Groves & Greenberg, 1978). The resulting protein fragments were diluted with water and lyophilized 3 times to remove the remaining CNBr and formic acid. CNBr or Ip-CNBr fragments were separated by gel filtration on Sephadex G-50 in 0.05 M ammonium bicarbonate, pH 8.0 (10 mg on a 2 × 120 cm column). The eluant was monitored at 206 nm; appropriate fractions were combined and lyophilized. CNBr or Ip-CNBr fragments were digested with chymotrypsin as described above. Peptide fragments were subjected to amino acid analysis and PAGE in 4 M urea at pH 8.9.

Tryptic or chymotryptic peptides were separated by RP-HPLC on a Brownlee C_{18} (0.4 × 25 cm, 5-μm particle size) column with a Varian System 54 liquid chromatograph and a UV-50 detector. The mobile phase was 0.1% TFA (Pierce) in water as solvent A and 0.1% TFA in acetonitrile (Burdick & Jackson) as solvent B. A 10–80% linear gradient of solvent B in solvent A was developed over 30 min at a flow rate of 1 mL/min. Experiments were repeated 6–8 times, using higher than normal sample loading, and collecting peak material for N-terminal and amino acid analysis. N-Terminal groups of the peptides were identified by TLC as the (dimethylamino)azobenzeneisothiocyanate derivatives (Chang, 1983).

To identify specific peptides, the amino acid composition of each peak, in nanomole form, was entered onto a standard microcomputer spread sheet, from which the total concentration of each amino acid could easily be verified, and comparisons were made between peak compositions and possible tryptic peptides of β-lg (Braunitzer et al., 1973) or chymotryptic peptides previously sequenced in this laboratory (R. Greenberg, unpublished data). Data from the N-terminal analysis and from chromatograms obtained by monitoring at

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

280 nm were used to confirm the choice of particular peptides especially when a peak contained more than one peptide. Published studies of the contributions of specific amino acids to the retention characteristics of peptides (Meek, 1980; Meek & Rossetti, 1981; Browne et al., 1983) were helpful in deciding the probable order in which peptides might be eluted. When a cursory inspection of the amino acid composition of a peak suggested a particular peptide, the highest concentration for the peptide (based on the lowest determined concentration of any of its constituent amino acids) was subtracted from the composition of the peak. This process was repeated until no identifiable peptides remained. The use of the spread sheet made it relatively easy to try different combinations until the best fit was realized.

Deuterium NMR Measurements. NMR spectra were obtained for Ip-β-lg, RCM-Ip-β-lg, or a hydrolyzed amino acid mixture prepared from Ip-β-lg dissolved in 0.02 M Tris, 0.08 M NaCl, and 0.001 M EDTA at pH 7.5, and for both multilamellar and vesicle complexes of Ip-β-lg with DPPC prepared as previously described (Brown et al., 1983). Protein concentration was determined spectrophotometrically and the concentration of isopropyllysyl residues from the amino acid analysis.

Deuterium NMR spectra were obtained by pulse Fourier transform spectroscopy initially at 60 MHz with a JEOL FX60Q FT/NMR spectrometer. To avoid heating effects, all experiments were run without proton decoupling. The frequency of observation for the low-field spectra was 9.17 MHz. A constant field was maintained by setting the lock channel to 24.2 MHz and locking on the ^{31}P resonance obtained from a sealed capillary containing triphenyl phosphite in the center of a precision 10-mm NMR tube. A 2-mL sample of 0.27 mM Ip-β-lg (3.2 mM deuterium) was then placed in the tube surrounding the capillary. Approximately 5K transients were required to obtain useful information using 40-μs (90°) pulses with a spectral width of 500 Hz, a recycle time of 5 s (greater than $10T_1$ for ^2H_2O and ^2H_3C resonances), and 4K data points. Sweep widths up to 20 kHz were used to assure that the entire resonance area was measured.

When a 9.4-T JEOL GX-400 FT/NMR spectrometer became available, high-field spectra were recorded at a deuterium frequency of 61.25 MHz with samples containing 0.16 mM Ip-β-lg (2.0 mM deuterium). Each spectrum was obtained from 500 transients using 68.5-μs (90°) pulses with a spectral width of 1000 Hz, a recycle time of 4.2 s, and 8K data points. The shorter ringdown time (15 vs 56 μs) and faster digitization of this instrument doubled the resolution at a constant acquisition time.

The apparent concentration of [$^2H_6$]isopropyllysyl residues was estimated by comparing the integrated area of the ^2H_3C resonance with that of the solvent ([^2H] = 17.2 mM, aqueous, or 15.2 mM, 6 M guanidine hydrochloride). The ^2H_3C chemical shifts were measured relative to the resonance for the natural-abundance ^2H in ordinary water, arbitrarily assigned a position of 4.5 ppm. The relative response for ^2H_3C was verified with a standard solution of 9.0 mM [$^2H_6$]acetone (2.0 ppm).

Line widths, full width at half-height, were measured directly from the spectrum (9.17 MHz) or obtained from the internal computer for 61.25-MHz spectra. In addition, each 61.25-MHz spectrum was fitted with a Gauss–Newton nonlinear regression program (developed in this laboratory for the Modcomp Classic minicomputer) to determine line shape. This program allowed us to fit the data to multiple Gaussian and/or Lorentzian functions and from the deviation plot evaluate the goodness of the fit. The nonlinear regression was not used with the 9.17-MHz data because of the relatively poor resolution in these spectra.

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                10                  20
L I V T Q T M K G L D I Q K V A G T W Y S L A M A
    30                  40                  50
A S D I S L L D A Q S A P L R V Y V E E L K P T P
                60                  70
E G D L E I L L Q K W E N D E C A Q K K I I A E K
    80                  90                  100
T K I P A V F K I D A L N E N K V L V L D T D Y K
                110                 120
K Y L L F C M E N S A E P E Q S L V C Q C L V R T
    130                 140                 150
P E V D D E A L E K F D K A L K A L P M H I R L S
                160
F N P T Q L E E Q C H I
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FIGURE 1: Sequence of bovine β-lactoglobulin A (Braunitzer et al., 1973; Preaux et al., 1979; Eigel et al., 1984) in single-letter notation.

