Hayes, M. B., Hagenmaier, H., & Cohen, J. S. (1975) *J. Biol. Chem. 250*, 7461-7472.
Jones, B. N., Vigna, R. A., Dwulet, F. E., Bogardt, R. A., Lehman, L. D., & Gurd, F. R. N. (1976) *Biochemistry 15*, 4418-4422.
Jones, W. C., Jr., Rothgeb, T. M., & Gurd, F. R. N. (1976) *J. Biol. Chem. 251*, 7452-7460.
Kirby, E. P., & Steiner, R. F. (1970) *J. Biol. Chem. 245*, 6300-6306.
Konosu, S., Hashimoto, K., & Matsuura, F. (1958) *Nippon Suisan Gakkaishi 24*, 563-566.
Kovacs, A. L., Antonini, E., Brunori, M., Giacometti, G. M., & Tentori, L. (1977) in *Myoglobin* (Scheneck, A. G., & Vandecasserie, C., Eds.) pp 53-74, Editions de l'Université de Bruxelles.
Kronman, M. J. (1976) in *Biochemical Fluorescence* (Chen, R. F., & Edelhoch, H., Eds.) Marcel Dekker, New York and Basel.
Kuntz, I. D. (1975) *J. Am. Chem. Soc. 97*, 4362-4366.
Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379-400.
Lehman, L. D., Dwulet, F. E., Bogardt, R. A., Jones, B. N., & Gurd, F. R. N. (1977) *Biochemistry 16*, 706-709.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254-3263.
Longworth, J. W. (1966) *Biopolymers 4*, 1131-1141.
Longworth, J. W. (1977) *Biophys. J. 19*, 71-82.
Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1978) *Biochem. Biophys. Res. Commun. 81*, 410-415.
Ptitsyn, O. B., & Rashin, A. A. (1975) *Biophys. Chem. 3*, 1-20.
Puett, D. (1972) *Biochim. Biophys. Acta 257*, 537-542.
Puett, D. (1973) *J. Biol. Chem. 248*, 4623-4634.
Rossi-Fanelli, A., Antonini, E., & Caputo, A. (1958) *Biochim. Biophys. Acta 30*, 608-615.
Shen, L. L., & Hermans, J., Jr. (1972) *Biochemistry 11*, 1845-1849.
Shinitzky, M., & Goldman, R. (1967) *Eur. J. Biochem. 3*, 139-144.
Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974a) *Biochemistry 13*, 2967-2974.
Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974b) *Biochemistry 13*, 2974-2979.
Slifkin, M. A. (1971) *Charge Transfer Interactions of Biomolecules*, Academic Press, London and New York.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482-495.
Takano, T. (1977) *J. Mol. Biol. 110*, 535-568.
Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543.
Teale, F. W. J. (1960) *Biochem. J. 76*, 381-388.
Watson, H. C. (1968) *Prog. Stereochem. 4*, 299-333.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303-390.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697-701.

Nuclear Magnetic Resonance Characterization of Aromatic Residues of α-Lactalbumins. Laser Photo Chemically Induced Dynamic Nuclear Polarization Nuclear Magnetic Resonance Studies of Surface Exposure†

Lawrence J. Berliner*,‡ and Robert Kaptein*

ABSTRACT: The α-lactalbumins are involved as modifier proteins in lactose biosynthesis. Lactalbumins from different mammalian species are cross-reactive with the galactosyltransferases from others because of their homologous primary and tertiary structure. We have studied the surface exposure of several α-lactalbumins (bovine, goat, human, guinea pig, and rabbit) by the laser photo-CIDNP technique, an NMR method which measures the access of a photoexcited flavin dye to surface exposed Tyr, Trp, and His residues. An exposed histidine-68 residue exists in the bovine and goat species but is missing in other species and is replaced with a His-10 in the guinea pig species. The only exposed tryptophan residue was Trp-104, which cross-relaxed to nearby Trp-60. Cross-polarization to the latter residue was proven by taking free induction decays after extremely short light pulses (0.1 s). Furthermore, this cross-polarized Trp-60 resonance was absent in the guinea pig species, which has a substitution at position 60. The chemical shifts of these Trp residues were extremely similar in all species but rabbit, which has substituted His for a Tyr at position 103, situated close to both Trp-104 and -60. Three of the four tyrosines in bovine, goat, human, and guinea pig α-lactalbumin were exposed (the latter species has an extra Tyr in its sequence which was apparently not accessible). The rabbit species contains only two Tyr residues, the one which was not accessible (Tyr-50) being common to all species.

α-Lactalbumin, a principal milk whey component, functions as a "modifier" protein in lactose biosynthesis. The protein-enzyme lactose synthase complex between αLA[1] and galactosyltransferase (UDPgalactose:D-glucose 4-β-galactosyltransferase, EC 2.4.1.22) exhibits over a 1000-fold stronger affinity for glucose than in the absence of α-lactalbumin. Thus, the unique structural changes which accompany the association of one molecule each of galactosyltransferase and of αLA manifest themselves in an altered specificity from *N*-acetylglucosamine to glucose acceptors. The primary structures for several αLA species have been reported, in-

† From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and the Department of Physical Chemistry, University of Groningen, Groningen, The Netherlands 9747AG. *Received August 7, 1980.* This research was supported in part by the National Institutes of Health, U.S. Public Health Service Grant GM 21923, and U.S. National Science Foundation Grant PCM 77-24650. The National NMR Facility at Groningen was supported by The Netherlands Foundation for Chemical Research (SON) and The Netherlands Organization for the Advancement of Pure Research (ZWO).
‡ Established Investigator of the American Heart Association.

[1] Abbreviations used: αLA, α-lactalbumin; CIDNP, chemically induced dynamic nuclear polarization; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; FID, free induction decay.

0006-2960/81/0420-0799$01.00/0

cluding bovine (Brew et al., 1970), goat (MacGillivary et al., 1979), human (Findlay & Brew, 1972), guinea pig (Brew, 1972), and rabbit (Hopp & Woods, 1979).

The α-lactalbumins alone are interesting from a structural viewpoint for several reasons. First, the primary structure homologies between differing α-lactalbumin species are high,[2] while also striking is their homology to lysozyme, an enzyme with glycosidase function (Hill & Brew, 1975). There has been additional evidence to support the existence of unique conformational homologies directly amongst the differing α-lactalbumin species by virtue of their cross-reactivity with galactosyltransferases of other species (Ley & Jenness, 1970; Quarfoth & Jenness, 1975; Hopper & McKenzie, 1974). On the other hand, immunological cross-reactivity between various α-lactalbumins has been ambiguous and perhaps has not shown as much homology, especially between evolutionarily distant species (Tanahashi et al., 1968; Prieels et al., 1975). The low levels of cross-reactivity between some species imply subtle differences in surface determinants for antigenic specificity (Tanahashi et al., 1968; Prieels, et al., 1975). While specifically, Tanahashi et al. (1968) observed no immunoprecipitation between bovine α-lactalbumin antisera and pig, guinea pig, or human α-lactalbumins, Prieels et al. (1975) found evidence for *soluble* complexes between bovine or goat and human α-lactalbumin.

