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A distance measurement between specific sites on the cytoplasmic surface of bovine rhodopsin in rod outer segment disk membranes

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Abstract

Structural information on mammalian integral membrane proteins is scarce. As part of work on an alternative approach to the structure of bovine rhodopsin, a method was devised to obtain an intramolecular distance between two specific sites on rhodopsin while in the rod outer segment disk membrane. In this report, the distance between the rhodopsin kinase phosphorylation site(s) on the carboxyl terminal and the top of the third transmembrane helix was measured on native rhodopsin. Rhodopsin was labeled with a nuclear spin label (^{31}P) by limited phosphorylation with rhodopsin kinase. Major phosphorylation occurs at serines 343 and 338 on the carboxyl terminal. The phosphorylated rhodopsin was then specifically labeled on cysteine 140 with an electron spin label. Magic angle spinning ^{31}P-nuclear magnetic resonance revealed the resonance arising from the phosphorylated protein. The enhancement of the transverse relaxation of this resonance by the paramagnetic spin label was observed. The strength of this perturbation was used to determine the through-space distance between the phosphorylation site(s) and the spin label position. A distance of 18 ± 3 Å was obtained. © 1997 Elsevier Science B.V.

Keywords: Rhodopsin; Rod outer segment disk; Distance measurement; (Bovine)

1. Introduction

High resolution structural information for mammalian membrane proteins is lacking. The only integral membrane proteins for which high resolution structures are available are bacterial porin [1], bacteriorhodopsin [2], the photoreaction center from *Rhodopseudomonas viridis* [3] and from *Rhodobacter sphaeroides* [4], and cytochrome oxidase from *Paracoccus denitrificans* [5] and from bovine heart [6]. Thus only one high resolution structure is available for mammalian integral membrane proteins. There is a crucial need for alternative approaches to the structure of membrane proteins.

No high resolution structure is available for any G-protein receptor, including rhodopsin. Limited low resolution data on this structure are available. Based on circular dichroism (CD) measurements [7] and primary sequence [8], a bundle of seven transmem-

Abbreviations: CTAB, Cetyltrimethylammoniumbromide; 4,4-DTP, 4,4-Dithiopyridine; MAS ^{31}P-NMR, magic angle spinning ^{31}P-nuclear magnetic resonance; ROS, rod outer segment; spin label 1, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfate

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brane helices has been suggested as part of the structure for bovine rhodopsin. Electron diffraction studies have revealed the orientation of most of the transmembrane α-helices of rhodopsin [9,10]. FTIR measurements have revealed the presence of some β-sheet in the carboxyl terminal domain of rhodopsin [11]. Crosslinking experiments have revealed some nearest-neighbor interactions [12]. Spin label experiments have suggested the extent and rigidity of the third cytoplasmic loop [13].

Here we report a measurement of the distance between the rhodopsin kinase phosphorylation site(s) on the carboxyl terminal and the top of the third transmembrane helix on native rhodopsin in the rod outer segment disk membrane. This method should be of use for distance measurements on other membrane proteins.

2. Materials and methods

ATP was obtained from Sigma Chemical Co. (St. Louis, MO). (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfate was obtained from Reanal Fine Chemical Co. (Budapest, Hungary). 4,4′dithiopyridine was obtained from Aldrich Chemical Co. (Milwaukee, WI). Frozen, dark-adapted bovine retinas were obtained from J.A. and W.L. Lawson (Lincoln, NE). All experimentation was carried out, under dim red light, unless indicated otherwise.

2.1. Preparation of bovine rod outer segment (ROS) disk membranes

Retinal rod outer segment disk membranes were prepared from frozen bovine retinas as described [14]. Rhodopsin levels were determined from the absorbance at 500 nm, using the extinction coefficient of 40,000. Disks typically exhibited a ratio of the absorbance at 280 nm to that at 500 nm of 2.2. Unless otherwise stated, all manipulations of the rod outer segment disk membranes were performed under a Kodak 1A red filter. The isolated disks were washed and resuspended in 100 mM NaCl, 20 mM Hepes, 0.1 mM EDTA, pH 7.5 (buffer A) to a final rhodopsin concentration of 4–6 mg/ml. The buffers used were perfused with nitrogen or argon to reduce lipid oxidation [15].

2.2. Light-stimulated phosphorylation of rhodopsin in bovine retinal disks

Phosphorylated disks were obtained by an integration of the phosphorylation of ROS procedure described [16] and the isolation and purification of osmotically intact disks by Ficoll floatation procedure described [14]. The phosphate buffer used throughout the phosphorylation procedure was 100 mM NaHPO4, 1 mM MgCl2, 0.1 mM DTT, pH = 7.5. ROS were phosphorylated using 3 mM ATP, in phosphate buffer, with a 30 second bath sonication, followed by a 15 min preincubation at 37°C, before light stimulation to facilitate ATP entry into the ROS. Illumination was done for 30 min, at room temperature, using a 100 watt, incandescent light bulb. After phosphorylation, rhodopsin was regenerated by adding a 2 fold, mole excess of 11-cis retinal and incubating for 2 h at room temperature and then overnight at 4°C. Phosphorylated disks were pelleted in 10 mM Hepes, pH = 7.5. or were analyzed for the extent of phosphorylation, on isoelectric focusing gels, as described [16]. Rhodopsin concentrations and the extent of bleaching and regeneration was determine by spectral differences at 500 nm, in CTAB buffer. Greater than 95% regeneration was typically observed. Experimental conditions were optimized for the production of the most limited phosphorylated rhodopsin possible, in which the predominant phosphorylated species contained two phosphates per rhodopsin, as judged by the analysis of Aton et al. [17]. Attempts at producing more limited phosphorylation produced too little material for the NMR experiments.