Longitudinal relaxation times (T_1) were measured by the inversion recovery method (Vold et al., 1968) with a 180°–τ–90° pulse sequence. Variable delay times ranged from 1 to 25 s. Transverse relaxation times (T_2) at 9.17 MHz were determined from spin-locking measurements of $T_{1\rho}$, the longitudinal relaxation time in the rotating frame, with an offset frequency of 78.60 Hz (Farrar & Becker, 1971). In dilute solutions of low viscosity, at 9.17 MHz, $T_{1\rho}$ is equal to T_2 if $T_{1\rho}$ is independent of the spin-locking radio-frequency field. $T_{1\rho}$ proton experiments were performed to show that these relaxations were independent of the radio-frequency field used. At 61.25 MHz, T_2 values were measured by the Carr–Purcell–Meiboom–Gill spin–echo method. Relaxation times were evaluated by a least-squares two-parameter exponential fit as previously described (Kumosinski & Pessen, 1982).

RESULTS

Characterization of Ip-β-lg. The primary structure of bovine β-lg (Figure 1) has been published (Braunitzer et al., 1973; Preaux et al., 1979; Eigel et al., 1984). Among the 162 amino acid residues are 2 Trp, 4 Tyr, 4 Phe, 15 Lys, 3 Arg, and 4 Met. Lysine modification was carried out according to the conditions of Fretheim et al. (1979). Amino acid analysis showed that 80% of the 15 lysine residues of β-lg were reductively isopropylated (Table I). Monoisopropyllysine, quantitated by the decrease in concentration of lysine from native β-lg (Table I), was the only modified amino acid residue detected. Dansylation of β-lg, Ip-β-lg, and Ip-RCM-β-lg produced dansylleucine from β-lg and Ip-β-lg, but not from Ip-RCM-β-lg, indicating that the N-terminal leucine was not isopropylated, unless the disulfide bonds were broken before the protein was reductively isopropylated. Ip-RCM-β-lg prepared under the conditions used for the preparation of Ip-β-lg showed no increase in the number of N^{ϵ}-isopropyllysyl residues. However, when 6 M guanidine hydrochloride was included to unfold the RCM-β-lg, 95% of the lysine residues and the N-terminal α-amino group were labeled.

Sedimentation coefficients ($s_{20,w}$) of 2.3 ± 0.05 and 2.4 ± 0.05 S were obtained at pH 7.5 for Ip-β-lg and β-lg, respectively, with 7 mg/mL samples. The conformation of Ip-β-lg as determined from the 200–260-nm circular dichroism spectrum was essentially the same as that of β-lg (Brown et al., 1983) and suggests predominently β-structure with about 10% helix. Migration of β-lg and Ip-β-lg in a non-SDS–PAGE experiment was identical.

Table I: Amino Acid Composition[a] of β-lg, Ip-β-lg, and Peptides Produced by CNBr Cleavage

amino acid	β-lg, Ip-β-lg (1–162)[b]	I, Ip I (25–107)	II, Ip II (108–145)	III, Ip III (146–162, 8–24)
Lys[c]	14.9, 2.9 (15)	9.4, 1.6 (10)	3.3, 0.9 (3)	2.2, 0.5 (2)[d]
His	2.0, 2.2 (2)	0.4, 0.8 (0)	0.3, 0.5 (0)	1.6, 1.7 (2)
Arg	2.9, 3.1 (3)	1.2, 1.3 (1)	1.0, 0.9 (1)	1.4, 0.9 (1)
CM-Cys	4.1, 4.1 (5)	1.8, 1.9 (2)	1.2, 0.8 (2)	0.6, 0.1 (1)
Asp	15.5, 15.1 (16)	9.3, 8.1 (10)	4.0, 3.6 (4)	1.9, 3.3 (2)
Thr	7.2, 7.1 (8)	2.9, 3.0 (3)	1.1, 1.6 (1)	2.6, 3.4 (2)
Ser	6.5, 6.2 (7)	2.9, 2.9 (3)	1.7, 1.8 (1)	1.7, 2.5 (2)
Glu	24.7, 26.0 (25)	11.9, 12.4 (11)	7.7, 6.6 (8)	5.3, 6.2 (5)
Pro	7.7, 8.9 (8)	4.1, 4.0 (4)	2.8, 2.2 (3)	1.0, 1.4 (1)
Gly	3.1, 3.2 (3)	1.0, 1.4 (1)	0.4, 0.8 (0)	1.8, 2.2 (2)
Ala	13.6, 12.6 (14)	6.7, 6.9 (8)	3.6, 3.2 (4)	1.7, 2.3 (2)
Val	9.0, 9.2 (10)	5.0, 5.0 (5)	2.8, 2.5 (3)	1.5, 2.0 (1)
Met	3.6, 4.3 (4)	(1)	(1)	(1)
Ile	8.3, 9.3 (10)	4.6, 4.2 (6)	0.7, 1.0 (0)	3.2, 3.1 (3)
Leu	22.0, 22.2 (22)	11.4, 11.0 (12)	5.1, 5.0 (5)	4.3, 4.7 (4)
Tyr	4.0, 4.3 (4)	2.1, 2.3 (2)	0.3, 0.5 (0)	1.0, 1.0 (1)
Phe	4.0, 4.0 (4)	2.0, 2.0 (2)	1.0, 1.0 (1)	1.0, 1.0 (1)
Trp[e]	+, + (2)	+, + (1)	−, − (0)	+, + (1)

[a] Values are the average of two hydrolyses. Compositions are in residues per mole of peptide or protein calculated relative to PHE, except that for III; after peptide 146–162 was calculated relative to PHE, it was substracted from the peak composition, and peptide 8–24 was calculated relative to Tyr; the concentrations suggest a 55/45% ratio of (146–162)/(8–24) in this peak. The N-terminal fragment, 1–7, was omitted from the table. [b] Position of peptide in sequence (Figure 1). [c] Unmodified lysine residues. [d] Integral values in parentheses are based on the sequence of β-lg A (Braunitzer et al., 1973). [e] Presence or absence of tryptophan was verified spectrophotometrically, but not quantitated in these samples.