While at the time of this writing a three-dimensional α-lactalbumin structure has not been reported, a quite convincing model-building study has been published for the bovine α-lactalbumin structure based on the atomic model for lysozyme (Browne et al., 1969). With some fairly minor changes for residue differences between the two proteins, a quite excellent fit was reported as well as a rather detailed description and comparison of side-chain environments in lysozyme vs. α-lactalbumin. Independently, Warme et al. (1974) have reported energy minimization calculations for the bovine α-lactalbumin conformation which agreed reasonably well with the model-building results.

While one might attempt to describe surface exposure of a protein molecule from its X-ray determined atomic model, it is more convincing if these properties are characterized in the dynamic (solution) state. The approaches employed thus far have utilized solvent perturbation spectroscopic studies (UV difference and fluorescence spectroscopy) and chemical modification techniques.

Much of the previous surface exposure work involving chemical modification of aromatic residues has been inconsistent from one study to the next, due to both the uncertainty of the reagent's accessibility to the protein interior and the many subtle differences in reactivity between different reagents. While all of the previously published works are too numerous to describe here, a good review is found in Hill & Brew (1975); some specific examples will be described under Discussion as they pertain to results from this work. When all previous modification as well as spectroscopic results are summarized, the number of surface exposed aromatic side chains for bovine αLA range from one to three His, one to three Trp, and three to four Tyr residues (Hill & Brew, 1975).

We have taken note of the strongly inferred structural homologies between the various αLA species and the occasional substitution of one or two aromatic residues from one species to another. These homologies can serve as a valuable aid in both characterizing and assigning many His, Trp, and Tyr residues while describing surface residue homologies as well. Table I summarizes the specific aromatic residues in five α-lactalbumins, as well as some comments about their exposure derived from the "lysozyme" model of bovine α-lactalbumin (Browne et al., 1969). While αLA species differences will be discussed in greater detail for each residue type, it is obvious, for example, that comparison of Trp residues of human and guinea pig αLA with the remaining species allows one a potential identification of Trp-26 or Trp-60 by deduction.

Experimental Procedures

Materials

Proteins. Bovine α-lactalbumin (lot 75C8110) was from Sigma Chemical Co. Goat α-lactalbumin was a generous gift of Dr. Martin J. Kronman. Dr. R. K. Craig and Professor P. N. Campbell generously provided both guinea pig and human α-lactalbumin, and Dr. I. Kiyosawa also provided human α-lactalbumin. Rabbit α-lactalbumin was purified from whey by the procedure of Hopp & Woods (1979).

Chemicals. Deuterium oxide (99.95%), DSS, DCl, and KOD were from either Wilmad or Merck Isotopes. 3-*N*-(Carboxymethyl)lumiflavin was a gift from Dr. F. Muller (University of Wageningen).

Instrumentation. The NMR instrumentation was a Bruker Instruments HX360 spectrometer operating in the ^1H Fourier transform mode. The irradiation apparatus, employing a Spectra Physics Model 171 argon ion laser and computer-controlled software, has been described earlier (Kaptein, 1978). Protein samples were preexchanged in D_2O for approximately 30–60 min at room temperature and lyophilized before use. All pH values are uncorrected. Temperature was maintained with the Bruker variable temperature accessory at 30 °C. Photo-CIDNP spectra were taken of protein solutions containing 0.4 mM flavin dye. Alternating "light" and "dark" free induction decays were taken, which after Fourier transformation and subtraction result in the photo-CIDNP difference spectra.

Methods

Laser Photo-CIDNP Method. Chemically induced dynamic nuclear polarization (CIDNP) effects can be generated in amino acid residues by light irradiation of a protein solution in the presence of a dye (Kaptein et al., 1978). Photo excitation of the dye is accomplished by laser irradiation of the solution directly in the NMR probe. The CIDNP effects arise from reversible electron or hydrogen atom transfer reactions of the photoexcited dye with aromatic amino acids. Back reaction of the intermediate radical pairs leads to nuclear spin polarization. This, in turn, results in strong and specific intensity enhancements in the ^1H NMR spectrum of the *native protein.* With the flavin dye used in this study the side-chain protons of histidine, tyrosine, and tryptophan can be polarized, while those of the other aromatic residue, phenylalanaine, remain CIDNP silent.

The polarization characteristics in the aromatic part of the ^1H NMR spectrum are as follows. Tyrosine shows emission (negative intensity) for the 3,5 protons (ortho with respect to the OH group), while the 2,6 protons may show a weak indirect emission effect (see below). Histidine shows an enhanced absorption effect for the C-2 and C-4 protons. Tryptophan also shows positive polarization for the C-2, C-4, and C-6 protons on the indole ring. Since the His proton positions are markedly pH dependent in the neutral pH range,

[2] The overall sequence homology amongst the species bovine, goat, human, and guinea pig αLA is 58% overall and better than 70% for residues 69–123, while rabbit αLA is ~20% less homologous over this sequence by comparison (Hill & Brew, 1975; Hopp & Woods, 1979).

whereas the Trp resonances are not, assignments of photo-CIDNP lines as to type of residue usually follow directly from the sign and pH dependence of the polarizations.

Photo-CIDNP and Surface Accessibility. Since for the photoreaction contact is necessary between the photoexcited dye and the amino acid side chains, the method tests for side-chain accessibility. Compared to other surface probes it has the high intrinsic resolution of NMR and the capability of identifying individual amino acid residues. Thus, it has been confirmed for several proteins (such as BPTI, ribonuclease, and lysozyme), for which both the X-ray structure and NMR assignments are known, that surface residues can be distinguished from those lying in the interior of a protein (Kaptein, 1978; Lenstra et al., 1979).

For a detailed interpretation of side-chain accessibility as reflected by the CIDNP intensities, a better understanding of all factors involved in the rather complex polarization process is required. While this is still under study some preliminary conclusions can already by drawn, especially with regard to the primary steps of the photoreactions. From model studies it appears that in the case of tryptophan the primary step involves electron transfer to triplet flavin, whereas for both tyrosine and histidine hydrogen atom abstraction is involved. As a consequence His and Tyr can only be polarized when the ring NH or OH groups are freely accessible. When these groups are involved in hydrogen bonding, the CIDNP effect seems to be suppressed (Bolscher et al., 1979). For Trp the stereochemical requirements are probably less severe. Any contact with the indole ring may lead to the electron transfer reaction.

For residues lying in the cleft region it should be realized, of course, that the size of the cleft should be large enough to allow penetration of the dye. This is an important consideration when comparing the photo-CIDNP results with those from other surface probes such as solvent perturbation or fluorescence quenching. Finally, it is important to note that the polarization process requires separation of the intermediate radicals and takes place in a short time interval of approximately 10^{-9}–10^{-7} s [for a review, see Kaptein (1977)]. Thus, strongly bound dyes that might disturb the protein structure would not be expected to give rise to CIDNP. However, the effects of weak dye binding are not known, and this possibility cannot always be excluded.

