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Laser chemically induced dynamic nuclear polarization studies in proteins: α-lactalbumin

Estudios de dinámica de polarización nuclear inducidos químicamente por laser en proteínas: α-lactalbúmina

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The 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.*, 1982). Photo excitación 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.*

The side chain protons of histidine, tyrosine, and tryptophan can be polarized, while those of the other aromatic residue, phenylalanine, 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 (ortho) 3,5 protons, while the 2,6 protons may show either an absorption or a weak indirect emission effect. Histidine shows an enhanced absorption effect for the C-2 and C-4 protons. Tryptohan also shows positive polarization for the C-2, C-4, and C-6 protons on the indole ring. Since His proton chemical shifts are pH dependent, especially in the neutral pH range (whereas 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 (see Kaptein, 1982).

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. Some preliminary conclusions can be 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 a cleft region, it should be realized that 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 of fluorescence

quenching. Finally, it is important to note that the polarization process requires separation of the intermediate radicals, taking place in a short time interval (approximately 10^{-9} - 10^{-7} s). 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 cannot always be excluded.

Cross-polarization effects. Besides the direct polarization outlined above, indirect processes exist whereby protons in close proximity to a primary polarized nucleus can acquire spin polarization. The most important is the 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). In proteins the polarization is transferred with retention of sign, as opposed to the sign reversal that occurs in small molecules (Kaptein, 1982).

The rate at which polarization is transferred via 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. Thus, 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 of 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.

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. While the primary polarization buildups and decays with a time constant approximately equal to the proton T_1, the cross-polarized proton shows a sigmoidal buildup curve. In initially lags behind and, after the light is switched off, then continues to grow and then decays. This behavior has been verified experimentally by Kaptein & Edzes (1979) and suggests a way to

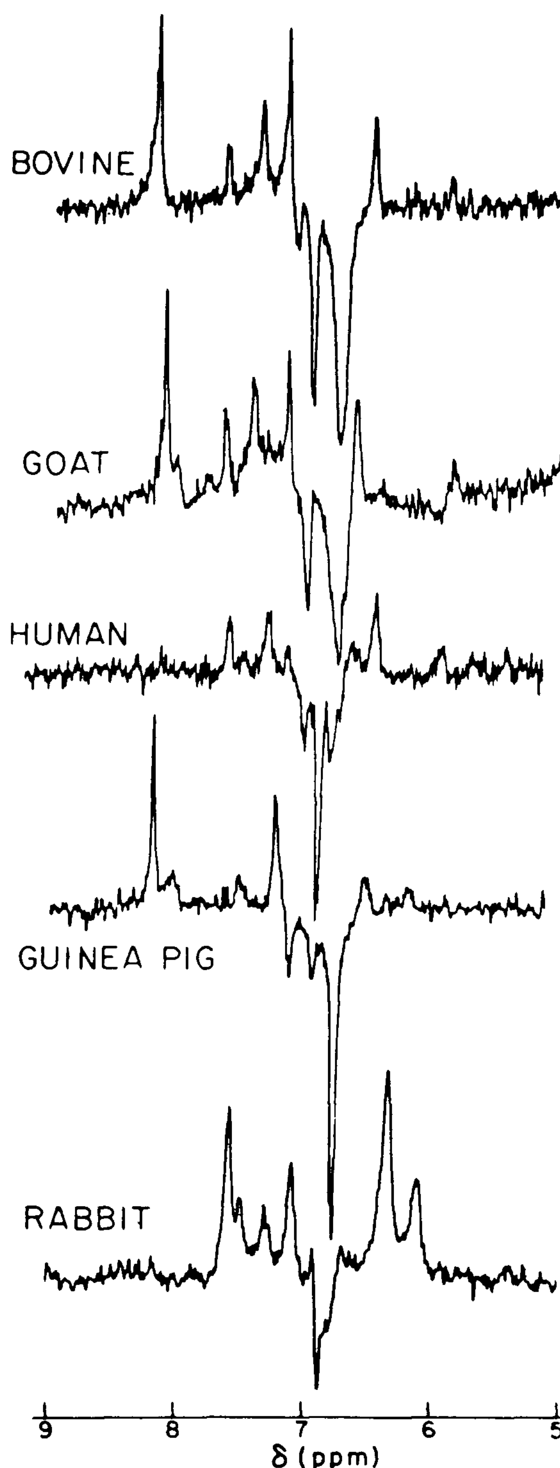

Fig. 1: Time dependence of the photo-CIDNP intensity after switching light on and off: (a) direct polarization; (b) cross-polarization. From Berliner and Kaptein (1981).

discriminate between direct and cross-polarization effects. This may be done in two ways: 1), by using short light pulses (usually 0.1 s is short enough), the cross-relaxation effect will then be relatively suppressed; 2) by inserting a delay between the end of the light pulse and recording of the FID, the cross-relaxation effect will be relatively enhanced. Examples of both experiments are discussed in this paper.

