Photochemically Induced Dynamic Nuclear Polarization NMR Study of Yeast and Horse Muscle Phosphoglycerate Kinase†

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ABSTRACT: A photochemically induced dynamic nuclear polarization (photo-CIDNP) study of yeast and horse muscle phosphoglycerate kinase with flavin dyes was undertaken to identify the histidine, tryptophan, and tyrosine resonances in the aromatic region of the simplified ^{1}H NMR spectra of these enzymes and to investigate the effect of substrates on the resonances observable by CIDNP. Identification of the CIDNP-enhanced resonances with respect to the type of amino acid residue has been achieved since only tyrosine yields emission peaks and the dye 8-aminoriboflavin enhances tryptophan but not histidine. By use of the known amino acid sequences and structures derived from X-ray crystallographic studies of the enzymes from the two species, assignment of the specific residues in the protein sequences giving rise to the CIDNP spectra was partially achieved. In addition, flavin dye accessibility was used to probe any changes in enzyme structure induced by substrate binding. The nine resonance peaks observed in the CIDNP spectrum of yeast phosphoglycerate kinase have been assigned tentatively to five residues: histidines-53 and -151, tryptophan-310, and tyrosines-48 and -195. The accessibility of a tyrosine to photoexcited flavin is reduced in the presence of MgATP. Since the tyrosine residues are located some distance from the MgATP binding site of the catalytic center, it is proposed either that this change is due to a distant conformational change or that a second metal–ATP site inferred from other studies lies close to one of the tyrosines. Horse muscle phosphoglycerate kinase exhibits seven resonances by CIDNP NMR. None of the four tyrosines of the horse enzyme (three are conserved in the yeast enzyme) is observed in the CIDNP spectra. The resonances have been tentatively assigned to tryptophan-310 and to histidines-172, -294, and -390. It is concluded that the conserved residues in the two enzymes do not necessarily have equal accessibility to flavin. Binding of 3-phosphoglycerate to the horse muscle enzyme shifts the resonances assigned to histidine-172 upfield due to either a conformational change or a pK_a change of the histidine. The addition of 3-phosphoglycerate and MgATP results in the appearance of two additional resonances in the CIDNP spectrum due to a histidine residue that is inaccessible to flavin in both the enzyme alone and its binary complex with 3-phosphoglycerate. This histidine residue is tentatively assigned to residue 390 which would become accessible to flavin upon loss of the hydrogen bond to glutamic acid residue 192. Weakening of the hydrogen bond between histidine-390 and glutamic acid residue 192 in the hinge region of the bilobal protein structure has been implicated from crystallographic studies as an important factor in the domain movement required to bring the substrates in close enough proximity for direct catalytic transfer of the phosphoryl moiety. The CIDNP spectra are consistent with the suggestions that binding of 3-phosphoglycerate alone is insufficient to effect domain movement and that binding of both substrates are required for conversion of the horse muscle enzyme to its catalytically active form.

Pronounced enhancements in the intensity of the ^{1}H NMR resonances of some His, Trp, and Tyr protein residues appear following reaction with photoexcited flavin dye, an example of chemically induced dynamic nuclear polarization (CIDNP).[1] The enhancements, due to spin polarization of the aromatic amino acids and flavin, result from a cyclic reaction, with intermediate formation of a dye–protein free radical pair (Kaptein, 1982), in which there is no net chemical change. Triplet-state flavin is easily generated by photoexcitation of the dye with an argon ion laser in an energy region relatively safe from destructive protein photochemistry. The radical pair theory of CIDNP has been reviewed by Kaptein (Kaptein, 1982), and the conditions that lead to signal enhancement are discussed. The reaction with triplet flavin involves a reversible electron transfer for Trp (McCord et al., 1981) and hydrogen atom abstraction for His and Tyr (Kaptein, 1982). The amino acids must not be sterically hindered for reaction with flavin, and the hydrogen on the N1 nitrogen of His or C4 hydroxyl of Tyr must be available for reaction, i.e., not hydrogen bonded (Bolscher et al., 1979).

In the CIDNP spectrum, spin-polarized Tyr residues are observed as emissions, in contrast to His and Trp which exhibit enhanced absorption. The CIDNP NMR spectrum is obtained by subtracting a normal or "dark" NMR spectrum from a "light" spectrum of the same sample. With the enhancement

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[1] Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; 3-PGA, 3-phosphoglycerate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; N^{10}-CELF, N^{10}-(carboxyethyl)lumiflavin; 8-NH_2-RF, 8-aminoriboflavin.

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of proton resonances by the CIDNP technique, experimental time and/or the quantity of sample required for an experiment is reduced. The CIDNP spectrum, which is limited to those protein residues that react with flavin, is greatly simplified when compared to the dark ^1H NMR spectrum. Resonances of single protons can be observed in the aromatic region which are normally difficult to observe due to insufficient resolution of overlapping lines, a particularly serious problem with large proteins.

A secondary mechanism exists whereby amino acid residues may be spin-polarized through dipolar cross-relaxation from a directly polarized nucleus to a nearby nucleus on the same amino acid residue or a different residue (deKanter & Kaptein, 1979). Secondary polarizations of this type can be distinguished from direct enhancements by varying the time of irradiation or the delay between light irradiation and the radio-frequency pulse (Kaptein & Edzes, 1979). Under conditions of short times of irradiation and of delay, cross-polarization will not be observed.

The first level of assignment, namely, identification of each type of amino acid residue in the aromatic region, presents no problems in CIDNP spectra. Phenylalanine resonances do not appear in the CIDNP spectrum, Tyr resonances are readily identified as the sole emission peaks. His exhibits enhancements of absorption for both the C2 and C4 protons while Trp may show absorption enhancements for the C4-H doublet, C6-H triplet, and C2-H singlet resonances. Where three-dimensional crystal structures are known, it is often possible on the assumption that the solution structure is basically equivalent to make assignments on the second level, i.e., to a given residue in the sequence, from considerations of solvent accessibility and hydrogen bonding.

Interpretation of changes in the NMR profile of a protein upon binding of substrate, inhibitor, or cofactor is usually ambiguous, because effects from a conformational change cannot be distinguished from those due to direct contact. In the CIDNP spectrum the interpretation of a decrease in the intensity of aromatic amino acid enhancements upon ligand binding is similarly ambiguous. However, appearance of a new resonance or large increases in flavin accessibility to amino acid residues upon ligand binding can be ascribed unequivocally to conformational changes.