Cross-Polarization Effects. Apart from the direct polarization process indirect processes exist, by which protons in close proximity to a primary polarized nucleus can acquire spin polarization (de Kanter & Kaptein, 1979). The most important of these is the so-called cross-polarization effect based on dipolar cross-relaxation transitions. Cross-relaxation is also responsible for nuclear Overhauser effects and spin diffusion in proteins (Kalk & Berendsen, 1976; Sykes et al., 1978). In proteins the polarization is transferred with retention of sign, as opposed to the sign reversal that occurs in small molecules (de Kanter & Kaptein, 1979).

The rate at which polarization is transferred by cross-relaxation is proportional to $\tau_c r^{-6}$ where τ_c is the correlation time for the tumbling motion of the protein and r is the internuclear distance. Therefore, the cross-polarization effect is particularly strong in larger (slowly tumbling) proteins and has a pronounced distance dependence. Cross-polarization may occur within the same residue or between different residues. Thus, the tyrosine 2,6 ring protons generally show weak emission due to transfer of polarization from the neighboring 3,5 protons (Kaptein & Edzes, 1979). Transfer between different residues is potentially very useful in establishing proximity relations between residues in a protein.

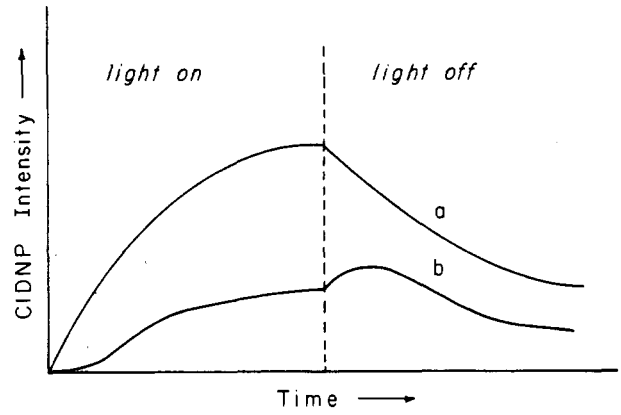

FIGURE 1: Time dependence of the photo-CIDNP intensity after switching light on and off: (a) direct polarization; (b) cross-polarization.

It is interesting to consider the time dependence of the effect. The buildup and decay after switching the light on and off is schematically shown in Figure 1. Whereas the primary polarization would build up and decay with a time constant approximately equal to that of the proton T_1, the cross-polarized proton shows a sigmoidal buildup curve. It lags behind initially and, after the light is switched off, first continues to grow and then decays. This behavior, which has been verified experimentally (Kaptein & Edzes, 1979), suggests a way to discriminate between direct and cross-polarization effects. In fact, this can be done in two ways: first, by employment of short light pulses (usually 0.1 s is short enough), the cross-relaxation effect will then be relatively suppressed; secondly, by employment of a delay between the end of the light pulse and recording to FID, the cross-relaxation effect will be relatively enhanced. Examples of both experiments will be discussed below.

This paper describes and compares the surface exposed aromatic residues of bovine, goat, human, guinea pig, and rabbit α-lactalbumins. A subsequent paper will cover other assignments, including those of internal residues as well.

Results

CIDNP Spectra. Figure 2 depicts photo-CIDNP difference spectra of the aromatic region for the five species: bovine, goat, human, guinea pig, and rabbit α-lactalbumins. These are designated as normal spectra, i.e., a light pulse of 0.6–0.8 s and a delay of 0.05 s before FID acquisition. All are characterized by one or two strong Tyr 3,5 ortho proton (negative) emission lines in the 6.8–7.03-ppm range. All of the species, except human αLA, gave a pH dependent pair of positive absorptions for the C-2 and C-4 protons of an exposed histidine. These show up with bovine, goat, and guinea pig in this figure at 8.16–8.25 ppm and 7.2–7.26 ppm for the C-2 and C-4 protons, respectively. It is interesting to note from Table I that the human, guinea pig, and rabbit species lack His-68, while the latter two species each contain an additional His residue. While complete assignments will be discussed later, it is straightforward from Figure 2 and Table I that human αLA was missing an exposed His.

The remaining (positive) absorption lines in Figure 2, which may be assigned to polarized tryptophan residue(s), appeared as four to five lines in all species *except* guinea pig over the range 5.9–7.7 ppm. The very high-field shifted weak resonance at 5.9 ppm which appeared in bovine, goat, and human αLA was missing in guinea pig and showed up slightly more downfield (6.14 ppm) in rabbit αLA. With progression to the

Table I: α-Lactalbumin Species Differences[a]

bovine	goat	human	guinea pig	rabbit	comments[b]
			His-10		exposed
His-32	His-32	His-32	His-32	His-32	partly buried; near Phe-31
			His-47		partly buried
His-68	His-68	*Gln*	*Gln*	*Gln*	exposed
				His-103	see Tyr-103 below
His-107	His-107	His-107	His-107	His-107	most buried of all His
Trp-26	Trp-26	*Leu*	Trp-26	Trp-26	buried
Trp-60	Trp-60	Trp-60	*Phe*	Trp-60	inaccessible; near surface; almost completely buried in Tyr-103, Asn-56, Asp-97, and Cys-73–Cys 91[c]
Trp-104	Trp-104	Trp-104	Trp-104	Trp-104	surface, cleft
Trp-118	Trp-118	Trp-118	Trp-118	Trp-118	possibly partially solvent accessible; two other buried conformations are energetically equivalent[c]
Tyr-18	Tyr-18	Tyr-18	Tyr-18	Tyr-18	surface; easily solvent accessible[c]
Tyr-36	Tyr-36	Tyr-36	Tyr-36	*Leu*	near surface, hydroxyl group exposed; mostly buried[c]
Tyr-50	Tyr-50	Tyr-50	Tyr-50	Tyr-50	most buried,[c] hydroxyl group exposed
Tyr-103	Tyr-103	Tyr-103	Tyr-103	*His-103*	surface, close to Trp-60; solvent accessible[c]
			Tyr-119		surface, but covered by Leu-25, Val-29, and Leu-129

[a] The numbering system is based on bovine α-lactalbumin after Brew et al. (1970). [b] Summarized from Browne et al. (1969) plus additional footnotes below. [c] Warme et al. (1974).