The potential applications of this technique appear to be manifold; in particular, those residues in a specific binding site on a protein surface should be diminished or eliminated in photo CIDNP spectrum in the presence of site specific ligands (substrates, inhibitors, cofactors, other proteins, etc.). An excellent review covering biological applications appeared recently (Kaptein, 1982).

α-Lactalbumin (α-LA), a principal milk whey component, functions as a modifier protein in lactose biosynthesis. The protein-enzyme lactose synthase complex between α-LA and galactosyltransferase exhibits >1000-fold stronger affinity for glucose than in the absence of α-LA. The unique structural changes which accompany the association of one molecule each of galactosyl-transferase and the α-LA manifest themselves in an altered specificity from N-acetylglucosamine to glucose acceptors. A three-dimensional α-lactalbumin structure has not yet been reported, however a model-building study has been reported for bovine α-LA based on the atomic model for lysozyme (Browne *et al.*, 1969). Independently, Warme *et al.* (1974) have reported energy minimization calculations for the bovine α-LA conformer. Our work with several α-LA species showed the extremely strong binding of calcium and lanthanide ions to a single specific site, causing a unique conformational change as monitored by a distinct blue shift and quenching of the instrinsic fluorescence (Murakami *et al.*, 1982).

EXPERIMENTAL

Briefly, the method involves the inducement of nuclear polarization at surface Tyr, Trp and His residues resulting from radical pair formation between the exposed residue(s) and a laser photoexcited dye molecule (3-N-carboxymethyllumiflavin). Fortuitously, the overall reaction is principally cyclical (to yield the native residue and monomeric dye molecule). The contributions of other polarized side reaction products are minimal. Experimentally, the method involves the irradiation of a solution containing a protein (>100 μM) and dye (~100 μM) by an argon-ion laser (Spectra Physics Model 171) in the probe of a high resolution NMR spectrometer in the proton pulse Fourier transform mode. Gating of light and rf pulses (computer-controlled) are collected in alternating light and dark free induction decays. Recently an inexpensive method was reported for adapting any NMR spectrometer for CIDNP using fiber optics coupling (Scheffler *et al.*, 1985). Typically protein concentrations in the 100 μM to 1 mM range yield sufficient sensitivity after 10 scans to identify all (or most) surface aromatic residues which have come in contact with the dye. A typical enhancement over the conventional NMR spectral line of a particular residue runs conservatively in the 50 to 100 fold range.

RESULTS AND DISCUSSION

Fig. 2 depicts photo-CIDNP difference spectra for the aromatic region of five α-LA species: bovine, goat, human, guinea pig, and rabbit (Berliner and Kaptein, 1981). All are characterized by one or two strong Tyr 3,5 ortho proton (negative) emission lines in the 6.8 - 7.08 ppm range. All of the species, except human α-LA, gave a pH dependent pair of positive absorptions for the C-2 and C-4 protons of an exposed His. These showed up 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 that the human, guinea pig, and rabbit species lack His-68, although the latter two species each contain an additional His residue. It is straightforward that human α-LA was missing an exposed His. The remaining (positive) absorption lines in Fig. 2, which may be assigned to

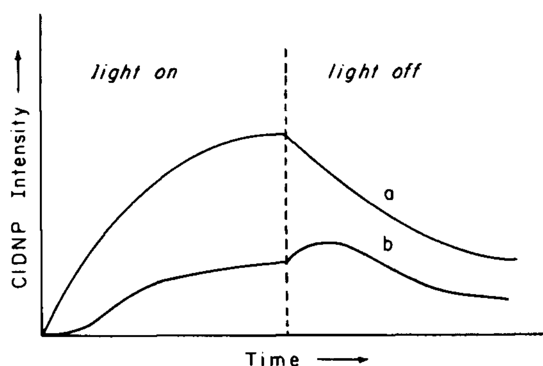

Fig. 2: Laser photo-CIDNP proton spectra of spectra of aromatic region of α-LA species. Instrumental conditions were as follows; 10 or 20 scans; light pulse, 0.6 or 0.8 s; delay before FID, 0.05 s; laser power, 4-7 W; 2 x 4 K data points (quadrature detection); acquisition time, 1 s; total time per light-dark 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. From Berliner and Kaptein (1981).