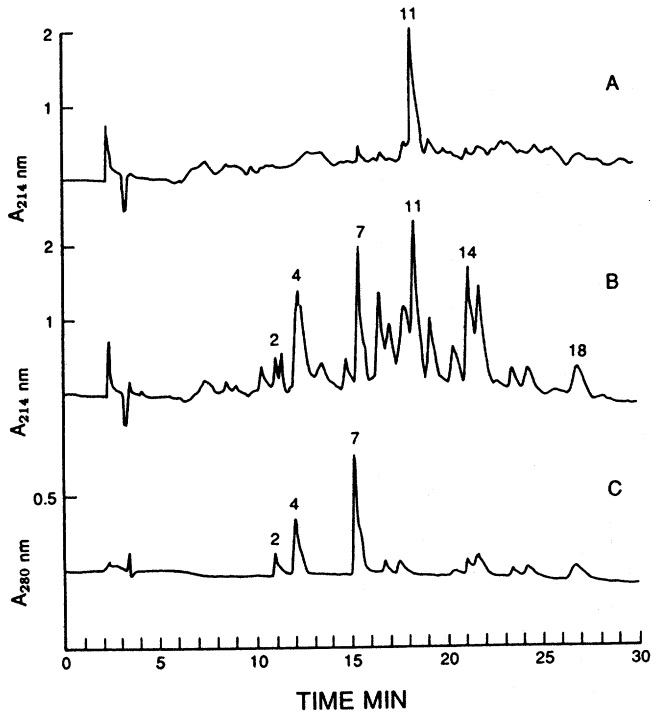

FIGURE 2: Reversed-phase HPLC of tryptic digests of Ip-β-lg (A) and β-lg (B and C). Chromatograms A and B were obtained by monitoring the absorbance at 214 nm to observe all peptides eluted from the column. Tryptophan- or tyrosine-containing peaks were located in chromatogram C. The relative absorbance scales are indicated for each chromatogram. Detailed analysis of the peptides from β-lg will be published elsewhere.

Peptide Maps. The resistance of Ip-lysine residues to tryptic hydrolysis (Brown & Greenberg, 1984) led us to expect fewer tryptic peptides from Ip-β-lg than from β-lg. In fact (Figure 2A), the 214-nm chromatogram of the tryptic digest of Ip-β-lg shows a single well-resolved peak, in sharp contrast with the 18 resolved peaks from β-lg (Figure 2B). The 280-nm chromatogram of the tryptic digest of β-lg (Figure 2C) was useful for identifying peptides containing aromatic residues and confirming that the digest was equivalent to that reported by Braunitzer et al. (1973), including the splitting of Trp_{61} into peaks T2 (residues 61–69) and T4 (residues 61–70). The resolved tryptic peak from Ip-β-lg (Figure 2A, T11) contained

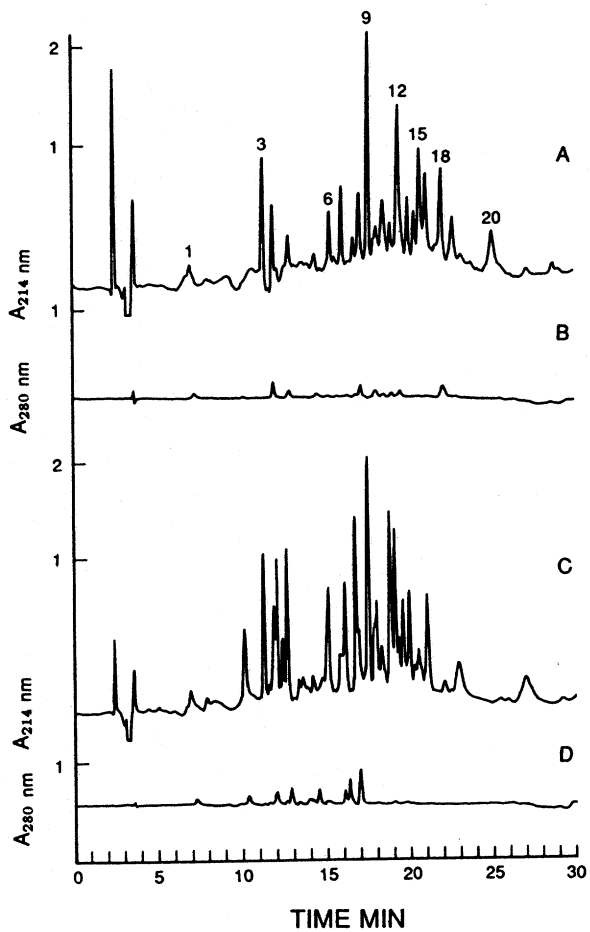

FIGURE 3: Reversed-phase HPLC of chymotryptic digests of Ip-β-lg (A and B) and β-lg (C and D). Chromatograms A and C were obtained at 214 nm, B and D at 280 nm. Numbers are included as an aid in correlating with the text.

the carboxy-terminal peptide, residues 149–162, resulting from cleavage of the Arg_{148}–Leu_{149} bond. In addition, three large peptides separable by PAGE (data not shown) were present in the tryptic digest of Ip-β-lg.

Chymotryptic maps of Ip-β-lg (Figure 3A) and β-lg (Figure 3C) contained similar numbers of peaks. Tryptophan, tyrosine, phenylalanine, and some but not all leucine residues in both

Table II: Location of Lys_{47}[a] in Chymotryptic Peptides of Ip-CNBr I

minutes[b] (peak no.)	peptides[c]	nmol[d]	% peak material[e]
12.0 (4)	47–61	2.7	40
	40–42	16.2	44
18.2 (10)	40–69	1.8	90
19.0 (12)	43–61	2.3	34
	76–93	4.6	64
19.2 (13)	47–57	5.2	47
	40–46	2.7	16
	83–87	5.3	17
20.1 (15)	43–95	2.5	54
	43–61	1.9	13
	43–54	1.9	11
20.6 (17)	43–82	1.3	30
	83–93	7.4	33
	55–58	10.5	21
22.1 (19)	40–61	1.5	40
	62–87	1.8	60

[a] Total concentration of identified peptides containing unlabeled Lys_{47} is 21.1 nmol, from a sample in which the average concentration per residue in Ip-CNBr I was 22.6 nmol. [b] Elution times and peak numbers (in parentheses) are given to correlate data with Figure 3. [c] Position of peptide in sequence (Figure 1). [d] Concentration of the identified peptide. [e] Peaks may contain some background material, or unidentified peptides.