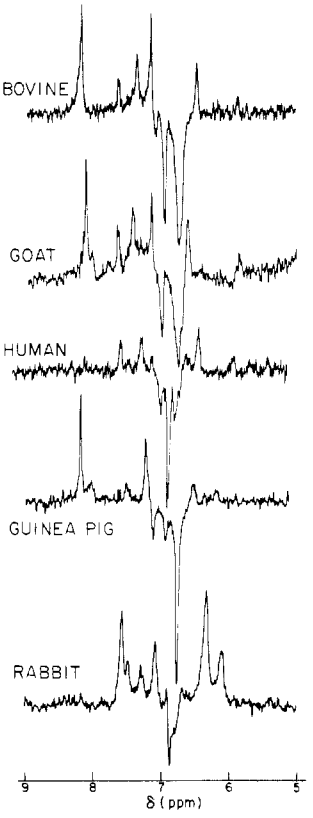

FIGURE 2: Normal laser photo-CIDNP proton spectra of aromatic region of α-lactalbumin species. Instrumental conditions were as follows: 10 or 20 scans; light pulse, 0.6 or 0.8 s; delay before FID, 0.05 s; laser power, 4–7 W; 2 × 4 K data points (quadrature detection); acquisition time, 1 s; total time per light–dark cycle, 15 s. Chemical shifts are relative to internal DSS. Protein concentration and pH in 0.1 M potassium phosphate, D_2O, 30 °C, were as follows: bovine, 0.61 mM, pH 6.37; goat, 0.42 mM, pH 6.57; human, 0.93 mM, pH 6.37; guinea pig, 0.99 mM, pH 6.78; rabbit, 0.55 mM, pH 6.27.

left (larger chemical shifts), a stronger upfield-shifted Trp line appeared in *all* species at 6.3–6.6 ppm and then two resonances in the vicinity of 7.1–7.4 ppm and 7.47–7.65 ppm, respectively. With the assumption at this juncture that these were all Trp resonances, both the number of lines and their large chemical shift range suggest two tryptopahn residues. It is remarkable to note the almost complete identity of the bovine, goat, and human species in these resonance line positions, especially with regard to the unique upfield-shifted resonances at 6.5 and 5.9 ppm, while rabbit αLA differs essentially only by a slight shift in the position of these two resonances, at 6.3 and 6.1 ppm (vs. 6.5 and 5.9 ppm for the other species). On the other hand, guinea pig αLA specifically lacks the high field shifted line at 5.9 ppm, while the remaining resonance lines (attributed to Trp polarizations) at 6.54 and 7.53 ppm are again remarkably similar to the corresponding lines in the other species (Figure 2).

Perhaps the most characteristic resonances which *distinguish* the species from one another (with the exception of the almost complete identity between bovine and goat αLA) are the (negative) Tyr emission lines noted earlier. The broader emission line for bovine and goat αLA at 6.8 ppm (called Tyr-1) actually represents two exposed Tyr residues, while the next (downfield) line at 7.0 ppm is attributed to an additional single Tyr-2. The small emission, most clearly shown for bovine αLA at 7.1 ppm, represents the (cross-polarized) 2,6 protons which are connected with the Tyr line at 7.0 ppm. Cross-polarization phenomena for these and other resonances are presented in the later figures (Figures 3–5). The human species also showed three exposed Tyr, two falling together at 6.8 ppm, similar to Tyr-1 above, yet the single Tyr-2 at 6.95 ppm was stronger. The very weak emission at 7.04 ppm was the cross-polarized 2,6 proton related to the 6.95-ppm line. Guinea pig αLA showed three emissions at 6.78, 6.96, and 7.13 ppm, the strong upfield line (Tyr-1) attributed to one (or possibly two) residues, the center emission (6.96 ppm) to one Tyr, and the lowest field emission (6.78 ppm) again cross-polarized 2,6 Tyr protons coupled to the center line. The 6.88-ppm polarized emission line in rabbit αLA was probably due to a single strongly polarized Tyr. One notes from Table I that rabbit αLA contains only two Tyr compared to four Tyr for the remaining species (except five Tyr for guinea pig αLA).

Cross-Polarization Studies. As discussed under Methods, exposed residues which interact with the flavin should give directly polarized resonance lines in an FID taken immediately after a short light pulse, while the buildup of any cross-polarized nucleus requires a time period related to the nuclear spin–lattice relaxation time (T_1) of the directly polarized residue with which it interacts, the cross-relaxation time being related to the internuclear distance. Therefore, the time range for the spectra measured in Figure 2 under normal CIDNP conditions (0.6–0.8-s light pulse, 0.05-s delay before FID

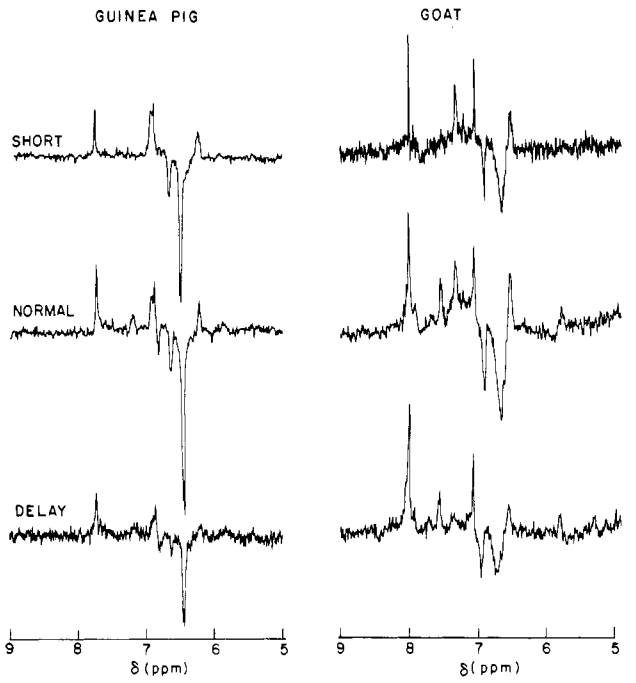

FIGURE 3: Variation in light pulse and preacquisition (FID) delay on aromatic region CIDNP spectra. Short pulse (50 scans), light (14 W) 0.1 s, delay 0.05 s; normal pulse (20 scans), light (7 W) 0.6 s, delay 0.05 s; delay pulse (20 scans), light (7 W) 0.6 s, delay 0.3 s: (a) guinea pig α-lactalbumin, 0.48 mM, pH 7.07 (0.044 M potassium phosphate); (b) goat α-lactalbumin, 0.42 mM, pH 6.58 (0.1 M potassium phosphate). All other conditions were as described in Figure 2.

acquisition) may involve cross-polarization processes as well, which could appear in the resultant Fourier-transformed spectra. Therefore, we subsequently examined each species under a variety of conditions encompassing primarily only direct polarization (short pulse, 0.1-s light, 0.05-s preacquisition delay), normal pulse (as in Figure 2), and primarily cross-polarization (delay pulse, 0.6-s light, 0.3-s preacquisition delay). Figure 3 depicts spectra taken under the short, normal, and delay pulse conditions, respectively, for guinea pig and goat α-lactalbumins. In the case of guinea pig αLA (left), the weakest Tyr emission line at 7.13 ppm, shown here in the normal or delay spectra (as well as in Figure 2), was missing in the short spectrum above where cross-polarization was minimized. This can be assigned to cross-polarized 2,6 protons which sould be related to the Tyr-2 3,5 proton emission at 6.96 ppm.