Yeast 3-PGA kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) is composed of a single polypeptide chain of 415 amino acids and M_r ~45 000. the enzyme contains eight His, seven Tyr, and two Trp residues (Larsson-Raznikiewicz, 1970). The amino acid sequence has been completed (Perkins et al., 1983), and a detailed three-dimensional structure has been constructed from X-ray crystallographic data (Watson et al., 1982). Tanswell et al. (1976) have reported an ^1H NMR study of yeast 3-PGA kinase. The sequence (Banks et al., 1979; Merrett, 1981) and high-resolution crystallographic structure (Blake & Rice, 1981; Rice & Blake, 1984) for horse muscle 3-PGA kinase have been available for some time but the enzyme from this species has not previously been studied by ^1H NMR. The horse muscle enzyme consists of 416 amino acids, 4 Trp (2 conserved), 4 Tyr (3 conserved), and 6 His (5 conserved) residues. It was anticipated that the availability for the two 3-PGA kinase species of the complete amino acid sequences with 63% homology and of their highly homologous crystal structures would make possible some unequivocal assignments to individual aromatic amino acids in both proteins. Furthermore, the CIDNP technique might yield information on substrate–enzyme interactions that would shed light on substrate-induced domain movement postulated from crystallographic studies.

Experimental Procedures

Materials. ATP, 3-PGA, and bovine α-lactalbumin were purchased from Sigma Chemical Co. D_2O was obtained from MSD Isotopes, diethylmalonic acid from Calbiochem, and Sephadex from Pharmacia. N^{10}-CELF was generously supplied by Dr. David Porter of the University of Pennsylvania, and 8-NH_2-RF and 8-α-[(ω-aminooctyl)amino]riboflavin were gifts from Dr. Donald B. McCormick of Emory University. All other chemicals were of the highest grade available commercially.

Preparation of 3-PGA Kinase. The enzyme from yeast was purified by the modified (Jaffe & Cohn, 1980) method of Scopes (Scopes, 1971). 3-PGA kinase from horse muscle was a generous gift from Dr. Robert Scopes of La Trobe University. The enzymes were assayed spectrophotometrically with glyceraldehyde-3-phosphate dehydrogenase (Scopes, 1971).

Preparation of NMR Samples. The enzymes were stored at 4 °C in ammonium sulfate which was removed by extensive dialysis against 10 mM Tris-HCl buffer at the appropriate pH. The protein was concentrated for NMR experiments to 100–200 mg mL^{-1} in an Amicon B15 membrane concentration cell (5-mL capacity) and dialyzed against 50 mM KCl using a dialysis stick. The enzyme was either lyophilized and dissolved in 20 mM KCl in D_2O or passed over a Sephadex G-25 column equilibrated with 20 mM KCl in D_2O. The pH of the NMR samples was adjusted by the addition of small amounts of dilute DCl or NaOD. In some cases diethylmalonic acid was used as a buffer since, unlike Tris, 2-(*N*-morpholino)-ethanesulfonic acid, and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, diethylmalonic acid did not interfere with CIDNP enhancements under our experimental conditions. The pH values reported represent readings on a Radiometer 26 pH meter without correction.

NMR Spectra. ^1H NMR spectra were recorded on a Bruker WH-360 spectrometer equipped with a special Bruker 5-mm ^1H CIDNP probe having a quartz light guide parallel to the probe. The Coherent Model CR2 argon ion laser used to irradiate samples at 488 nm delivers 0.7 W at that wavelength. A mirror at the base of the magnet was used to focus the laser beam on the end of a quartz rod that directed a diverged light beam to the sample in the region of the radio-frequency coil. A computer-interfaced shutter controlled sample irradiation. Alignment of the photo-CIDNP optical system was achieved by using the photometer system described by McDonald (McDonald, 1983). Typically, samples were irradiated for 0.1–1.0 s with a variable delay (1–50 ms) following the light pulse, after which free induction decays were collected with a 90° flip angle (pulse width = 6.5 μs). A 10-s delay between each scan prevented heating of samples. During the delay time the H_2O resonance was selectively irradiated, to reduce its intensity. If a sample was used for more than one spectrum, samples were routinely mixed between recording of spectra to prevent signal attenuation due to bleaching of the flavin dye. Dark and light spectra were accumulated by using identical pulse sequences, transformed, and subsequently subtracted. The CIDNP spectrum is defined as the difference spectrum, light minus dark. The chemical shifts were determined with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as reference at 0 ppm.

Cross-Polarization Studies. To determine whether the laser used in these experiments provides sufficient power at short irradiation times to observe direct polarization and at longer irradiation times to observe direct and indirect polarizations, bovine α-lactalbumin was tested under our experimental

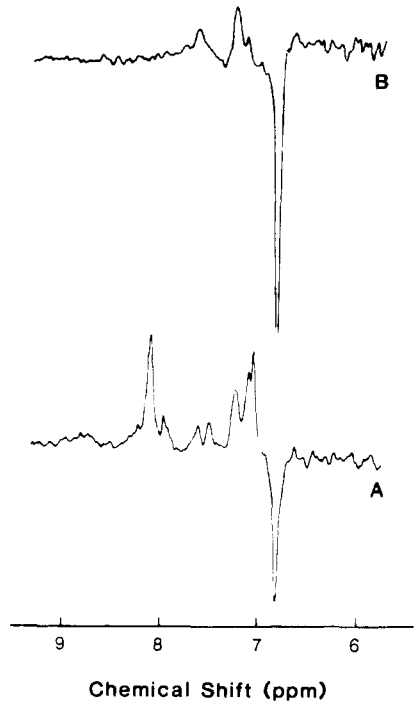

FIGURE 1: CIDNP spectra of partially inactivated yeast 3-PGA kinase, 1 mM, pH 7.0. (A) Riboflavin, 0.4 mM; (B) 8-NH_2-RF, 0.4 mM. The spectra represent the difference between 32 scans obtained after a 0.3-s irradiation followed by a 10-ms delay and 32 dark scans.

conditions. The cross-polarization of Trp-60 and another unidentified resonance by Trp-104 in the α-lactalbumin were observed as previously described with a high-power laser (Berliner & Kaptein, 1981) and were observed only after the longer irradiation times.