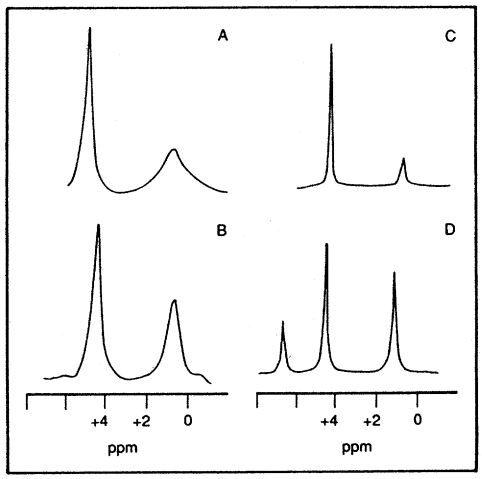

FIGURE 4: ^2H NMR spectra of N^{ϵ}-[$^2H_6$]Ip-β-lg (A) at 9.17 MHz in Tris buffer, pH 7.5, (B) at 9.17 MHz in 6 M guanidine hydrochloride, (C) at 61.25 MHz in Tris buffer, pH 7.5, and (D) at 61.25 MHz in 6 M guanidine hydrochloride. The figure was prepared by manually digitizing actual spectra so that they could be scaled appropriately.

modified and unmodified protein were cleaved. Direct comparison of the chymotryptic maps derived from whole protein was complicated by the large number of peaks, several containing more than one peptide, and by the general shift toward longer retention times for peptides containing isopropyllysyl residues. This shift was most readily seen in the 280-nm chromatograms (Figure 3B,D).

Methionine residues occur at positions 7, 24, 107, and 145 in β-lg. Chromatography of the CNBr fragments of β-lg or Ip-β-lg on Sephadex G-50 gave four major peaks. The amino acid compositions of these peaks, listed as CNBr I–IV, are given in Table I. On the basis of those compositions, the following assignments were made: CNBr I, 83 residues, 25–107; CNBr II, 38 residues, 108–145; CNBr III, both 17-residue fragments, 8–24 and 146–162; and CNBr IV, the N-terminal fragment, 1–7. CNBr IV was devoid of lysine and was not studied further. From the amino acid analysis, the 2.9–3.0 residues of unmodified lysine per molecule of Ip-β-lg were determined to be distributed among the Ip-CNBr fragments as follows: 1.6 residues in Ip-CNBr I; 1.0 residue in Ip-CNBr II; and 0.5 residue in Ip-CNBr III (Table I). Although these data suggest the possibility of a completely unmodified residue in either Ip-CNBr I or Ip-CNBr II, finding an exact location would be simplified by smaller fragments.

Chromatograms of the chymotryptic digests of CNBr fragments of β-lg and Ip-β-lg contained fewer, better separated, peaks which were more easily collected and analyzed. Ip-CNBr I, residues 25–107, contained 10 lysine residues with the equivalent of 1.6 residues not modified. One residue, Lys_{47}, was easily monitored because in any chymotryptic peptide it was associated with two proline residues, 48 and 50, and one glycine residue, 52. Digestion of Ip-CNBr I or Ip-β-lg with chymotrypsin resulted in several peptides containing this combination of amino acids (Table II). The amino-terminal analysis of material from a chromatographic peak was helpful in deciding which peptides were not present, but less useful in determining which were present because of the occurrence of more than one peptide fragment with the same N-terminal residue. For example, chymotryptic cleavage of Tyr_{42}–Val_{43} was nearly complete, but cleavage at any of the several leucine residues between Tyr_{42} and Phe_{82} was less complete. Nevertheless, more than 90% of all lysines at position 47 were accounted for through the peptides listed in Table II. Minor amounts of unmodified lysine at other positions in Ip-CNBr I were detected and ranged from 10% at Lys_{60} to 1% at Lys_{101}.

Ip-CNBr II, residues 108–145, contained the equivalent of one unmodified lysine residue (Table I). Peaks 7, 9, and 12 at 15, 17, and 19 min in the chromatogram of the chymotryptic digest of β-lg (Figure 3A) contained residues 141–143 (Lys_{141}), 137–140 (Lys_{138}), and 123–136 (Lys_{135}), respectively. In chromatograms of Ip-β-lg (Figure 3A), these peaks were present, but reduced in area. Analysis of the chymotryptic digests of CNBr II and Ip-CNBr II suggested that the label was nearly equally distributed among the three lysine residues.

A peptide composed of residues 8–20 containing Ip-lysine eluted at 20.4 min from the chymotryptic digest of Ip-CNBr III, while from Ip-β-lg residues 1–20 which contained Ip-lysine were eluted at 22.7 min. The unmodified lysine at residues 8 and 14, amounting to not more than 0.5 residue (Table I), was not identified in specific peptides, suggesting that part of this may be background material, characteristic of the analysis system.

^2H Nuclear Magnetic Resonance. Figure 4A,C shows ^2H NMR spectra obtained at 9.17 and 61.25 MHz for Ip-β-lg in 0.02 M Tris, 0.08 M NaCl, and 0.001 M EDTA (pH 7.5). Relative to the 4.5 ppm position for deuterium in ordinary water, the resonance due to Ip-β-lg was observed at 1.1 ppm in 9.17-MHz spectra, and at 0.9 ppm in 61.25-MHz spectra. These positions are slightly different because of variation in the ambient temperature (21–23 °C) of the probe of these two instruments. At 9.17 MHz, a single solvent peak (4.5 ppm) was observed for Ip-β-lg in buffered 6 M guanidine hydrochloride (Figure 4B). However, at 61.25 MHz (Figure 4D), the solvent peak was split, with the relative areas of the peaks indicating that the 4.5 ppm peak results from the NH_2 group of guanidine and water, while the 6.6 ppm peak is due to the $C(NH_2)_2$ group of guanidine. Spectra of buffered guanidine hydrochloride without protein (not shown) contain a single 4.5 ppm peak at 9.17 MHz, and two peaks (4.5 and 6.6 ppm) at 61.25 MHz.