polarized Trp residue(s), appeared as four to five lines in all species *except* guinea pig over the range 5.9 - 7.7 ppm. The upfield weak shifted resonance at 5.9 ppm in bovine, goat, and human α-LA was *missing* in guinea pig. A stronger upfield-shifted Trp line appeared in all species at 6.3 - 6.6 ppm and as two resonances in the vicinity of 7.1 - 7.4 ppm and 7.47 - 7.65 ppm; their large chemical shift range suggest two tryptophan residues.

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, we subsequently examined each spectra under conditions encompassing only direct polarization (short pulse), *normal* pulse (as in Fig. 2), cross-polarization (*delay* pulse). In Fig. 3 for guinea pig α-LA (left) the weakest Tyr emission line at 7.13 ppm was missing in the *short* spectrum. This can be assigned to cross-polarized 2,6 protons, related to the Tyr 3,5 proton emission at 6.96 ppm. 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 grew at the expense of the other Trp resonances at 7.5 and 6.64 ppm, respectively. We can rigorously state that the 7.67 ppm resonance was a cross-polarized proton from the Trp lines at 7.45 and 6.64 ppm; its assignment as one of the nonpolarized protons (7.67 ppm) on the same Trp moiety is reasonable from its chemical shift. The 5.86 ppm resonance in goat α-LA was missing but grew in the *normal* and *delay* spectra at the expense of the line at 6.64 and 7.45 ppm. The identical behavior occurred with human and bovine α-LA with the *exception* of guinea pig α-LA where it was absent.

Effects of calcium binding

If one monitors bovine α-LA structure by high resolution 500 MHz proton NMR, slight shifts are noted for almost every resolvable proton including those Trp residues which have been assigned above (Trp 60, 104) (K. Koga and L.J. Berliner, manuscript in preparation). A striking result was observed from our CIDNP studies of calcium binding which indicated that the two overlapping Tyr resonances at 6.8 ppm (Fig. 2) totally disappeared (Berliner *et al.*, 1987). Except for a possible increase in the degree of cross-polarization to Trp 60 at 5.9 ppm, the remainder of the CIDNP spectrum remained unaffected. Thus the changes in Trp residue conformation associated with the fluorescence quenching and blue shift were not associated with solvent exposure to the flavin dye.

CONCLUSIONS

At least one Trp is quenched as a result of the metal induced conformational change. Several pieces of evidence point to Trp 118 as this residue. Specifically, we found that rabbit α-LA lacks a C-