2.3. Spin labeling of cysteine 140 with (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfate

Cysteine-316 of the phosphorylated rhodopsin was blocked, in the dark, with 4,4-DTP using a modified procedure of Chen and Hubbell [18]. Phosphorylated disks were pelleted in 100 mM NaCl, 10 mM Hepes Buffer, pH = 6.8, in an SS34 rotor, at 17 000 rpm, for 20 min, at 5°C. The pelleted disks were resuspended, at a concentration of 1 mg/ml, in ice-cold 1 mM 4,4-DTP, 10 mM Hepes Buffer, pH = 6.8. The phosphorylated disks were incubated with the 4,4-DTP for 2 h, on ice. Following incubation, the phosphory-

lated, blocked disks were washed repeatedly, first in 10 mM Hepes Buffer, pH = 6.8, then in 50 mM TRIS-HCl, 1 mM MgCl2, pH = 7.3.

Spin labeling of phosphorylated, (cys 316) blocked disks was accomplished in the dark by using a modified procedure as described by Rowntree and Watts [19]. Phosphorylated, blocked disks were resuspended in 50 mM TRIS-HCl, 1 mM $MgCl_2$, pH = 7.3, at a concentration of 5 mgs/mL. Spin label I, in a minimal amount of ethanol (100 μl), was added to the disks, at 0.3 mgs spin label/mg of rhodopsin. The disks were incubated 18 h, under nitrogen, at 4°C. The phosphorylated, blocked, spin labeled disks were then washed several times to remove excess spin label. The final wash, in which the disks were pelleted, was in 10 mM Hepes, pH = 7.5. This results in a specific spin labeling of cysteine 140 [20]. Previous data showed that the extent of labeling in the dark was independent of the presence of opsin [18]. Furthermore in our experiments in the dark, 95% or more of the protein was in the rhodopsin form.

2.4. Magic angle spinning (MAS) ^{31}P-NMR

MAS ^{31}P-NMR spectra were obtained on a MSL400 NMR spectrometer at 161.98 MHz in 4 mm sample rotors with a normal acquisition sequence ($\pi/2$ pulse width of 5 ms) using a total delay of 5s. Spinning rates of 5 kHz were used. Approximately 10,000 transients were collected with 4 k data points. Partially relaxed spectra were obtained using an inversion-recovery sequence. Chemical shifts were determined relative to external phosphoric acid.

3. Theory

Paramagnetic enhancement of nuclear relaxation by spin labels has proven in the past to be an effective means to determine distances in molecules. Because the magnetic moment of the electron is nearly two orders of magnitude larger than the nuclear magnetic moment, an unpaired electron can have a strong dipolar interaction with the nuclear magnetic moment which can be observed over distances up to 20 Å. Relaxation effects can be observed in both longitudinal relaxation (T_1) and in transverse relaxation (T_2). In this study, only T_2 could be used for the distance measurement.

The observed T_2 in the presence of a paramagnetic center is:

$$\frac{1}{T_{2\text{obs}}} = \frac{1}{T_2} + \frac{1}{T_{2\text{M}}} \tag{1}$$

The paramagnetic enhancement of transverse relaxation, T_{2M}, is by dipolar relaxation, and is given as:

$$\frac{1}{T_{2\text{M}}} = \frac{1}{15}\,\frac{S(S+1)h^{2\gamma}I^{2\gamma}S^2}{\boldsymbol{r}^6}(4\tau_c) \tag{2}$$

when $\omega_S \gg w_I$, $\omega_S^2\tau_c^2 \gg 1$, and $\omega_I^2\tau_c^2 \gg 1$. Here, S is the spin of the electron, ω_S is the Larmor frequency for the electron, λ_S is the electron magnetogyric ratio, ω_I is the Larmor frequency for the phosphorus nucleus, γ_I is the nuclear magnetogyric ratio, τ_c is the effective correlation time for the dipolar interaction between the electron and the phosphorus nucleus, and **r** is the through-space distance between the electron and the phosphorus nucleus. Since the distance, **r**, is not short (see Section 4), the scalar term has been left out of Eq. (2), following Wien et al. [22].

$$\frac{1}{\tau_c} = \frac{1}{\tau_e} + \frac{1}{\tau_r} \tag{3}$$

where τ_e is the longitudinal relaxation of the electron on the spin label and τ_r is the rotational correlation time of the protein. τ_e is of the order of 10^{-5}–10^{-6} s [23], while τ_r is 2–4×10^{-5} s [24]. Therefore τ_c can be approximated by τ_e. The following expression can then be derived for **r**, the distance between the unpaired electron and the phosphorus atom, from Eq. (2) and Eq. (3):

$$\boldsymbol{r} \approx 35(T_{2\text{M}})^{1/6} \tag{4}$$

4. Results

The goal of these experiments was to measure the distance between two sites on intact rhodopsin by using the dipolar interaction between the electron spin label specifically bonded to cys 140 at the top of helix three of the transmembrane domain of rhodopsin and the phosphorus nuclear spin label at either ser 338 or ser 343. The paramagnetism of the electron spin label will perturb the nuclear relaxation of the

phosphorous nuclei in a manner dependent upon $\mathbf{r}^{-6}$ where **r** was the distance between the nucleus and the spin label.