Noting next the Trp resonances in both proteins, we see that the lowest field Trp resonance at ~7.5–7.6 ppm in both species was missing in the short pulse spectra, began to appear in the normal spectra, and, particularly in the delay spectra with goat αLA, grew at the expense of the other Trp resonances at 7.45 and 6.64 ppm, respectively. Actually, we can only rigorously state that the low-field resonance at 7.67 ppm was a cross-polarized proton from the Trp residues with lines at 7.45 and 6.64 ppm; however, its assignment as one of the nonpolarized protons (7.67 ppm) on the same Trp moiety is reasonable both from its chemical shift and previous observations of similar phenomena (R. Kaptein, unpublished results). Probably the most important resonance to note is the high-field line at 5.86 ppm observed for bovine, goat, and human αLA (Figure 2). Note that for the short-pulse goat αLA spectrum (Figure 3), the 5.86-ppm resonance was missing but grew in the normal and delay spectra at the expense of the lines at 6.64 and 7.45 ppm, respectively, similar to the description for the low-field cross-polarized resonance at 7.67 ppm. The identical behavior

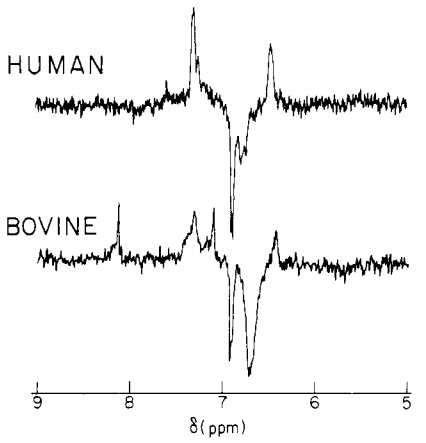

FIGURE 4: Short-pulse photo-CIDNP spectra. Instrumental conditions were as follows: light pulse (14 W), 0.1 s; delay, 0.05 s. Each spectrum represents 50 scans. Protein concentration and pH (0.1 M potassium phosphate) were human, 0.64 mM, pH 6.37, and bovine, 0.61 mM, pH 6.34. All other conditions were as described in Figure 2.

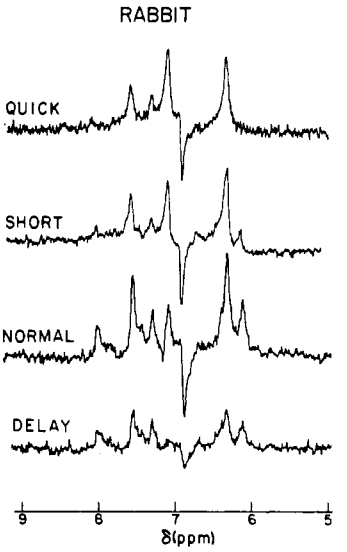

FIGURE 5: Effect of pulse variation on aromatic region of rabbit α-lactalbumin. Instrumental conditions were as follows: (a) quick pulse (100 scans), light (14 W) 0.05 s, delay 0.01 s; (b) short pulse (50 scans), light (14 W) 0.1 s, delay 0.05 s; (c) normal pulse (10 scans), light (7 W) 0.8 s, delay 0.05 s; (d) delay pulse (10 scans), light (9 W) 0.6 s, delay 0.3 s. Protein concentration was 0.61 mM, pH 7.4 (0.13 M potassium phosphate). All other conditions were as described in Figure 2.

occurred with human and bovine αLA as well, as depicted in the short-pulse spectra (Figure 4), to be compared with the normal spectra in Figure 2. In all cases, with the *exception* of guinea pig αLA, the high-field line at ~5.9 ppm was a cross-polarized resonance by virtue of its absence in the short-pulse spectra (Figures 3 and 4). It is important to note at this point the unique omission in guinea pig αLA of Trp-60, compared with the bovine, goat, and human species (Table I). Last, we note that the small emissions (Figure 2) at 7.04 ppm in human and 7.07 ppm in bovine αLA, respectively, were missing in the short-pulse spectra (Figure 4), confirming their identification as cross-polarized Tyr 2,6 protons of the Tyr 3,5 emissions discussed earlier in Figure 2.

Rabbit α-Lactalbumin. Our results with rabbit αLA showed many qualitative similarities to the other species above, while some unique features also appeared which were probably attributable to distinct differences in primary structure between this and the other species above.[2] Examining first the short-, normal-, and delay-pulse spectra in Figure 5, we note that the

upfield-shifted Trp line at 6.14 ppm grows analogously to the corresponding line at 5.9 ppm in the bovine, goat, and human species (Figures 3 and 4); however, it was not completely suppressed in the short-pulse spectrum (Figure 5). Recalling that cross-polarization is a function of nuclear relaxation times, we realized that, in this case, the cross-relaxation times may be shorter than in the other species. By applying an extremely short light pulse and delay (0.05 s and 0.01 s, respectively), we obtained the "quick-pulse" spectrum shown in Figure 5 (top) where the 6.14-ppm resonance was now absent. In looking at this series in general (Figure 5), we note that the relative intensity of the 6.14-ppm line increased at the expense of the 6.34-ppm line. The two additional positively polarized lines at 7.58 and 7.31 ppm in Figures 2 and 5 did not, however, show behavior analogous to the other species. One notes from the pulse series of Figure 5 that these two lines were present from the quick-pulse through the normal-pulse spectra with little, if any, change in intensity. These lines (7.58 and 7.31 ppm) must have been contributed by a *directly* polarized residue which may be assigned to a Trp residue by virtue of their positive polarization, pH-independent chemical shifts, and the observation of an additional directly polarized emission doublet in the aliphatic region (3.1 ppm, not shown) corresponding to β-CH_2 protons. By contrast, human αLA, which had only one directly polarized Trp (see Figure 4), produced only one β-CH_2 emission doublet in the aliphatic region (not shown). By comparison, we recall the results above with the other species, where the absorption line in the 7.5–7.6-ppm range was a cross-polarized proton from a Trp residue, which for rabbit αLA would correspond to the 7.10- and 6.34-ppm lines. The corresponding cross-polarized line would be the resonance at 7.58 ppm discussed above. It is conceivable that this latter resonance may be the coincidental superposition of a directly polarized Trp and a cross-polarized Trp, accounting for its relatively strong intensity in the rabbit αLA spectra.

The second unique feature about the rabbit αLA series (Figure 5) was the line at 8.01 ppm in the normal-pulse spectrum (pH 7.4) which decreased in intensity at lower pH and was already absent at pH 6.27 (Figure 2). It is clear from the pulse series in Figure 5 that this resonance was from a cross-polarized residue by virtue of its gradual appearance from the quick- through delay-pulse spectra, while the 7.10-ppm Trp resonance *decreased* over the same spectral series.

pH-Dependent Surface Exposure. In general, these lactalbumin species show very small changes, if any, in aromatic residue chemical shifts except, of course, for His residues over the neutral pH range. On the other hand, the degree of exposure (i.e., accessibility to the flavin dye) appeared to change slightly, for example, with bovine αLA as depicted in Figure 6. The two spectra shown at pH 6.0 and 7.6, respectively, were subtracted to yield the difference spectrum (bottom, Figure 6) where the two sharp positive resonances (8.4 and 7.25 ppm) and two sharp negative resonances (7.75 and 7.02 ppm) represent the C-2 and C-4 protons of the single exposed His residue at the two pH values, respectively. The remaining differences (positive line at 6.76 ppm and negative line at 6.95 ppm, respectively) arise purely from the polarized Tyr resonances. It should be emphasized that the changes detected here are subtle, probably local, surface exposure alterations which would likely be passed over by fluorescence or difference spectroscopic methods which are more sensitive to global changes (Sommers & Kronman, 1980).