RESULTS

Flavin Dyes. A variety of flavin dyes can be employed with the photo-CIDNP technique including riboflavin or its 5-phosphate, N^3-(carboxymethyl)lumiflavin, N^{10}-CELF, and 8-NH_2-RF. The sign and intensity of amino acid enhancements with each dye is dependent upon the free radical g values and several other factors (Kaptein, 1971). Upon examination of the CIDNP properties of yeast 3-PGA kinase with a variety of flavins, 8-NH_2-RF was found to be unique in that it does not enhance His residues. An aged sample of 3-PGA kinase of low activity (specific activity = 140 units mg^{-1}) was used for a comparison of flavins; in Figure 1A the flavin was riboflavin, and in Figure 1B, the flavin was 8-NH_2-RF. The selective behavior of 8-NH_2-RF is also observed when the CIDNP spectra are obtained with solutions of free amino acids (not shown). This property of 8-NH_2-RF is extremely useful in unambiguously distinguishing His and Trp resonances in a protein by photo-CIDNP.

The intensity of each peak (see Figure 1) normalized to the intensity of the low-field Trp (7.60 ppm) for four dyes is given in Table I. With the exception of 8-NH_2-RF, which does not enhance His (see Figure 1), the relative CIDNP enhancements for His and Tyr as reflected in the ratio of Tyr/His (C2-H) intensities varies only between 0.9 and 1.3. It should be noted that in the native enzyme (specific activity = 1035 units mg^{-1}) shown in Figure 2B, the Tyr/His (C2-H) intensity ratio with N^{10}-CELF as dye at pH 7.0 is less than 0.1 compared to 0.9 for the partially inactivated species under the same conditions shown in Figure 1. The intensity of the Tyr emission peak has been found to be inversely proportional to the activity of the enzyme and is a good measure of the retention of the native structure.

Table I: Low Activity Yeast 3-PGA Kinase, pH 7.0, T = 20 °C

	relative CIDNP signal enhancements				
flavin dye	Trp-1	Trp-2	Tyr	His C2-H	Tyr/His
8-aminoriboflavin	1.0	1.68	9.4		
riboflavin	1.0	2.36	5.6	4.2	1.3
N^{10}-(carboxyethyl)lumiflavin	1.0	0.73	2.4	2.6	0.92
8-α-[(ω-aminooctyl)-amino]riboflavin	1.0	2.13	6.9	7.0	0.99

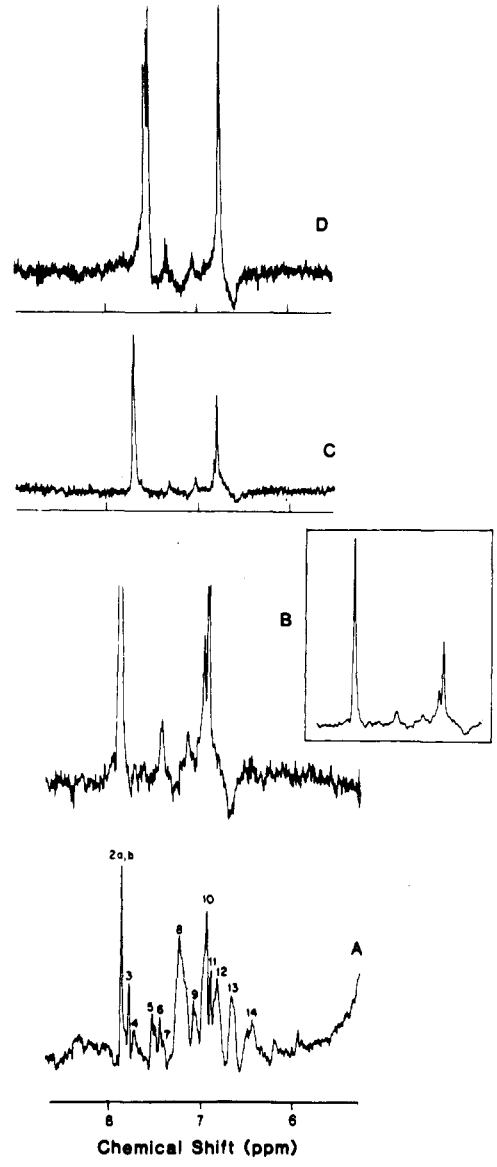

FIGURE 2: CIDNP spectra of yeast 3-PGA kinase at different pH values. (A) Dark spectrum, 1 mM enzyme, 0.4 mM N^{10}-CELF, 10 mM sodium diethylmalonate buffer, pH 7.0; (B) CIDNP spectrum, same conditions as (A); (C) CIDNP spectrum, pH 7.6; (D) CIDNP spectrum, pH 8.5. The dark spectrum in (A) is resolution-enhanced by convolution difference techniques after accumulation of 1500 scans. The CIDNP spectra are the differences of 64 light and 64 dark scans after a 0.3-s irradiation followed by a 10-ms delay.

CIDNP of Yeast 3-PGA Kinase. Earlier ^1H NMR studies of yeast 3-PGA kinase in the presence of ammonium sulfate at 40 °C recorded at 270 MHz (Tanswell et al., 1976) revealed 15 resolved peaks[2] in the aromatic region of the spectrum

[2] We have employed the numbering system for the ^1H NMR spectrum of yeast 3-PGA kinase introduced previously (Tanswell et al., 1976).

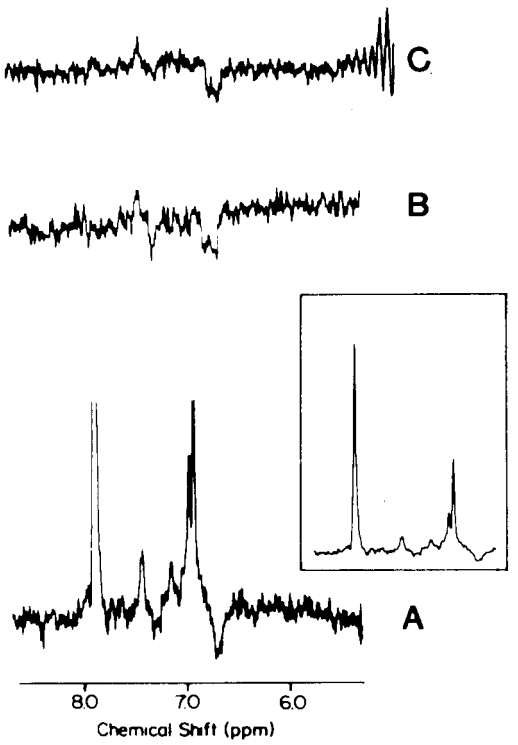

FIGURE 3: CIDNP spectra of yeast 3-PGA kinase with two flavin dyes. (A) 1 mM enzyme, 0.4 mM N^{10}-CELF, and 10 mM diethylmalonate buffer, pH 7.0; (B) same as (A) except dye, 0.4 mM 8-NH_2-RF; (C) same as (B) plus 2 mM MgATP. CIDNP spectra obtained as in Figure 2.