The apparent numbers of labeled lysine residues obtained from a comparison of the integrated areas of the ^2H_3C resonance and the solvent resonance are given in Table III. For Ip-β-lg, under nondenaturing conditions at 9.17 MHz, a surprisingly small portion of the expected ^2H resonance, based on the amino acid analysis, was observed. Increases in the

Table III: Summary of NMR Data Analyzed as a Single Lorentzian

sample	MHz	apparent[f] residues	$\Delta\omega$ (Hz)	$\tau_{c,eff}$ (ps)
Ip-β-lg[a]	9.17	5.7	7.1	52
	61.25	9.7	10.4	76
RCM-Ip-β-lg[a]	9.17	8.0	6.9	51
	61.25	9.2	6.8	50
Ip-β-lg, 6 M Gdn·HCl[b]	9.17	8.8	5.3	39
	61.25	12	7.6	56
RCM-Ip-β-lg, 6 M Gdn·HCl[b]	9.17	12.1	7.9	58
	61.25	12.2	7.6	56
Ip-β-lg (hydrolysate)[c]	61.25	12.1	4.2	31
Ip-β-lg–DPPC (ves)[d]	9.17	0.6	5	37
Ip-β-lg–DPPC (lam)[e]	9.17	3	20	147

[a] In 0.02 M Tris, 0.08 M KCl, and 0.001 M EDTA, pH 7.5. [b] 6 M guanidine hydrochloride in buffer *a*. [c] Ip-β-lg was hydrolyzed overnight in 6 M HCl, the acid was removed by rotatory evaporation, and the hydrolysate was redissolved in buffer *a*. [d] Vesicle preparation of Ip-β-lg in buffer *a* sonicated with DPPC. [e] Lamellar preparation of Ip-β-lg in buffer *a* vortexed with DPPC. [f] Determined from the ratio of the ^2H_3C resonance to the ^2H resonance for solvent.

sweep width to 20 kHz did not reveal increases in the observed resonance area. The apparent number of modified lysine residues did increase when spectra were obtained for RCM-Ip-β-lg, or when 6 M guanidine hydrochloride was used as a denaturing solvent. Only when RCM-Ip-β-lg was dissolved in 6 M guanidine hydrochloride was the total resonance area, commensurate with the amino acid analysis, observed. Because such small resonance areas were obtained for aqueous buffered solutions of Ip-β-lg, samples were sent to other laboratories for confirmation. The data were consistent in that under nondenaturing conditions the deuterium resonance observed at 13.8 MHz[3] corresponded to seven isopropyllsyl residues, and at 76.8 MHz[4] to nine isopropyllysyl residues. The greater sensitivity available when we obtained a higher field (61.25 MHz) instrument allowed us to observe a larger portion of the ^2H resonance of Ip-β-lg under a variety of conditions, but denaturation was still necessary to obtain a resonance area equivalent to 12 isopropyllysyl groups per molecule.

In the high-field spectra, short-probe dead times and fast digitization allow one to observe a large portion of the broad components associated with restricted lysines. The loss of signal intensity in the lower field spectra is not related to any change in line width, but to the ability to visualize the broad components. Instrumental characteristics (lower sensitivity, longer dead times, and slower digitization) combine to edit out more of the resonance response of the slow-moving lysines at the low field.

The observed line shapes for all of the ^2H NMR spectra were Lorentzian in nature, satisfying the criteria $\omega_{o(^2H)} \ll 1/\tau_c$ where $\omega_{o(^2H)}$ is the observed ^2H Larmor frequency and τ_c is the reorienting correlation time. Thus, the effective correlation time ($\tau_{c,eff}$) for [$^2H_6$]isopropyl groups incorporated into the protein could be calculated directly. Line widths at both fields were measured, and effective correlation times under various conditions were calculated from the relationship:

$$\tau_{c,eff} = \frac{8/3\pi\Delta\omega_{1/2}}{(e^2qQ/\hbar)^2}$$

where the quadrapole coupling constant ($e^2qQ/\hbar$) is 170 kHz,

[3] Data at 13.8 MHz from Dr. Lynn Jelinski (Bell Labs, Murray Hill, NJ).

[4] Data at 76.8 MHz from Dr. Sunney Chan of the California Institute of Technology.

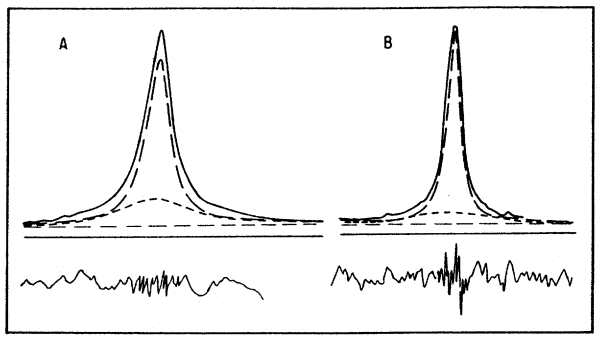

FIGURE 5: 61.25-MHz ^2H NMR spectrum (solid line) of N^ϵ-[$^2H_6$]-Ip-β-lg (A) in Tris buffer, pH 7.5, and (B) in 6 M guanidine hydrochloride, pH 7.5. Each spectrum was fitted with a Gauss–Newton nonlinear curve-fitting program to obtain the individual functions (dashed lines), the sum of which is the original spectrum. Deviation patterns shown below the spectra are magnified by a factor of 10.

the average value for an aliphatic deuteron–carbon bond reported by Glasel et al. (1973), and $\Delta\omega_{1/2}$ is the line width (full width at half-height) in hertz. The average values obtained for τ_c are summarized in Table III; the 61.25-MHz values can be expected to be more accurate than the lower field values because of the greater accuracy with which the line width could be determined.

