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LASER PHOTO-CIDNP NMR OF PYRIDOXAL PHOSPHATE AND PYRIDOXYLLYSINE RESIDUES: AN EXTRINSIC PROBE FOR NON-AROMATIC AMINO ACID RESIDUES IN PROTEINS

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SUMMARY

The proton on C-6 of the pyridine ring of pyridoxal phosphate gives rise to a prominent emission in the photo-CIDNP NMR spectrum upon irradiation of a flavin-containing solution at an appropriate wavelength. When pyridoxal phosphate is covalently bound to bovine pancreatic ribonuclease by sodium borohydride reduction of a mixture of the two compounds, the CIDNP emission from H-6 is considerably more intense than that from H-3,5 of the tyrosines, and can be observed readily even with low light intensity. The signal is a composite of several lines, demonstrating that the pyridoxyl groups of the modified lysines retain useful information in the form of chemical shift dispersion.

INTRODUCTION

The induction of CIDNP[2] effects in the NMR spectrum of histidine, tyrosine, and tryptophan protein residues accessible to certain dyes by irradiation of protein solutions containing such dyes has recently been exploited in investigations of protein conformational change and aggregation (1-9). For tyrosine, a strong nuclear polarization is observed for the protons ortho to the aromatic hydroxyl group, due to hyperfine interactions with the unpaired electron in the transient phenoxyl radical (2,4). It seemed likely to us that a proton para to an aromatic hydroxyl group would undergo a similar effect. The proton on C-6 of pyridoxal or its derivatives would thus be a useful CIDNP probe for various enzymatic reactions and for protein structure when covalently attached to lysine residues by borohydride reduction of the Schiff

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2) Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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base. This sequence of modification and photo-CIDNP would extend the scope of the NMR technique to an aliphatic amino acid. Moreover, the fact that the NMR signal from H-6 in pyridoxal phosphate is a singlet should make possible greater resolution of different environments than for the multiplets in the CIDNP spectra of tyrosine and tryptophan.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease was covalently modified by a method similar to that of Papas, et al. (10). Ribonuclease, pyridoxal phosphate, and HEPES were obtained from Sigma. Sodium borohydride was from Fisher. Five ml of a solution containing 4.8 mM pyridoxal phosphate and 0.24 mM ribonuclease in 20 mM potassium HEPES buffer, pH 7.5, were incubated in the dark at room temperature for 83 min. The pH was dropped to 4.7 by adding 30 µl of 4.4 M acetic acid. A fresh 1% solution (900 µl) of sodium borohydride in 0.1 M KOH was added in eleven portions, alternating with additions of 4.4 M acetic acid to keep the pH between 4.7 and 5.0. After the final hydrogen evolution had subsided, the pH was raised to 7.2 with 1 M KOH. After exhaustive dialysis of the resulting solution against water, the preparation was assayed by absorbance at 280 nm and 325 nm (10-12), and was found to contain 2.3 moles of pyridoxyl groups per mole of protein. The sample was lyophilized, then redissolved in 500 µl of D_2O. The material was presumed to be heterogeneous with respect to the ten possible lysine sites of modification. No separation of differently modified species was attempted, since our primary objective was to determine whether CIDNP could effect resolution of various lysine residues.

The light source for CIDNP experiments was a Spectra-Physics 171-01 krypton laser in a location remote from the Bruker 360 MHz NMR spectrometer. The laser beam was focused onto one end of a 30 m length of quartz optical fiber, 0.4 mm in diameter (Math Associates QSF-400). A computer-controlled shutter was placed in the beam. The other end of the fiber was placed directly in the sample tube in the NMR magnet. The laser was operated in single-line mode at 476 nm. Experiments at other wavelengths revealed that CIDNP-inducing effectiveness roughly parallels the fluorescence excitation spectrum of the flavin (λ_{max} = 467 nm for emission at 540 nm), and that irradiation above 500 nm, even with high power, was almost totally ineffectual. It was thus advantageous to eliminate the long wavelength light to limit sample heating. Reported light power measurements refer to output of the fiber at the sample; this was typically 50-60% of the laser output. The method of data acquisition was similar to that described by Kaptein (2,4). Reported chemical shifts are parts per million downfield from external sodium 4,4-dimethyl-4-silapentanesulfonate.