under some experimental conditions. Similar spectra have been observed in the current investigation. It was proposed from chemical shifts and pH titrations that resonances 1–6 correspond to the C2-H for seven of the eight His residues in the enzyme and that resonances 9–11 correspond to C4-H of His residues. The resonances designated 2a,b (a and b are resolved above pH 8) and resonances 10 and 11 in Figure 2A have pK_a values of 7.05 (Tanswell et al., 1976) and are the only two His residues observed in CIDNP spectra (as will be discussed). The CIDNP spectra of yeast 3-PGA kinase at pH 7.0, 7.6, and 8.5 and a reference dark NMR spectrum at pH 7.0 are shown in Figure 2. The CIDNP spectra are greatly simplified relative to the dark spectrum, and the changes of chemical shifts of His residues with pH are the same as seen in the dark spectra (Tanswell et al., 1976). However, in the CIDNP spectra the His C4-H protons (peaks 10 and 11) are not obscured by other aromatic peaks. There are eight enhanced resonances in the aromatic region for which assignment on the first level is possible.

The resonances at 6.73 ppm in Figure 2B–D and at 7.31 ppm in Figure 3A,B correspond to peaks 14 and 8 in the dark spectrum, respectively. These emission peaks can be unequivocally assigned to the C3,5-H resonance of two Tyr residues. In order to distinguish unequivocally between His and Trp among the absorption peaks, a CIDNP spectrum was obtained with photoexcited 8-NH_2-RF (Figure 3B). A comparison of the CIDNP spectra obtained with the N^{10}-CELF (Figure 3A) and with 8-NH_2-RF (Figure 3B) demonstrates that the most enhanced resonances with the former dye are completely missing with the latter dye. The positive enhancement at 7.47 ppm in Figure 3B,C must necessarily be a Trp resonance corresponding to peak 7 in the dark ^{1}H NMR spectrum (Figure 2A) since His is not observed with this dye. The 7.47 ppm resonance is assigned to the C4-H of a Trp residue from its chemical shift and its doublet structure observed in Figure 2D. Examination of the CIDNP spectra with N^{10}-CELF (Figure 2B–D) reveals a second enhanced resonance at 7.18 ppm (peak 9) which probably arises from the same Trp residue as peak 7 and may be assigned to C2-H of that Trp residue. The C6-H of Trp (6.84 ppm, peak 12 in the dark spectrum, Figure 2A) is probably obscured by the intense resonances due to His C4-H (peaks 10 and 11) seen in Figure 2. The observable Trp resonances are therefore assigned as the C4-H (7.47 ppm) and C2-H (7.18 ppm) based on the chemical shifts of Trp in random-coil peptides, C4-H and C2-H at 7.504 and 7.244 ppm, respectively (Bundi & Wuthrich, 1979).

Table II: CIDNP of Yeast 3-PGA Kinase

peak	chemical shift[a]	enhancement[b] ^{10}N-CELF	enhancement[b] 8-NH_2-RF	assignments first level	assignments second level
2a	7.87	+	0	His C2-H	(151)[c]
2b	7.87	+	0	His C2-H	53
7	7.47	+	+	Trp C4-H	310
8	7.31	–	–	Tyr C3,5-H	(48)
9	7.18	+	0	Trp C2-H	310
10	6.97	+	0	His C4-H	(151)
11	6.94	+	0	His C4-H	53
12	6.84	+	0	Trp C6-H	310
14	6.73	–	–	Tyr C3,5-H	(195)

[a] pH 7.0. [b] (+) indicates positive enhancement, (–) indicates negative enhancement, and (0) indicates no enhancement. [c] Residue numbers in parentheses are tentative assignments.

The remaining resonances of the CIDNP spectra (Figure 2B–D) can be assigned to enhanced His residues. The most intense His resonances occur at 7.87 and 6.94 ppm (pH 7.6) corresponding to peaks 2b and 11 of the dark spectrum. They have been assigned as the C2-H and C4-H of a single His residue. The smaller His signals at 7.87 and 6.97 ppm observed at pH 7.6 (Figure 2C) correspond to peaks 2a and 10 of the dark protein spectrum, i.e., the C2 and C4 protons of a second His which is apparently somewhat less accessible to flavin than the first one. Assignment of the aromatic resonances of yeast 3-PGA kinase are summarized in Table II.

Effect of Substrates of Yeast 3-PGA Kinase. In Figure 3C it can be seen that upon the addition of MgATP to the yeast enzyme the Tyr C3,5-H at 7.31 ppm (peak 8) is no longer enhanced. Either the substrate binds very close to this Tyr residue, thus blocking its interaction with flavin, or MgATP binding produces a change in protein conformation which renders the Tyr residue less accessible to flavin. It is unlikely that the disappearance of Tyr (peak 8) in the presence of MgATP is due to competition of MgATP for reaction with flavin (Kaptein et al., 1979) since Trp resonance 7 and Tyr-14 do not appear significantly attenuated. A direct steric effect on accessibility is unlikely since there are no Tyr residues in the region of the nucleotide binding site (Watson et al., 1982).

CIDNP of Horse Muscle 3-PGA Kinase. The CIDNP spectrum of horse 3-PGA kinase (Figure 4) contains at least seven enhanced proton resonances that are numbered sequentially with increasing magnetic field. No Tyr emissions were observed under any conditions examined (Figures 4 and 5). The four CIDNP resonances 1, 2, 6, and 7 (Figure 4A,B) at pH 8.0 and 7.4, respectively, observed with N^{10}-CELF as the flavin component disappeared (Figure 4C) when 8-NH_2-RF was substituted. Both the pH dependence of resonances 2 and 6 and the dye specificity of all four identify them as His protons. By a comparison of the spectrum at pH 8.0 (Figure 4A) and at pH 7.4 (Figure 4B), it is clear that peaks 2 and 6 shift downfield by 0.17 and 0.12 ppm, respectively, as the pH is decreased and are consequently assigned to a

Table III: CIDNP of Horse Muscle 3-PGA Kinase

peak	chemical shift pH 7.4	chemical shift pH 8.0	enhancement N^{10}-CELF	enhancement 8-NH_2-RF	first level	second level
1	7.86	7.82	+	0	His-1 C2-H	(172)[c]
2	7.86	7.69	+	0	His-2 C2-H	294
3	7.46	7.48	+	+	Trp-1 C4-H	310
4	7.33	7.35	+	+	Trp-1 C2-H	310
5		7.10	+	+	Trp-1 C6-H	310
6	7.08	6.96	+	0	His-2 C4-H	294
7	6.94	6.90	+	0	His-1 C4-H	(172)
8[b]	7.79		+	ND[a]	His-3 C2-H	(390)
9[b]	6.70		+	ND	His-3 C4-H	(390)

[a] ND = not determined. [b] Observed only in the presence of 3-PGA and MgATP, pH 7.84. [c] Residue numbers in parentheses are tentative assignments.