Discussion

The laser photo-CIDNP results allow an unequivocal assessment of the number and possible assignment of solvent

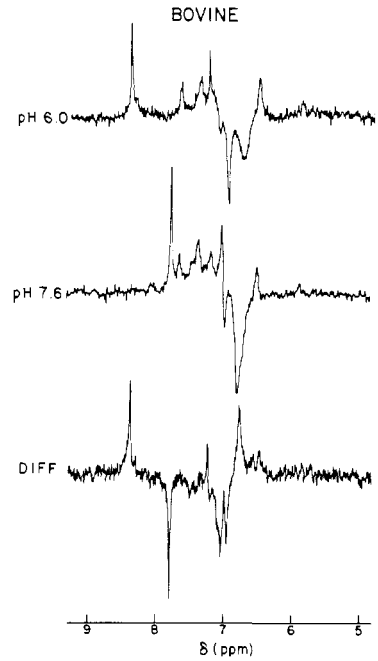

FIGURE 6: Effect of pH on tyrosine exposure in bovine α-lactalbumin. Photo-CIDNP spectra of the aromatic region are shown for 0.62 mM bovine αLA at pH 6.0, pH 7.61, and their difference, respectively. All other conditions were as described in Figure 2.

(flavin dye) accessible aromatic residues. Furthermore, the cross-polarization studies allow some insight about nonexposed residues which are close enough to polarized nuclei for efficient spin diffusion.

Histidine Exposure. Comparing the CIDNP spectra for bovine, goat, and human αLA (Figure 2), we note the distinct absence of any His polarization in human αLA, while bovine and goat show the C-2 and C-4 protons, respectively, of a single exposed His residue. Their sequence comparisons (Table I) clearly show that the only difference in His content is at position 68 where human αLA has substituted a Gln residue. Furthermore, correlation of this His resonance in bovine αLA with previous assignments (Bradbury & Norton, 1975) serves as double confirmation of the only exposed histidine residue in bovine αLA (and by homology, goat αLA) as His-68. These results shed doubts on surface-accessibility conclusions drawn from chemical modification studies. In particular, Prieels et al. (1979), in an extension of the earlier work of Schindler et al. (1976) on diethyl pyrocarbonate inactivation of human αLA, reconfirmed the specific ethoxyformylation of N-1 of His-32. With the bovine species, at concentrations favoring only monomeric species ($\sim$1 mg/mL, pH 6.15), they suggested that His-68 reacted first at a 10-fold excess of reagent while His-32 was blocked at higher reagent concentrations (Prieels et al., 1979). Of most note, however, were their results at bovine αLA concentrations of 46 mg/mL, where dimer or higher aggregates were suspected to occur (Kronman & Andreotti, 1964; Kronman et al., 1967; Berliner & Kaptein, 1980), which showed that only His-32 was accessible to the diethyl pyrocarbonate.

While chemical modification studies are actually kinetic phenomena, the implied accessibility of His-32 by these results above cannot be properly judged unless the extent and specificity of modification were monitored with time and compared to model compounds containing moieties of well-established exposure. Secondly, a great deal of caution must be applied to the results at high concentrations of bovine αLA since CIDNP studies in earlier work specifically addressed the associative behavior of bovine αLA and showed only His-68

as a surface-exposed residue over a broad pH and concentration range. His-32 polarization was never observed at any concentration (Berliner & Kaptein, 1980; L. J. Berliner and R. Kaptein, unpublished results).

On the other hand, earlier work by Castellino & Hill (1970) showed that carboxymethylation of bovine αLA with iodoacetic acid preferentially modified His-68 over His-32 at about a 3-fold faster rate. Bradbury & Norton (1975) found that the ^1H–^2H exchange rate was slightly faster for the most buried His-107 than for His-68 but realized that both the lower pK_a of His-107 and its proposed environment could account for such a result as an exception to the carboxymethylation data and, most importantly, the proposed three-dimensional model which shows without a doubt that His-68 is the only completely exposed residue (Browne et al., 1969).

Results from the photo-CIDNP studies are summarized here as follows: bovine and goat αLA have an exposed His-68; guinea pig αLA also contains only one exposed histidine whose assignment at this juncture is suggested from model building (Table I) to be the most exposed His-10 probably requires further characterization.

Tryptophan Exposure. The photo-CIDNP evidence accounted for only one accessible Trp for all but rabbit αLA. As discussed in Figure 2, the additional upfield resonance (~5.9 ppm) has been assigned to a Trp-60 proton, since its *absence* in guinea pig αLA correlated specifically with the deletion of Trp-60; i.e., it was not a directly polarized residue and was therefore not exposed. Yet, if we compare previous chemical modification work, anywhere from one to three Trp residues have been modified in bovine αLA, the most reactive Trp varying with modification technique. Barman (1970, 1972) and Barman & Bagshaw (1972) found that 2-hydroxy-5-nitrobenzyl bromide reacted with Trp-26, as well as Trp-104 and -118, despite the fact that the analogous tryptophan in lysozyme and the proposed environment for Trp-26 in the bovine αLA model showed it to be completely buried. Bell et al. (1975) showed that Trp-26 was also modified with *N*-bromosuccinimide along with the faster reacting Trp-118. On the other hand, while Trp-60 was unreactive with the reagent above, treatment of bovine αLA with 2-nitrophenylsulfenyl chloride resulted in modification of Trp-60 as well as Trp-118 (Schechter et al., 1974). Photooxidation using proflavin as a sensitizer resulted in the modification of three Trp residues, two of which reacted faster than the third (Tamburro et al., 1972). Again here the inconsistencies among the various modification experiments point to the fact that these results are a complex function of kinetic factors as well as (possible) conformational changes associated with reagent binding. On the other hand, the CIDNP phenomenon is not manifested if strong dye binding occurs, since the radical pair diffusion step must occur in a nanosecond to microsecond time domain.

Sommers & Kronman (1980) have analyzed Trp fluorescence and solvent perturbation measurements on bovine αLA with much the same conclusions drawn from the CIDNP data. Past solvent perturbation data (Kronman et al., 1965, 1972; Takase et al., 1978) showed from 1.4 to 1.8 exposed Trp groups. Quenching by I^- was quite weak, supporting their conclusions drawn from the "lysozyme model" that none of the four Trp in bovine αLA was fully exposed and possibly some were partially exposed to the external solvent. Furthermore, they correctly reinterpreted the fluorescence red shift of the excitation spectrum of *N*-bromosuccinimide-oxidized Trp-118 (Bell et al., 1975) to conclude that this moiety is most likely *not* solvent exposed which was also consistent with the calculations of Warme et al. (1974). From analysis of the molecular model for αLA, this left only Trp-60 and -104 as potentially solvent-accessible residues, the former of which is almost completely shielded by Tyr-103, Asp-97, and disulfide bridge 73–91 (Warme et al., 1974; Browne et al., 1969). Furthermore, the 6-Å or less separation between Trp-104 and Trp-60 was optimal for Forster energy transfer as a very efficient mechanism for energy transfer quenching of Trp-104 by Trp-60. Guinea pig αLA, which is missing Trp-60, had a much higher quantum yield since this energy transfer quenching was no longer possible (Sommers & Kronman, 1980). Recalling that a Trp resonance in the 6.3–6.6-ppm range appeared in all species (Figure 2) and was coupled via cross-relaxation to a higher field resonance at 5.9–6.1 ppm in the four species which contained Trp-60 (Table I), we can conclude that Trp-104 was the exposed Trp residue in all species (6.3–6.6 ppm); this was also consistent with the results of Warme et al. (1974). Cross-relaxation to a neighboring tryptophan (i.e., Trp-60, 5.9–6.1 ppm) was in total agreement with the data of Sommers & Kronman (1980) which supported energy-transfer quenching of Trp-104 by nearby Trp-60. These cross-relaxation and quenching contributions were absent in guinea pig αLA which lacks this (internal) Trp-60 (see Figures 2 and 3; Sommers & Kronman, 1980).