RESULTS

In a CIDNP experiment with 10 mM pyridoxal phosphate and 0.2 mM 7,8-dimethyl-N^{10}-carboxyethylisoalloxazine (hereafter referred to as "flavin"), using pulsed irradiation with 290 mW of light at 476 nm, the sharp singlet due to H-6 at δ 7.65 is the only proton NMR signal that exhibits a substantial CIDNP effect. In the light spectrum, it gives an emission with an intensity about twice that of the dark absorption. This result is similar to the effects see

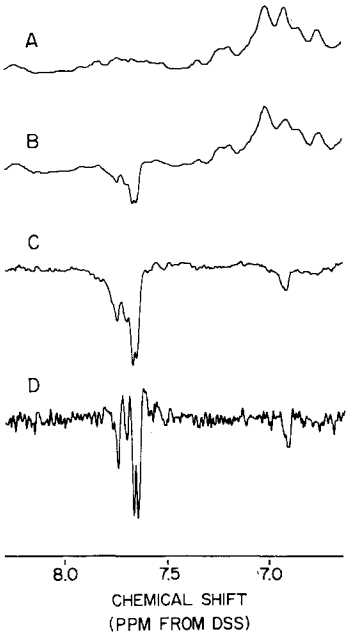

Figure 1. CIDNP experiment on 1 mM pyridoxal phosphate-modified ribonuclease plus 0.2 mM flavin at 25°C, pH 6.0. The sample was irradiated with 360 mW at 476 nm. NMR parameters were: memory size, 8K (4K real spectral data points); spectral width, 5000 Hz; 100 transients; flip angle, 90° (pulse width, 7 μs; presaturation, 1 s; light pulse, 2 s; acquisition time, 0.82 s; recovery, 3 s; (A) dark spectrum; (B) light spectrum; (C) light spectrum minus dark spectrum; (D) difference spectrum with resolution enhancement as described in text.

on the protons ortho to the hydroxyl group of tyrosine model compounds under comparable low power conditions. Within the ranges accessible to us, the magnitude of the CIDNP effect increases linearly with the light intensity at a given wavelength.

The results with the modified protein are more dramatic. The aromatic regions of the dark, light, and CIDNP difference spectra of a solution with 1 mM modified ribonuclease and 0.2 mM flavin are shown in Figure 1. The sample was irradiated with 360 mW at 476 nm. Two sets of negative signals (emission) are clear, one near δ 6.9 due to the tyrosines, and a stronger, multiline one from δ 7.6 to 7.8; the latter set must necessarily be due to the H-6's of the pyridoxyl groups since only a single histidine peak of ribonuclease shows a relatively very small positive CIDNP effect (enhanced absorption) in this region of the spectrum (4). Figure 1D shows the CIDNP difference spectrum

after resolution enhancement by convolution difference (13) with τ_1 = 0.5 s, τ_2 = 0.125 s, and K = 1. The pyridoxyl CIDNP shows at least four, and perhaps five, different environments for the pyridoxyl groups.

DISCUSSION

The use of pyridoxal phosphate or pyridoxyllysine residues as extrinsic CIDNP probes complements and extends the recent use of intrinsic aromatic amino acid residues. In certain situations, it may even offer one or more of the following significant advantages. (1) In some enzymes for which pyridoxal or its derivatives are substrates, cofactors, or inhibitors, the active site may prove to permit enough access to flavin to observe CIDNP of productively bound pyridoxyl groups, with the added possibility of polarization transfer to nearby active site residues. (2) Proteins lacking histidine, tyrosine, or tryptophan residues with sufficient solvent access for CIDNP could be studied via their lysines. Since the lysine side chain is the most highly charged of all of these at neutral pH, it is the one most likely to be found on the surface of proteins. (3) The CIDNP signals from protein-bound pyridoxyl groups appear to be rather narrow relative to those of the tyrosines. The reduced linewidth is probably due to (a) the lack of coupling to other ring protons, and (b) increased mobility of the long arm of the lysine side chain. This effect permits the improved resolution of lysine residues in different environments on the same protein. (4) The CIDNP sensitivity of the pyridoxyl groups in the modified protein is evidently considerably greater than that of the aromatic residues, at least in bovine pancreatic ribonuclease. This effect may be due in part to the lysine arm, providing extra accessibility to flavin. The practical benefit is that very low light power can be used, reducing sample heating and dye bleaching. The reduction of the light power requirement may also make the CIDNP technique more accessible.

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