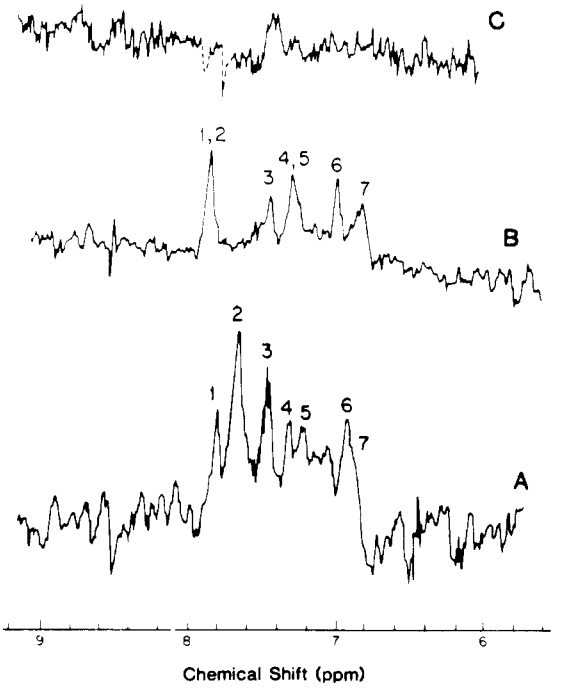

FIGURE 4: CIDNP spectra of horse muscle 3-PGA kinase with two flavin dyes. (A) 1 mM enzyme, 0.4 mM N^{10}-CELF, and 10 mM diethylmalonate buffer, pH 8.0, difference spectrum between 64 light and dark scans; (B) same as (A), pH 7.4; (C) same as (A) except flavin, 0.4 mM 8-NH_2-RF, pH 7.5, difference spectrum between 32 light and dark scans. All light spectra were obtained after a 0.3-s irradiation followed by a 10-ms delay. The dashed lines represent flavin proton emissions.

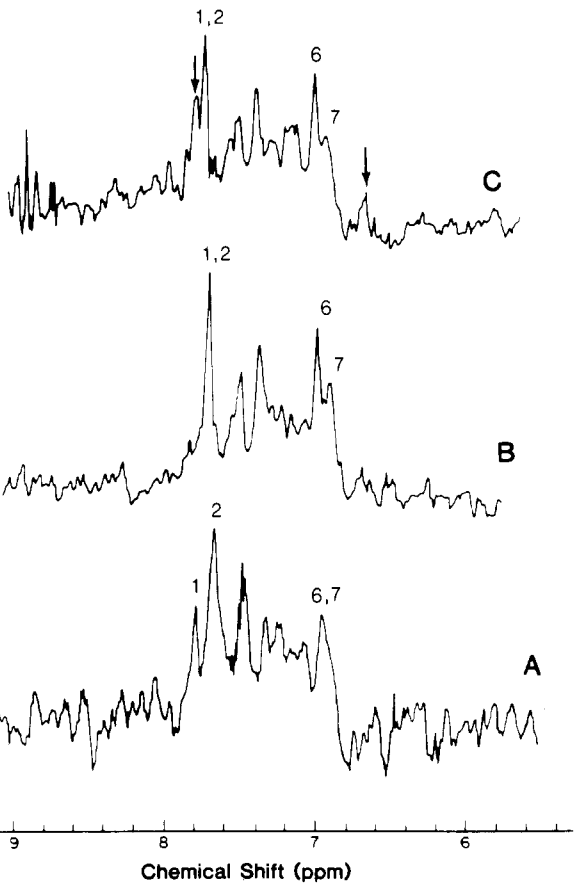

FIGURE 5: CIDNP spectra of horse enzyme–substrate complexes. (A) 1 mM enzyme, 0.4 mM N^{10}-CELF, 10 mM diethylmalonate buffer, pH 8.0; (B) same as (A) plus 2 mM 3-PGA, pH 7.82; (C) same as B plus 4.9 mM MgATP, pH 7.84. CIDNP spectra were obtained as in Figure 2.

C2-H and a C4-H of a single His. On the other hand, peaks 1 and 7 barely shift with pH in this pH range and are assigned to the C2-H and C4-H of a second His. Only two weak enhancements are observed with 8-NH_2-RF at 7.48 and 7.35 ppm (pH 7.5) and correspond to peaks 3 and 4 in Figure 4A,B. These peaks may be assigned to the C2 and C4 protons of a single Trp residue. In summary, two His, one Trp, and, within the sensitivity of our measurements, no Tyr residues are observed in the CIDNP spectrum of horse 3-PGA kinase (Table III).

Effect of Substrates of Horse PGA Kinase. When 3-PGA is added to the enzyme, no new CIDNP enhancements are observed (Figure 5B). The only significant changes in the CIDNP spectrum are the upfield shifts of peaks 1 and 7 which correspond to C2-H and C4-H of the His-1 residue. The binding of 3-PGA apparently leads either to a change in protein conformation that introduces a ring-current shift on the His-1 protons or to a change in the pK_a of this His residue. The formation of the equilibrium mixture upon addition of MgATP to the sample already containing horse 3-PGA kinase and 3-PGA does not change the resonances observed with 3-PGA alone, but in the equilibrium mixture two new CIDNP peaks (indicated by the arrows in Figure 5C) appear at 7.79 and 6.70 ppm, respectively. The difference in chemical shift between the two new enhanced resonances suggests that they are the C2-H and C4-H of a third His (cf. Table III).

Cross-Polarization Studies. When the CIDNP spectra for yeast 3-PGA kinase were recorded by using a short or long light pulse identical with those used for α-lactalbumin (see Experimental Procedures), no cross-polarized enhancements were observed in the aromatic region. Similarly, no changes were observed with horse muscle 3-PGA kinase when the irradiation time was varied. Therefore, all of the CIDNP signals of horse and yeast PGA kinase may be ascribed to the direct interaction of the observed residues with N^{10}-CELF. In the yeast enzyme unlike the horse enzyme, three pairs of

Table IV: Sequence Numbers of His, Trp, and Tyr Residues in 3-PGA Kinase

	C/T^a									
Trp										
yeast	1/2	310	335							
horse	1/4	310	335	344	382					
Tyr										
yeast	2/7	48	56	75	123	160	195			382
horse	0/4			75		160	195		323	
His										
yeast	2/8	52	53	62	124	151	169	172		390
horse	3/6			62	124		169	172	294	390

$^a C/T$ = $CIDNP_{obsd}$/total.

aromatic residues, Tyr_{48}–His_{52}, His_{53}–His_{52}, and Tyr_{123}–His_{124}, could be candidates for polarization donor and acceptor pairs if one residue of a pair is accessible to flavin. However, no cross-polarization has been observed under our experimental conditions.