Figure 5A shows the 61.25-MHz spectrum of Ip-β-lg, in aqueous buffer, the functions chosen by the Gauss–Newton nonlinear curve-fitting routine to give the best fit, and the resulting deviation pattern. Spectral analysis of Ip-β-lg in 6 M guanidine hydrochloride is shown in Figure 5B. Attempts at fitting these data with one or two Gaussian functions or with a single Lorentzian function resulted in poor fits with nonrandom noise patterns. The sum of two Lorentzian functions with identical peak positions but different line widths produced the best fit for spectra of the intact protein at 61.25 MHz in either solvent. With nonlinear analysis, deviation plots furnish nonsubjective and sensitive criteria of the goodness of fit. The random distribution of noise seen in the bottom panels of Figure 5 is characteristic of an appropriate fit (Meites, 1979). Two populations of observable lysine residues were apparent in spectra obtained in either aqueous buffer or 6 M guanidine hydrochloride. In aqueous buffer, the equivalent of 9.7 modified residues were observed, 6.5 residues with a fast average correlation time (τ_c = 70 ps), 3.2 residues with a slow, but observable average correlation time (τ_c = 320 ps), and the remaining 2.3 residues with a $\tau_{c,eff}$ so long that they could not be resolved from the base line even at this field. In 6 M guanidine hydrochloride, where all of the modified lysine was observed, the equivalent of 8.7 residues had a fast average correlation time (τ_c = 20 ps) and 3.3 residues a slow average correlation time (τ_c = 320 ps).

Slow and fast correlation times were determined from the individual line widths obtained from the nonlinear regression analysis of the spectra; the numbers of residues corresponding to each τ_c were obtained from the ratio of the areas of the two Lorentzian curves. The best fit for the spectrum of Ip-β-lg after hydrolysis to individual amino acids (not shown) was a single Lorentzian; in this sample, all ^2H_3C groups were equally mobile; the value for τ_c (24 ps) obtained from the curve-fitting program may be a better estimate of the correlation time than that of 31 ps calculated from the observed line width (Table III).

All measurements were made under conditions of complete relaxation. In nondenaturing solution, T_1 and $T_{1\rho}$ values (16.67 and 17.24 ms) for 5.7 residues, measured at 9.17 MHz, were essentially equal, and at 61.25 MHz, T_1 (28.5 ms) was equal

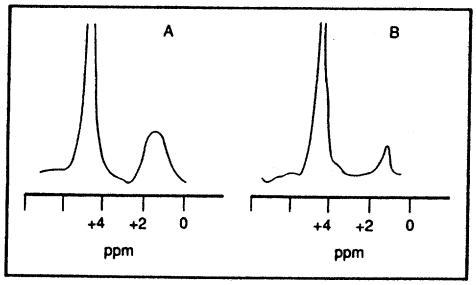

FIGURE 6: 9.17-MHz ^2H NMR spectra of Ip-β-lg complexed with DPPC (A) multilamellar liposome and (B) sonicated vesicles. The water peak has been truncated to make the ^2H_3C peak more visible.

to T_2 (28.2 ms) for 9.7 residues. In 6 M guanidine hydrochloride, T_1 and $T_{1\rho}$ were 10.3 and 8.5 ms for 8.8 residues measured at 9.17 MHz, while at 61.25 MHz T_1 = 31.0 ms and T_2 = 27.0 ms for 12 residues. Numerical values change as more residues are observed through protein unfolding, or with the greater sensitivity of the higher field instrument.

Lipid–Protein Interactions. Deuterium-labeled protein is useful for the NMR study of protein complexed with one or more lipid molecules whose properties may more readily be followed with ^{31}P or ^{13}C NMR. The ^2H NMR resonance for a multilamellar liposome of DPPC complexed with Ip-β-lg corresponded to only a small portion of the Ip-lysine residues in the sample. The resonance line (Figure 6A) was isotropic but significantly broadened relative to the protein in solution, suggesting a restricted environment for the observable lysines. Sonication of this complex to form small unilamellar vesicles resulted in a narrower line (Figure 6B), the area of which corresponded to an even smaller portion of the labeled lysine, approximately one residue per protein molecule.

DISCUSSION

Reductive methylation with [^{13}C]formaldehyde and cyanoborohydride in buffered solutions at pH 6.5–7.5 (Jentoft & Dearborn, 1979) has resulted in extensive labeling of lysine residues in ribonuclease (Jentoft et al., 1981), lysozyme (Gerken et al., 1982), concanavalin A (Sherry & Teherani, 1983), and α-lactalbumin (Gerken, 1984). *N*-Isopropyllysylfetuin was similarly prepared for NMR studies (Bernstein et al., 1979), but the incorporation of the label was not quantitated. The extent of reductive alkylation as a function of substituent size tends to correlate with the location and function of primary amino groups. When the lysine residues are at the protein surface as in chymotrypsin and lysozyme (Birktoft & Blow, 1972; Imoto et al., 1972), both methylation and isopropylation proceeded beyond the 95% level (Means & Feeney, 1968; Fretheim et al., 1979). Reductive isopropylation was less extensive than methylation in human serum transferrin (84% methylated, 29% isopropylated) (Means & Feeney, 1968) where some of the lysine residues are located on an amphipathic helix (Segrest & Feldman, 1977). Because of their smaller size, methyl groups are expected to label more residues than are isopropyl groups unless all lysyl side chains are completely exposed.

Reductive isopropylation by the cyanoborohydride method labeled only 25–45% of the lysine residues of β-lg, even when pH, buffer composition, concentrations of reagents, and reaction time were varied in attempts to maximize the alkylation. With sodium borohydride, we were able to completely isopropylate the lysine residues of ribonuclease (unpublished). However, 80% isopropyllysine formation was the maximum obtained for β-lg, despite variation of other conditions. This result was not entirely unexpected considering the report of Rowley et al. (1979) that reductive methylation of β-lg proceeded to 6%, 32%, or 80% depending upon the formaldehyde concentration. Other chemical modifications of β-lg have indicated limited availability of some lysines and other residues to an extent which depended on the substituent and type of reaction involved. Five lysine residues were found to be resistant to modification by methyl picolinimidate, and one of two tryptophan residues was resistant to modification by 2-hydroxy-5-nitrobenzyl bromide (Malin et al., 1979). Esterification or amidation was 55–85% effective at modifying carboxyl groups (Mattarella et al., 1983). Although Brinegar and Kinsella (1981) did report 95% acylation of lysine residues with citraconic anhydride, there appears to be a core of relatively unavailable amino acid residues in β-lg.