Tyrosine Exposure. For all of the αLA species which contain four or more Tyr in their sequence (see Table I) the CIDNP data have shown that three are exposed (with possibly one less in the case of guinea pig αLA). The CIDNP spectra for most of the αLA species (Figure 2) showed two almost unresolvable Tyr resonances at ~6.80 ppm, a chemical shift almost identical with those for tyrosine model compounds or highly exposed Tyr residues in other proteins (Kaptein, 1978), suggesting that two Tyr were possibly either completely solvated or in a physicochemical environment which highly resembles the solvent. The third Tyr was slightly downfield, around 7.0 ppm, in some site where some deshielding interactions may have been affected (ring current, hydrogen bonding, etc.). By deduction we may strongly infer that, where four Tyr were homologous in all αLA species (except rabbit αLA which contains only two Tyr), the one *buried* Tyr in bovine, goat, human, and rabbit αLA may be homologous to all species. This was consistent with the results of Warme et al. (1974) and should tentatively be assigned as Tyr-50 (see Table I).

Previous chemical modification data on bovine αLA are in more agreement although no characterization studies have been reported to date to our knowledge. The majority of the modification studies pointed to three reactive Tyr groups plus a fourth Tyr which reacted more slowly at higher reagent to protein ratios (Hill & Brew, 1975).

Lastly, it is interesting to address the many efforts at comparing α-lactalbumin with the highly homologous sequence for hen egg white (HEW) lysozyme. While the previous literature on this subject is too extensive to cite in detail here, it is sufficient to say that the combined results of optical spectroscopy, immunocross-reactivity, catalytic cross-reactivity, chemical modification, and various thermodynamic stability studies rarely gave sufficiently solid evidence for conformational homology. The same conclusions arose when comparing CIDNP data between HEW lysozyme (Kaptein, 1978) and the lactalbumins due, in great part, to key sequence changes in Trp, Tyr, and His residues both on the surface and near the purported active sites for each protein. In fact, at room temperature, lysozyme had only two polarized Trp residues, yet none of the three Tyr residues become exposed until tem-

peratures above 50 °C (R. Kaptein, unpublished experiments). However, this should not be too alarming since the major homologies between proteins should be with internal structure, which dictates polypeptide backbone folding, *not* surface structure which is sensitive to the distribution of charged residues as well.

Rabbit α-Lactalbumin Assignments. The two resonances at 7.10 and 6.33 ppm (Figures 2 and 5) which arose from a Trp residue which cross-relaxed the resonance at 6.14 ppm were totally analogous in the bovine, goat, and human αLA species to the cross-polarization of Trp-60 by Trp-104. The distinct chemical shifts for these residues in rabbit αLA vs. the other species (Trp-104, 7.10 and 6.33 ppm vs. 7.4–7.5 and 6.5–6.6 ppm; Trp-60, 6.14 vs. 6.5–6.6 ppm) can be easily understood by virtue of the substitution at position 103 of a histidine vs. Tyr in all of the other species. Tyr-103 lies close enough to both Trp-104 and -60 to expect substantive ring current effects. Thus replacement of Tyr-103 by a His in rabbit αLA would expectedly result in altered magnetic environments for neighboring residues. Thus the assignments of Trp-104 and -60 for the upfield Trp absorptions in Figures 2 and 5 are consistent with the results from the other species in Figures 2–4.

The additional Trp resonances in rabbit αLA at 7.58 and 7.31 ppm (Figures 2 and 5) arose from an additional directly polarized tryptophan by virtue of its chemical shift, pH invariance, and additional aliphatic emission doublet (see Results). From examination of Table I and the results with human αLA, there is little doubt that this directly polarized residue was Trp-118, which must have enjoyed a higher percentage of exposed conformations in the less homologous rabbit αLA than in the other species in the αLA family (Warme et al., 1974). Specifically, rabbit αLA is one residue shorter, missing the carboxy terminal Leu-123 (Gln-123 in guinea pig αLA) which forms a salt bridge to the ϵ-NH_3^+ of Lys-5 and helps to form the hydrophobic pocket conformations surrounding Trp-118. Deletion of Leu-123 in rabbit αLA correlates completely with the one completely exposed conformation, T2, where the carboxy terminal residues are away from Trp-118 (Warme et al., 1974).

Last, the 8.01-ppm cross-polarized absorption line in Figure 5, which was absent at pHs below 7.0, has been assigned to a histidine C-2 resonance, in part, on the basis of its chemical shift. Recalling that His-103 was substituted in rabbit αLA for a Tyr which is situated quite near Trp-104 and -60 and recalling that a cross-polarized proton retains the sign of its directly polarized donor (i.e., a positive absorption), it follows that the directly polarized donor must have been a tryptophan, specifically Trp-104.[3] The odd pH dependence of this cross-polarized resonance (His-103) may have been due in part to the pH dependence noted for histidine model compounds (R. Kaptein, unpublished results) but also must definitely involve subtle structural aspects of its orientation in the protein.

Conclusions

The photo-CIDNP results for five αLA species confirm a high degree of surface exposure homology from species to species, with only minor differences in chemical shift environment between them. The results of cross-polarization short-pulse studies (Figures 3–5) gave evidence for a homologous low-field resonance (approximately 7.5 to 7.7 ppm) in all species (except rabbit αLA) which grew at the expense of a single polarized Trp-104 with lines spanning approximately 6.5–7.4 ppm. (It is quite possible that this low-field cross-relaxed line arose from a nonpolarized proton, e.g., on C-5 or C-7 of the same Trp indole nucleus.) With the exception of guinea pig αLA, another upfield-shifted resonance appeared which probably arose from a nearby internal (Trp-60) residue in all species. The close identity in chemical shift for bovine, goat, and human αLA strongly suggests that this Trp side chain resides in an almost identical environment in each species. The correlation between Trp-60 cross-relaxation by Trp-104 for bovine, goat, and human αLA was in complete correspondence with the intramolecular energy-transfer quenching found by Sommers & Kronman (1980) for these residues; the guinea pig αLA species, which is missing Trp-60, showed no such effects as expected. The only exposed Trp was residue 104, with the exception of rabbit which also showed evidence for exposed conformations of Trp-118. In the case of rabbit αLA, on the other hand, the chemical shift predicted that this residue must reside in a slightly altered environment. Evidence for a single exposed histidine residue occurred for all but human and rabbit αLA; while Table I indicated that all species contain His-32 and -107, the three species displaying His polarization each contained one very exposed His residue from both the "lysozyme" model analysis and previous assignments (Bradbury & Norton, 1975). This would implicate His-68 on both bovine and goat αLA and His-10 on guinea pig αLA. External accessibility was found for three of the four Tyr residues in bovine, goat, and human αLA while guinea pig αLA showed at most three of the five Tyr in its sequence. Finally, the studies with rabbit αLA gave evidence for one exposed Tyr and one buried Tyr. Thus with the exception of two buried Tyr in guinea pig αLA (four Tyr are sequence homologous to the other species) all lactalbumins have one homologous Tyr-50 which is buried in all species.