Second-Level Assignments. The residue numbers of the aromatic amino acids (excluding Phe) of the yeast and horse muscle enzymes are summarized in Table IV. Because those His, Tyr, and Trp residues that exhibit CIDNP resonances must be accessible to flavin, a number of specific residues in the structure may be eliminated on the basis of the known sequences for the yeast (Perkins et al., 1983) and the horse muscle (Merrett, 1981) enzymes and on the crystallographic structures of the yeast (Watson et al., 1982) and the horse muscle (Blake & Rice, 1981) enzymes. There is 63% sequence homology between the two enzymes, and the X-ray analyses yield very similar three-dimensional structures. The coordinates of the yeast 3-PGA kinase taken from the Protein Data Bank and those of the horse muscle enzyme supplied by C. C. F. Blake have been used for an Evans–Sutherland graphics display of the structures on the VAX 11/780 computer at Fox Chase for the purpose of gauging accessibility of various residues.

Tryptophan. The CIDNP spectra of yeast and horse muscle 3-PGA kinase exhibit one Trp enhancement. The yeast enzyme contains only two Trp residues, both of which are conserved in the horse sequence and have similar structural environments. Trp-335[3] is buried and lies close to the ATP binding site. Trp-310 is a surface residue in an unstructured region distant from the active site. Therefore, resonances of the single Trp observed in the CIDNP spectra of the yeast enzyme (peaks 7, 9, and 12 of Figure 2) can be assigned to residue 310 (cf. Table II). Clearly, Trp-310 is accessible to flavin in both enzymes.

Histidine. The CIDNP spectra of both yeast and horse muscle 3-PGA kinase exhibit two strongly enhanced pairs of His resonances, each set corresponding to a C2-H and to a C4-H resonance of His, respectively. For the yeast enzyme, the variation of the chemical shift with pH corresponds closely to peaks 2a and 2b and peaks 10 and 11 of the dark spectrum (Tanswell et al., 1976). Examination of the crystal structure in a graphics display reveals that His-53 is on the surface, fully exposed to solvent, and is not hydrogen-bonded at either the ND or NE position. The most CIDNP-enhanced His residue corresponding to peaks 2b and 11 of the dark spectrum (Figure 2) are consequently assigned to His-53 (Table II). There is a second His residue with a normal pK_a in the yeast enzyme. The His residues appear accessible in the order 151 > 52 > 172, and the remaining His residues are more buried. In particular, His-390 is both hydrogen-bonded and fairly buried. His-151 is the tentative assignment for the enhanced CIDNP resonances of yeast 3-PGA kinase.

Neither His-53 nor His-151 is conserved in the horse muscle enzyme (Table IV). Of the two pairs of His resonances in the horse muscle enzyme, one pair, namely, peaks 2 (C2-H) and 6 (C4-H), has chemical shifts and titrates with pH in the same manner as expected for fully exposed His residues like His-53 in the yeast enzyme. The only fully exposed His residue in the horse muscle enzyme is 294, and therefore, resonances 2 and 6 are assigned to residue 294 (Table III). The second His residue observed in the CIDNP spectrum of the horse enzyme shows no significant change in chemical shift between pH 7.4 and pH 8.0 but does show an upfield shift upon addition of 3-PGA, indicating either a decrease in pK_a or a ring-current shift. Since the addition of 3-PGA does induce conformational changes in the crystal (Rice & Blake, 1984), it may move a His closer to an aromatic residue, thereby inducing a ring-current shift. On the other hand, if it is a pK_a shift, it is opposite in direction from what is expected upon binding a highly charged anion such as 3-PGA close to the His in question. Therefore, it is probably not a His residue in sufficiently close proximity to bound 3-PGA. The most accessible His residue other than His-294 is His-172, and peaks 1 and 7 (Table III) are tentatively assigned to His-172.

The third His residue which appears in the presence of the equilibrium mixture with the horse muscle enzyme could be tentatively assigned to His-390 for the following reason. In the crystalline state it is hydrogen-bonded to Glu-192 in the open form of the enzyme, but upon addition of both substrates, the movement of the two domains necessitates the breaking of this bond (Watson et al., 1982). The apparent weakness of this assignment, i.e., the absence of a CIDNP enhancement for this conserved residue in the yeast enzyme spectrum under the same conditions, disappears since His-390 is far less accessible in the yeast enzyme (both ND and NE positions may be hydrogen-bonded) than in the horse muscle enzyme, where the ND position of His-390 is exposed and only NE forms a hydrogen bond to Glu-192. Until it becomes possible to compare the two crystal structures in the "closed" form, i.e., the enzyme complexed with both substrates, this assignment remains tentative. It has been stated that the current crystallographic structure of the horse muscle enzyme represents an inhibited form of 3-PGA kinase (Rice & Blake, 1984).

Tyrosine. None of the resonances of the four Tyr residues of horse 3-PGA kinase appears to be enhanced by the photo-CIDNP technique. In the native yeast enzyme there are at least two Tyr residues that exhibit small enhancements. The low enhancement of the Tyr resonances compared to His resonances could be anticipated from the graphics display since none of the seven Tyr residues in yeast 3-PGA kinase (Table IV) is exposed as much as the two His residues. The least buried Tyr residue is 195 which has also been identified as the most reactive Tyr residue in nitration by tetranitromethane

[3] The residue numbers in the sequence for both the yeast and horse enzymes follow the published convention (Perkins et al., 1983).

(Bacharach et al., 1977). Peak 14 (Table II) is consequently assigned tentatively to residue 195. Tyr-195 in the horse muscle enzyme appears to be less buried than in the yeast enzyme. It is not clear why no Tyr CIDNP is observed in the horse enzyme. Possible explanations include overlap of the Tyr emission by the much larger His absorption and the lower signal to noise in the spectra of the horse muscle enzyme, and lastly, the conformation in solution may differ from that in the crystal.