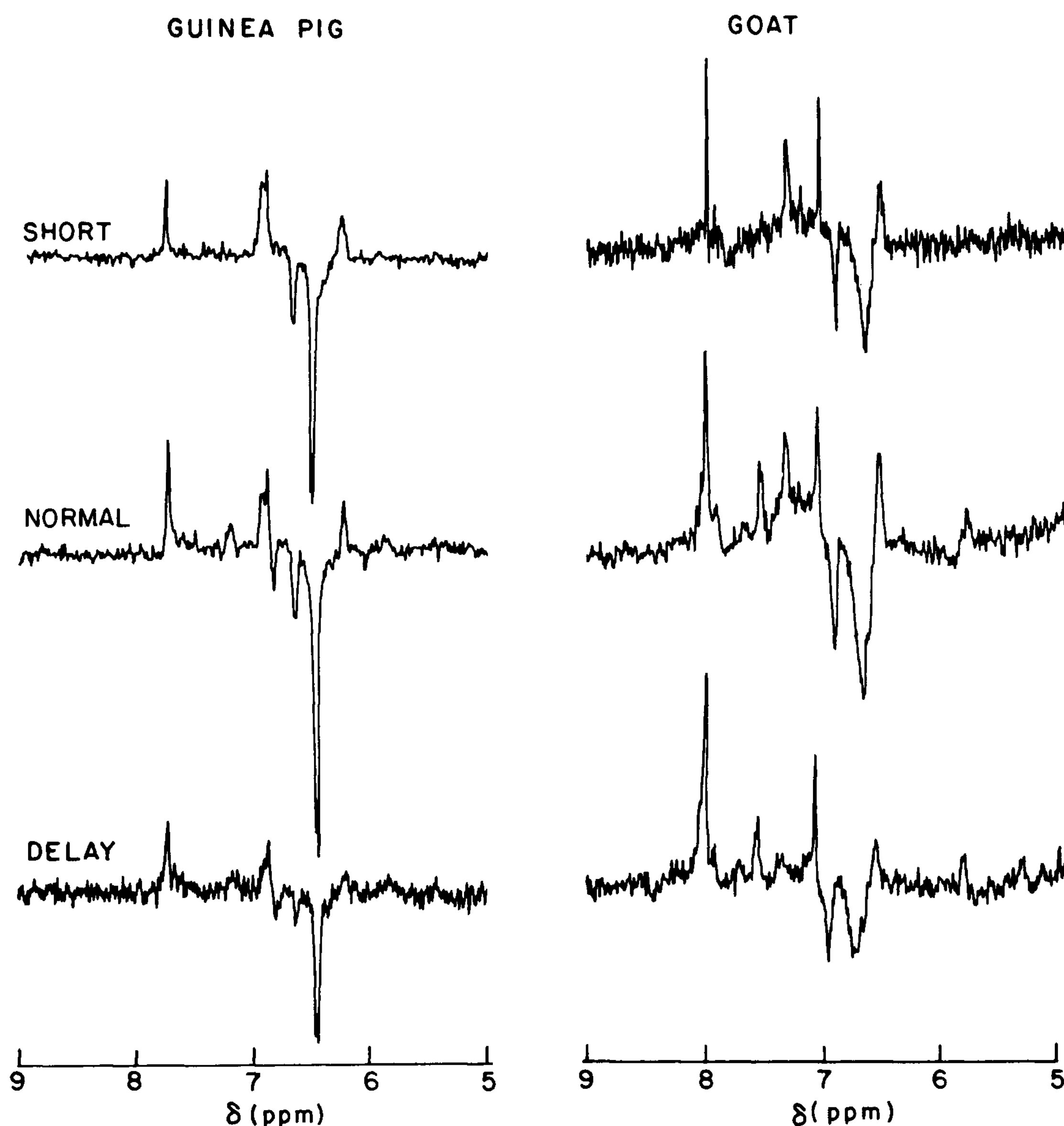

Fig. 3: Variation in light pulse delay on CIDNP spectra. *Short* pulse (50 scans), light (14 W) 0.1 s, delay 0.05 s; *normal* pulse (20 scans), light (7 W) 0.6 s, delay 0.05 s; *delay* pulse (20 scans), light (7 W) 0.6 s, delay 0.3 s. (a) guinea pig α-LA, pH 7.07; (b) goat α-LA, pH 6.58. All other conditions were as described in Fig. 2. From Berliner and Kaptein (1981).

terminal residue 123 which in the conformation calculated by Warme *et al.* (1974) salt bridges to ε-NH^{3+} of Lys-5, forming a hydrophobic pocket protecting Trp 118 from the solvent. The CIDNP spectrum of rabbit α-LA showed two additional directly polarized Trp protons at 7.58 and 7.31 ppm (see (Fig. 2). Furthermore, rabbit α-LA showed no fluorescence quenching upon Ca(II) binding (Murakami and Berliner, 1983). Consequently, since the other Trp residues (26, 60, 104) are structurally homologous in all α-LA species, the uniquely different residue Trp 118 appears to be most sensitive to Ca(II) binding. The physiological role of this calcium binding currently remains incompletely described. Lactose synthesis *in vitro* is kinetically identical in the presence of apo-or Ca(II)-lactalbumin. While it is difficult to estimate the free calcium concentration in the various organelles of the lactating mammary cell, calcium cells in milk and serum are certainly

sufficient to complex with lactalbumin. On the other hand, there is mounting evidence suggesting a hydrophobic surface on apo-α-LA whose affinity for apolar ligands decreases significantly on conversion to the calcium form (Lindahl and Vogel, 1984; Musci and Berliner, 1985). It is tempting to speculate that the cell stores α-LA in a tight membrane lipid complex. Experiments are currently in progress both *in vitro* and *in vivo* to unravel this intriguing function.

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