Native β-lg A exists as a monomer–dimer equilibrium mixture under physiological conditions. The association–dissociation properties of the protein have been thoroughly characterized (Timasheff & Townend, 1969). The dimer (M_r 36 000, $s_{20,w}$ = 2.9 S), which is the predominate form at pH 6.2, further associates to form an octamer at pH 4.65 under conditions of high concentration and low temperature (Townend & Timasheff, 1960). Significant dissociation to the monomer occurs as the pH is either raised above pH 6.2 or lowered below pH 4.65 so that $s_{20,w}$ = 2.1 S for solutions of moderate concentration at either pH 8.2 (McKenzie & Sawyer, 1972) or pH 2.46 (Beckerdite et al., 1983). The protein is essentially monomeric at pH 9.0 where reductive alkylation was performed, which may account for the higher levels of alkylation attained with $NaBH_4$ than with cyanoborohydride (normally used in more neutral solution). The resistance of lysines to modification by methyl picolinimidate (Malin et al., 1979) was also determined under neutral conditions. The addition of isopropyl groups to the lysine residues of β-lg did not significantly alter the ability to self-associate, as judged by the sedimentation coefficients obtained with equal concentrations of modified and unmodified β-lg. The single peak obtained for the monomer–dimer equilibrium mixture on ultracentrifugation suggests little alteration in the association constant from that of native β-lg.

Amino acid analysis, indicating 3.0 residues of unmodified lysine, led to the hypotheses that either three specific lysine residues were not modified or a percentage of all lysines were not modified. Tryptic maps intuitively appeared to be an effective way of locating unmodified lysine residues particularly in a protein such as β-lg for which the sequence was determined via tryptic peptides (Braunitzer et al., 1973). Because reductive alkylation of lysine residues makes them resistant to hydrolysis by trypsin (Brown & Greenberg, 1984; Poncz & Dearborn, 1983), only arginine and unmodified lysine residues would be cleaved. If the three unmodified lysine residues per molecule were unique, the tryptic map of Ip-β-lg should contain peaks resulting from cleavage at each of those residues and at the three arginine residues. Cleavage only at arginine residues would yield 4 peptides consisting of 40, 84, 24, and 14 residues. When chromatographed on an HPLC system optimized for the resolution of peptides up to 20 residues, only the 14-residue peptide could be resolved. However, cleavage at any three additional positions would be expected to produce more peptides of resolvable size. Conversely, if the isopropylation were uniformly distributed among the lysine residues, each lysyl peptide from β-lg should be present at a lower level in the tryptic digest of Ip-β-lg. The very small amounts of peptides other than the carboxy-terminal peptide, residues 149–162, which were resolvable in the RP-HPLC system, and the presence of three large peptides, resolved by

electrophoresis, were, at first glance, incompatible with either probability.

Chymotryptic digests of the whole protein and CNBr fragmentation both suggested some localization of unmodified lysine but were not sufficient for specifically locating residues. Because each lysine-containing CNBr fragment contained some unmodified lysine, the chymotryptic digests of these fragments were examined.

Essentially all of the unmodified lysines in Ip-β-lg could, however, be accounted for through the use of chymotryptic maps of Ip-β-lg and the Ip-CNBr fragments. Lys_{47}, identified as the major source of unmodified lysine, appears to occupy a unique conformational position in β-lg. The X-ray structure reported by Papiz et al. (1986) shows Lys_{47} as the first residue in a β-strand, residues 47–55, as well as the final residue in a reverse turn, residues 44–47. The Lys_{47}–Pro_{48} bond increases the rigidity of the conformation and may contribute to the factors which prevent tryptic cleavage (Braunitzer et al., 1973) and inhibit the formation of Ip-Lys_{47}. Resistance of the Lys–Pro bond to hydrolysis by trypsin is a general phenomenon (Keil, 1971), probably steric in origin. Limited reactivity of a lysine residue could also result from an altered pK_a; however, titration studies of β-lg (Basch & Timasheff, 1967), which showed anomalous pK_a's for two carboxyl groups in all genetic variants and for two histidyl residues in one variant, showed no evidence of anomalous pK_a's for any other groups. Although the Lys-Pro sequence is not particularly common, it does occur at residues 224–225 of elastase where identical pK_a's of 10.3 (well within the range for successful reductive alkylation) were reported for lysines-87 and -224 (Hartley & Shotton, 1971).

The equivalent of one unmodified lysine in residues 108–145 of β-lg appears to be split nearly equally among three residues, $Lys_{135,138,141}$. Comparable behavior may be expected for each of these residues because of their location in positions $n + 4$ from an acidic residue along an amphipathic helix (Segrest & Feldman, 1977; Sawyer et al., 1985). Thus, each of these lysine residues has an equal probability of forming a stable ion pair with a residue on the prior turn of the helix (Honig & Hubbell, 1984). Formation of such ion pairs can stabilize conformations in which reductive isopropylation is partially inhibited even in small peptides (Brown & Greenberg, 1983).

Reductive isopropylation with [$^2H_6$]acetone as a means of labeling lysine residues in a protein for NMR studies has several advantages. Extensive modification occurs in 30 min, under relatively mild conditions, leading to only one labeled form. A single isopropyl group containing six equivalent, nonexchangeable, deuterons is added to each accessible primary amino group. The concentration of labeled lysine residues can be determined by difference from an ordinary amino acid analysis or directly from the ^2H NMR spectrum of the protein hydrolysate without the use of radioactive isotopes such as the $^{14}C/^{13}C$ mixture commonly used for quantitating protein methylation (Jentoft & Dearborn, 1979). The excellent separation of resonances due to the natural abundance of deuterium oxide in water and the isopropyllysyl group makes it convenient to determine apparent concentrations of isopropyllysine by comparing the resonance areas of the two signals. Although ^2H does not have the chemical shift dispersion of ^{13}C to define each resonance position at a discrete frequency, its quadrapolar nature makes it a sensitive probe of mobility as a function of its relaxation responses, while the increased hydrophobicity of an added isopropyl group could be expected to affect the chemical shift of an amino acid residue in a carbon spectrum (Cohen et al., 1983). The identity of the chemical shifts for the isopropyl group in the amino acid hydrolysate and the intact protein in either aqueous buffer or denaturing solvent provides evidence that the deuterium spectrum is less sensitive to changes in the hydrophobicity of the residue.