From an examination of the graphics display of the remaining six Tyr residues in the yeast enzyme, Tyr-158 is most buried, and the others are all partially buried and may form hydrogen bonds. Tyr-48 appears most accessible to solvent, and Tyr-56 follows it in accessibility, but the OH of the latter is within 2.1 Å of the carbonyl of residue 154. Therefore, the second Tyr CIDNP resonance (peak 8, Figure 2) is tentatively assigned to Tyr-48 (Table II) of the yeast enzyme. Tyr-48 is not conserved in the horse muscle enzyme.

Discussion

The pitfalls of equating the intensities of the photoinduced CIDNP enhancements in the NMR spectra of all the His, Trp, and Tyr residues of a protein to their absolute or relative accessibility to flavins are many. The properties of a particular dye which are important for photoinduced CIDNP include charge (van Schagen et al., 1982), reaction rates, stability to bleaching, and solubility (Kaptein, 1982). For a protein there are additional considerations. His and Tyr residues exposed on the surface will not yield a CIDNP signal if they are hydrogen-bonded. The magnitude of the CIDNP enhancements of individual amino acid residues is determined not only by accessibility to the flavin and reactivity with different flavins but also by competition among the various target amino acids as well as other factors (Vogel, 1983; Vogel & Sykes, 1984). It has been found in this investigation that one dye, 8-NH_2-RF, exhibits a very high degree of specificity; it does not interact with His residues or with free histidine. As a consequence, this dye is useless for studying His residues but makes it possible to assign CIDNP-enhanced resonances of Trp unequivocally.

Thus, the observation of a CIDNP-enhanced resonance for a given amino acid residue signifies that it is accessible to flavin but the absence of enhancement does not necessarily indicate that the amino acid is buried. Although relative intensities of different aromatic amino acids are not meaningful, it is valid to compare the relative intensities for one type of amino acid residue. For example, the signal intensities of the two His residues of yeast 3-PGA kinase (Figure 2) do correspond to their relative accessibilities to flavin used in the experiment. Also the finding that the intensity of a Tyr resonance is greatly enhanced in an inactive enzyme relative to an active one when measured under the same conditions (cf. Figures 1 and 2) demonstrates the potentiality of CIDNP to follow the folding of proteins.

For some families of protein that exhibit a high degree of conservation, e.g., α-lactalbumin, the CIDNP spectrum is nearly identical for every species examined (Berliner & Kaptein, 1981). This result is not unexpected for lactalbumin since the residues exposed on the protein surface are involved in the regulatory function of this protein. The conserved residues of 3-PGA kinase on the other hand are important for substrate binding and catalysis and are not likely to be located on the enzyme surface. Hence, it is not surprising to observe large differences in the CIDNP spectra of the horse muscle and yeast enzymes in spite of the highly conserved structure. In fact, the two His residues observed in the yeast enzyme, 53 and 151, are unique to the yeast sequence, and His-294 is unique to the horse muscle enzyme structure.

The usual ^{1}H NMR spectra of the aromatic region of yeast (Tanswell et al., 1976) and horse muscle 3-PGA kinase are fairly complex, consisting of resonances from 8 His, 2 Trp, 7 Tyr, and 19 Phe residues for the yeast enzyme and from 6 His, 4 Trp, 4 Tyr, and 16 Phe residues for the horse muscle enzyme. The CIDNP spectra of both proteins are greatly simplified since all Phe are eliminated and less than one-third of the other aromatic residues appear in the CIDNP spectra. First-level assignments present no problems as shown in table II. The CIDNP-enhanced residues of the yeast enzyme include at least one Trp, two Tyr, and two His residues. There is sometimes a quantitative problem; since the area under a resonance does not correspond directly to the number of protons, there is no criterion to distinguish whether one resonance arises, for example, from the protons of one Tyr residue or of two or more Tyr residues with overlapping resonances. Therefore, the number of residues attributed to the CIDNP-enhanced resonances are in some cases minimum values. The two His CIDNP resonances correspond to those well resolved in the dark spectrum which had previously been attributed to the only two His residues fully exposed to solvent (Tanswell et al., 1976) on the basis of their pK_a values of 7.05 (close to that of *N*-acetylhistidine) and on the narrowness of the lines compared to other His residues in the protein. It should be noted that, from the CIDNP results, the two His residues are not equally accessible to flavin although it had been concluded from pH titrations that the two residues were equally exposed to solvent (Tanswell et al., 1976).

The assignment of one Trp residue to the appropriate CIDNP-enhanced resonances in the yeast enzyme also presents no problems since one of the two Trp residues, 335, is deeply buried (Watson et al., 1982). The quantitation of Tyr residues in the yeast enzyme does present a problem because the enhanced emission is low and the resonance falls close to a large absorption due to C4-H of His (see Figures 2 and 3). If the low degree of enhancement were attributable to unsuccessful competition with the flavin compared to other enhanced residues, the Tyr enhancements would be greater with 8-NH_2-RF or riboflavin (Vogel & Sykes, 1984) compared to N^{10}-CELF. However, the Tyr enhancement observed with all three dyes is identical. Moreover, in the partially inactivated enzyme (Figure 1), the Tyr enhancement is very large, greater than the His enhancement. Therefore, it may be concluded that the Tyr residues observed in Figure 3 for the native yeast enzyme are marginally accessible to flavin. In the horse muscle enzyme, no Tyr residues are observed to be enhanced as already discussed under Results. Modification studies done on yeast PGA kinase have shown that three of the seven Tyr residues are readily nitrated with tetranitromethane (Hjelmgren et al., 1976). These studies further attest to the inaccessibility of four of the protein tyrosines as indicated by CIDNP.

Assignments on the second level, i.e., to specific residues in the sequence, have been justified in detail under Results and are summarized in Tables II and III. The assignment of Trp-310 is firm for both enzymes. His-53 of the yeast and His-294 of the horse muscle enzyme are firm, but all others must be considered tentative. Of the six His residues in the horse muscle enzyme, only one, His-294, is not conserved in the yeast enzyme. Three His residues of the horse enzyme exhibit CIDNP enhancements, and therefore, at least two must be in the conserved group. Therefore, at least one of the conserved His residues which we have designated 172 differs

in the two enzymes in accessibility to flavin.