Most significantly, the laser photo-CIDNP method is definitely a surface probe technique by virtue of the short lifetime collisional nature of the flavin dye–protein interaction. As has been demonstrated here, photo-CIDNP sheds considerable light on the surface characteristics of a protein in solution, which also correlates well with information from atomic model studies. Again conclusions drawn from chemical modification must be evaluated with extreme caution when assessing solvent accessibility of specific amino acid side chains.

Acknowledgments

Several colleagues generously supplied purified lactalbumin samples without whose support and interest this project would have not proved so informative: Dr. M. J. Kronman, Upstate Medical Center, Syracuse (goat), Drs. R. K. Craig and P. N. Campbell, Courtauld Institute, London (human and guinea pig), and Dr. I. Kiyosawa, Morinaga Milk Industries, Tokyo (human). Dr. Carey Hanly, University of Illinois Medical Center, Chicago, kindly supplied rabbit whey. We are also grateful to Dr. M. J. Kronman for many helpful discussions and for a prepublication manuscript. L.J.B. also wishes to thank Drs. G. T. Robillard and K. Dijkstra for their help and hospitality in Groningen.

References

Barman, T. E. (1970) *J. Mol. Biol. 52*, 391–394.
Barman, T. E. (1972) *Biochim. Biophys. Acta 258*, 297–313.
Barman, T. E., & Bagshaw, W. (1972) *Biochim. Biophys. Acta 278*, 491–500.

[3] The only other possible donor, Trp-118, lies too distant (>15 Å) from this histidine (Browne et al., 1969). Furthermore, the resonances assigned to Trp-118 (7.58 and 7.31 ppm) did not lose intensity at the expense of this 8.01-ppm line (see Figure 5).

Bell, J. E., Castellino, F. J., Trayer, J. P., & Hill, R. L. (1975) *J. Biol. Chem. 250*, 7579–7585.

Berliner, L. J., & Kaptein, R. (1980) *J. Biol. Chem. 255*, 3261–3262.

Bolscher, B. G. J. M., Lenstra, J. A., & Kaptein, R. (1979) *J. Magn. Reson. 35*, 163–166.

Bradbury, J. H., & Norton, R. S. (1975) *Eur. J. Biochem. 53*, 387–396.

Brew, K. (1972) *Eur. J. Biochem. 27*, 341–353.

Brew, K., Castellino, F., Vanaman, T. C., & Hill, R. L. (1970) *J. Biol. Chem. 245*, 4570–4582.

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.

Castellino, F. J., & Hill, R. L. (1970) *J. Biol. Chem. 245*, 417–424.

de Kanter, F. J. J., & Kaptein, R. (1979) *Chem. Phys. Lett. 62*, 421–425.

Findlay, J. B. C., & Brew, K. (1972) *Eur. J. Biochem. 27*, 65–68.

Hill, R. L., & Brew, K. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 411–490.

Hopp, T. P., & Woods, K. R. (1979) *Biochemistry 18*, 5182–5191.

Hopper, K. E., & McKenzie, M. A. (1974) *Mol. Cell. Biochem. 3*, 93–108.

Kalk, A., & Berendsen, H. J. C. (1976) *J. Magn. Reson. 24*, 343–366.

Kaptein, R. (1977) *NATO Adv. Study Inst. Ser., Ser. C 34*, 1–16.

Kaptein, R. (1978) *Jerusalem Symp. Quantum Chem. Biochem. 11*, 211–229.

Kaptein, R., & Edzes, H. T. (1979) in *Magnetic Resonance and Related Phenomena* (Kundla, E., Ed.) p 158, Springer, New York.

Kaptein, R., Dijkstra, K., & Nicolay, K. (1978) *Nature (London) 274*, 293–294.

Kronman, M. J., & Andreotti, R. E. (1964) *Biochemistry 3*, 1145–1151.

Kronman, M. J., Cerenkowski, L., & Holmes, L. G. (1965) *Biochemistry 4*, 518–525.

Kronman, M. J., Holmes, L. G., & Robbins, F. M. (1967) *Biochim. Biophys. Acta 133*, 46–55.

Kronman, M. J., Hoffman, W. B., Jeroszko, J., & Sage, G. W. (1972) *Biochim. Biophys. Acta 285*, 124–144.

Lenstra, J. A., Bolscher, B. G. J. M., Stob, S., Beintema, J. J., & Kaptein, R. (1979) *Eur. J. Biochem. 98*, 385–397.

Ley, J. M., & Jenness, R. (1970) *Arch. Biochem. Biophys. 138*, 464–469.

MacGillivary, R. T. A., Barnes, K., & Brew, K. (1979) *Arch. Biochem. Biophys. 197*, 404–414.

Prieels, J. P., Poortmans, J., Dolmans, M., & Leonis, J. (1975) *Eur. J. Biochem. 50*, 523–527.

Prieels, J. P., Bell, J. E., Schindler, M., Castellino, F. J., & Hill, R. L. (1979) *Biochemistry 18*, 1771–1776.

Quarfoth, G. J., & Jenness, R. (1975) *Biochim. Biophys. Acta 379*, 476–487.

Schechter, Y., Patchornik, A., & Burstein, Y. (1974) *J. Biol. Chem. 249*, 413–419.

Schindler, M., Sharon, N., & Prieels, J. P. (1976) *Biochem. Biophys. Res. Commun. 69*, 167–173.

Sommers, P. B., & Kronman, M. J. (1980) *Biophys. Chem. 11*, 217–232.

Sykes, B. D., Hull, W. E., & Snijder, G. H. (1978) *Biophys. J. 21*, 137–146.

Takase, K., Niki, R., & Arima, S. (1978) *J. Biochem. (Tokyo) 83*, 371–378.

Tamburro, A. M., Jori, G., Vidali, G., Scatturin, A., & Saccomani, G. (1972) *Biochim. Biophys. Acta 263*, 704–713.

Tanahashi, N., Brodbeck, U., & Ebner, K. E. (1968) *Biochim. Biophys. Acta 154*, 247–249.

Warme, P. E., Momany, F. A., Romball, S. W., Tuttle, R. W., & Scheraga, H. A. (1974) *Biochemistry 13*, 768–781.