The dynamics of lysine residues in proteins are a function of the level of structure in the protein. In general, residues which are sufficiently mobile to contribute to the ^2H NMR spectrum have correlation times suggesting rapid segmental reorientation (Bernstein et al., 1979). Residues located in unordered, flexible portions of the protein or projecting outward from relatively sharp turns would be expected to have the greatest mobility. The 9.17-MHz NMR experiments provided a semiquantitative measure of protein denaturation, with increases in the deuterium resonance area when disulfide bonds were reduced or when the protein unfolded in 6 M guanidine hydrochloride. At higher field, 61.25 MHz, resolution of the spectra yielded different relaxation rates for groups of lysine residues. These relaxation rates were either not observable or not resolvable in the 9.17-MHz spectra because of long dead times of the probe and slow digitization of the latter instrument.

The single correlation time (24 ps) observed for all the Ip-lysine groups of the amino acid hydrolysate of Ip-β-lg is within experimental limits equal to the fast correlation time determined for groups in the intact protein. This value should be representative of an Ip-lysine located on the outer surface of the protein and freely accessible to the solvent. The slower correlation times then may be expected to represent more restricted environments.

β-lg appears to have at least four populations of lysine residues in distinctly different environments, including a population that is resistant to reductive isopropylation. Although lysine residues are most often located on the surface of a protein, our results suggest that in Ip-β-lg some of the labeled lysine residues are more exposed than others. It is tempting in light of the recently published X-ray data to speculate as to which residues may be responsible for which correlation times. In aqueous buffer at pH 7.5, the equivalent of six to seven residues appeared to be located at the outer surface of the molecule with little restriction to side-chain mobility (τ_c = 70 ps). These may well be $Lys_{8,14,60,77,100,101}$ which are located on turns or in unordered regions of the protein where they may be expected to have fully exposed side chains. $Lys_{135,138,141}$ are located along the amphipathic helix observed by Papiz et al. (1986). Location on an amphipathic helix favors the formation of ion pairs with oppositely charged residues located at $n \pm 4$. In the absence of denaturing solvents, these residues would then have a correlation time similar to that of the entire molecule, which for β-lg is approximately 10 ns (Kumosinski & Pessen, 1982). Hence, the resulting line width (456 Hz) for ^2H attached to these residues would be broader than the spectral window, and not observed in spectra of the nondenatured protein. While the disulfide bonds of the protein did not limit the number of lysine residues modified, three lysine residues (τ_c = 320 ps) did appear to have their motion restricted primarily by the disulfide bonds. β-Strand D, residues 62–76 (Papiz et al., 1986), includes $Lys_{69,70,75}$ as well as Cys_{66}, so that the mobility of these lysine residues may well be restricted by the presence of the disulfide bond Cys_{66}–Cys_{162}. The smaller disulfide loop, Cys_{106}–Cys_{119}, contains no lysine residues and probably has little effect on any of the modified residues.

The effects of an added isopropyl group on the location of a lysine side chain within the tertiary structure of the protein

are probably 2-fold. The increased hydrophobicity of the isopropyl group would favor folding of the side chain into the interior of the globular structure, while the increased bulk would favor a more exposed exterior position. An exposed hydrophobic group might enhance membrane attachment by allowing the protein to interact both electrostatically with the lipid head group and hydrophobically with the fatty acid tail. The isopropyl group, which increases the hydrophobicity of a lysine residue but has little effect on its electrostatic properties, should be a good probe of the ability of β-lg to act like an extrinsic membrane protein (Brown et al., 1983). Extrinsic proteins may be associated with membrane surfaces through electrostatic interactions of lysine and arginine residues with negatively charged lipid groups (Fraser & Deber, 1984). The narrow line widths observed for the vesicle suspensions, similar to those for the protein in isotropic solution, suggest that those very few N^{ϵ}-[$^2H_6$]isopropyllysine residues which were detected by NMR were at the surface of the protein and not involved in its attachment to the vesicle. The broadened isotropic line observed for the ^2H signals in the multilamellar DPPC–Ip-β-lg complex suggest that in this state, the modified lysine residues are restricted, and largely not observable. However, there did not appear to be significant ordering, of the observable residues, within the interior of the bilayer; that is, $\tau_{c,eff} \ll (e^2qQ/\hbar)^{-1}$. In the case of significant ordering, doublets derived from slow reorientation, $\tau_{c,eff} > 10^{-5}$ s/rad, within the axially sequestered environment of the bilayer should have been observed (Smith, 1979). Thus, we conclude that the protein–lipid interaction observed here is primarily extrinsic in nature.

Recent reports of similarity between β-lg and retinol binding protein (Pervaiz & Brew, 1985; Godovac-Zimmermann et al., 1985; Sawyer et al., 1985; Papiz et al., 1986) have stimulated interest in determining the biological function of bovine β-lg. The structural similarity with retinol binding protein includes a peculiar cone-shaped core in which both proteins (Newcomer et al., 1984; Fugate & Song, 1980) can bind retinol in vitro. The high concentration of β-lg in bovine milk and its absence from human milk suggest that its function may be directly related to ruminant nutrition. That is, it may function in the transport and absorption of vitamin A and other lipid-soluble nutrients at least some of which may bind to positively charged amino acid residues as they do to extrinsic membrane proteins. The similar lipid binding properties of modified and unmodified β-lg (Brown et al., 1983) suggest that the ability to act like an extrinsic membrane protein is not altered by a slight increase in hydrophobicity of the lysine residues. The ability to partially modify some residues may indicate that slightly different structures are required for carrying different small molecules.

ACKNOWLEDGMENTS

We thank R. T. Boswell and K. M. Valentine for NMR experiments, H. J. Dower for amino acid analyses, R. E. Seiler for gel electrophoresis, Dr. E. L. Malin for providing us with the analysis of the tryptic digests of tryptophan-modified β-lg prior to publication, and Dr. H. M. Farrell, Jr., and our collaborators at other institutions for helpful discussions.

Registry No. DPPC, 2644-64-6; L-Lys, 56-87-1.

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