The crystal structures of horse muscle and yeast 3-PGA kinase (Blake et al., 1974; Banks et al., 1979; Bryant et al., 1974; Watson et al., 1982) show that the enzymes consist of two compact globular domains joined together by a thin "waist" region. The metal–nucleotide site resides on the carboxyl-terminal domain, and the 3-PGA (1,3-diphosphoglycerate) site is located on the N-terminal domain. On the basis of X-ray crystallographic studies, it has been proposed that substrate binding induces the protein domains to rotate on the axis of the hinge region while forming a hydrophobic active cavity and positioning substrates for direct in-line phosphoryl transfer. In support of this hypothesis large conformational changes have been observed upon substrate binding in X-ray crystallographic studies (Banks et al., 1979; Watson et al., 1982), sedimentation equilibrium measurements (Roustan et al., 1980), X-ray scattering studies (Pickover et al., 1979), and ^{1}H NMR (Tanswell et al., 1976). While the "hinge-bending" change in protein structure is generally agreed upon, there exists some discrepancy as to the precise nature of the substrate requirements for producing the "closed form" of the enzyme. In their X-ray crystallographic studies of horse muscle 3-PGA kinase Banks et al. (1979) observed large conformational changes upon the binding of 3-PGA which were not significantly increased in the ternary, E–PGA–MgATP, complex. This led to the suggestion that 3-PGA alone could elicit rotation about the hinge region. However, one difficulty in a comparison of structures of this enzyme in solution and in the crystalline state is the presence in the latter of high concentrations of ammonium sulfate. Sulfate that binds at multiple sites on the enzyme has been implicated in large changes in protein conformation by ^{1}H NMR (Tanswell et al., 1976), ^{31}P NMR (Nageswara Rao et al., 1978), chemical modification studies (Roustan et al., 1980; Wrobel & Stinson, 1978; Meyer & Westhead, 1976), analytical ultracentrifugation experiments (Roustan et al., 1980), and kinetic studies (Larsson-Raznikiewicz et al., 1973; Scopes, 1978a,b).

In contrast to the finding of large conformational changes upon formation of the binary E–PGA complex, Roustan et al. (1980) found that the ternary E–PGA–MgATP complex must be formed before significant changes in the molecular structure of yeast 3-PGA kinase could be observed by ultracentrifugation studies. Small-angle X-ray scattering experiments of the enzyme in solution (Pickover et al., 1979) showed a significant decrease in the protein radius of gyration when both substrates were present while 3-PGA or MgATP alone had little or no effect. The results of CIDNP NMR suggest that for horse muscle 3-PGA kinase both substrates must be bound to observe the exposure of a new His residue, thus reinforcing the hypothesis that a critical change in the protein conformation occurs in the ternary complex leading to the formation of a catalytically competent active site.

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Deuterium Nuclear Magnetic Resonance Investigation of the Exchangeable Sites on Gramicidin A and Gramicidin S in Multilamellar Vesicles of Dipalmitoylphosphatidylcholine†

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ABSTRACT: Solid gramicidin A and S and their interaction with DPPC bilayers were examined by ^2H NMR as well as ^{31}P NMR and differential scanning calorimetry (DSC). The deuterium spectra arose from deuterons associated with the peptide through chemical exchange in ^2H_2O. The spectra from both peptides were characterized by a quadrupolar splitting parameter, $\omega_Q/2\pi \approx 150$ kHz, and an asymmetry parameter, $\eta \approx 0.17$. An additional 33 kHz, $\eta = 0$ component arising from deuterons on mobile ornithine side chains was present in gramicidin S. In the gel phase of dipalmitoylphosphatidylcholine liposomes the gramicidins gave spectra that had components identical with those obtained from the solids. In the liquid-crystalline phase gramicidin A containing samples gave multicomponent spectra with a maximum quadrupolar splitting value of 133 kHz, $\eta = 0$. A minimum in the T_{2e} was observed, coinciding with the onset of the broadened phase transition measured by DSC and ^{31}P NMR, due to the onset of axial rotation of the peptide in the bilayer. The different powder patterns in the liquid-crystalline spectra from gramicidin A probably arise from different amide sites along the transmembrane channel. The broad component of the ^2H NMR spectra from gramicidin S in liposome preparations was not affected by the lipid-phase transition. The T_{2e} was also constant over this temperature range. The results are consistent with a location of gramicidin S at the membrane surface.

The examination of lipid protein interactions in membranes is an active area of research. It has been stimulated by a large number of observations that indicate that the physical properties of membranes influence their function (McElhaney, 1982). Model membranes consisting of a few components have been used extensively for these studies to reduce the complexity of the interactions between membrane components and to allow systematic investigations to be made of the various types and strengths of these interactions. The use of model membranes also avoids the problems of instability and limited availability associated with membranes from natural sources. Various physical techniques especially electron spin resonance (ESR)[1] (Devaux, 1983) and NMR (Seelig & Seelig, 1980; Davis, 1983; Bloom & Smith, 1985) have been used in these investigations. The effects of proteins on the physical properties of the lipid matrix have been examined in some detail by these techniques, but the converse, i.e., the effects of the lipids on protein structure, has not received as much attention. Recent exceptions to this are studies of amino acid side-chain motion in several integral membrane proteins (Smith & Oldfield, 1984), a report of ^{15}N NMR spectra recorded from backbone sites of fd bacteriophage coat protein (Cross & Opella, 1982), and a ^2H NMR study of exchangeable sites on a synthetic polypeptide incorporated into DPPC bilayers (Pauls et al., 1985). The ^2H NMR study of the synthetic polypeptide in DPPC was the prototype for a class of experiments that can be carried out on membrane proteins to characterize their dynamical properties. Such experiments are based on the measurement of spectra arising from exchangeable hydrogen sites on polypeptide molecules in samples containing excess ^2H_2O. The ^2H_2O is necessary to avoid complications due to hydrogen exchange but makes the detection of the broad peptide spectrum difficult because it gives rise to an enormous solvent signal. A method for overcoming this solvent interference was published separately (Callaghan et al., 1984). In this paper we report the use of this type of experiment to examine the dynamical properties of gramicidin A and gramicidin S incorporated, separately, into liposomes prepared from DPPC.

An extensive body of literature indicates that the linear pentadecapeptide gramicidin A forms ion-permeable pores in natural or artificial membranes. Conductance studies (Hladky & Haydon, 1972; Andersen, 1983, 1984) and physical measurements (Urry, 1971; Urry et al., 1973, 1983; Wallace et

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[1] Abbreviations: ESR, electron spin resonance; NMR, nuclear magnetic resonance; FID, free induction decay; DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine; $[^2H]Me_2SO$, deuterated dimethyl sulfoxide; T_1, spin–lattice relaxation time; T_{2e}, spin–spin relaxation time; N_s, number of scans; T_R, time between repetition of pulse sequence; τ_2, time between 90° pulses of the quadrupolar echo sequence.

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