The phosphorylation sites of rhodopsin have been extensively studied and it is now well established that rhodopsin is initially phosphorylated at ser 343 and ser 338. We followed published procedures (Methods) to obtain the specific labeling of these residues. The isoelectric focusing gels of our product matched those published previously [17], showing that the predominant species contained two phosphates. Other published work had already established that under these conditions, the two phosphorylation sites were ser 343 and ser 338 [25].

Cysteine 140 can also be uniquely labeled with a derivative with a paramagnetic center by a modification of existing protocols [20]. When exposed to sulfhydral reagents, two cysteines (cys 140 and cys 316) on the cytoplasmic face of rhodopsin can readily react. To achieve this specific labeling, cysteine 316, the most reactive of the two modifiable cysteines, was initially blocked. Then cysteine 140 could be uniquely labeled with a spin label.

4.1. Specific blocking of cysteine 316 with 4,4-DTP and labeling of cysteine 140 with spin label I

As described in Section 2 [18], the reaction of 4,4-DTP with rhodopsin in disk membranes was followed over the course of 6 h. As previously reported, 4,4-DTP reacts rapidly with rhodopsin, modifying two cysteines, cys 316 and cys 140. It was reported that cys 316 reacted more quickly than cys 140, thus allowing the specific labeling of cys 316 with 4,4-DTP, if the reaction was stopped at an appropriate time. At room temperature in this laboratory, we observed the same labeling process as previously reported. In addition we found that lowering the temperature preferentially decreased the rate of reaction of cys 140, relative to the reaction of cys 316, making it easier to separate the two. In particular, when 4,4-DTP was reacted with rhodopsin in ROS disk membranes at 4°C, and the reaction followed as a function of time as described in methods, the kinetics of the reaction were significantly slowed as seen in Fig. 1. The reaction of 4,4-DTP with cys 316 is seen, but the reaction with cys 140 at this temperature is negligible. Upon raising the temperature to

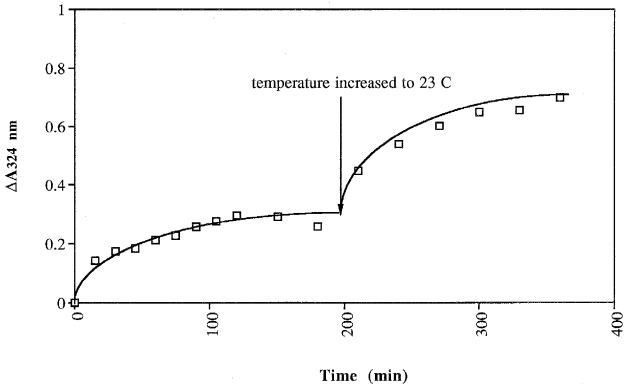

Fig. 1. Time-course of labeling by 4,4-DTP of rhodopsin in ROS disk membranes in the dark, following the procedures described in the text. The labeling was permitted to proceed at 4°C for 200 min, at which time the temperature was increased to 23°C. The extent of reaction was monitored by the change in absorbance at 324 nm. At 4°C, cys 316 was quantitatively labeled, with no significant labeling of cys 140 (see [20] for selective proteolysis experiments demonstrating the specificity of this labeling procedure). Increasing the temperature to 23°C led to labeling of cys 140.

23°C, labeling of cys 140 [18,20] was readily observed (Fig. 1). Therefore, through this modification of the published procedure we were able to more effectively separate the labeling of the two cysteines, and thus more specifically block cys 316, leaving cys 140 free to react with spin label. The specificity of this labeling was previously confirmed with selective proteolysis experiments [18].

Rhodopsin was phosphorylated in ROS disk membranes as described in the methods. After phosphorylation of rhodopsin, the reaction with 4,4-DTP was performed with ROS disk membranes at 4°C to specifically block cys 316. Phosphorylated rhodopsin blocked at cys 316 was further reacted with spin label I, as described in Section 2. This procedure specifically labels cys 140 with the spin label and produces a homogeneous ESR spectrum [20].

4.2. MAS ^{31}P-NMR of phosphorylated rhodopsin

Fig. 2A shows the MAS ^{31}P-NMR spectrum of rhodopsin in ROS disk membranes phosphorylated according to the protocol described in Section 2. The most intense resonances arise from the phospholipids in the disk membrane. The most upfield resonance comes from phosphatidylcholine, and the most in-

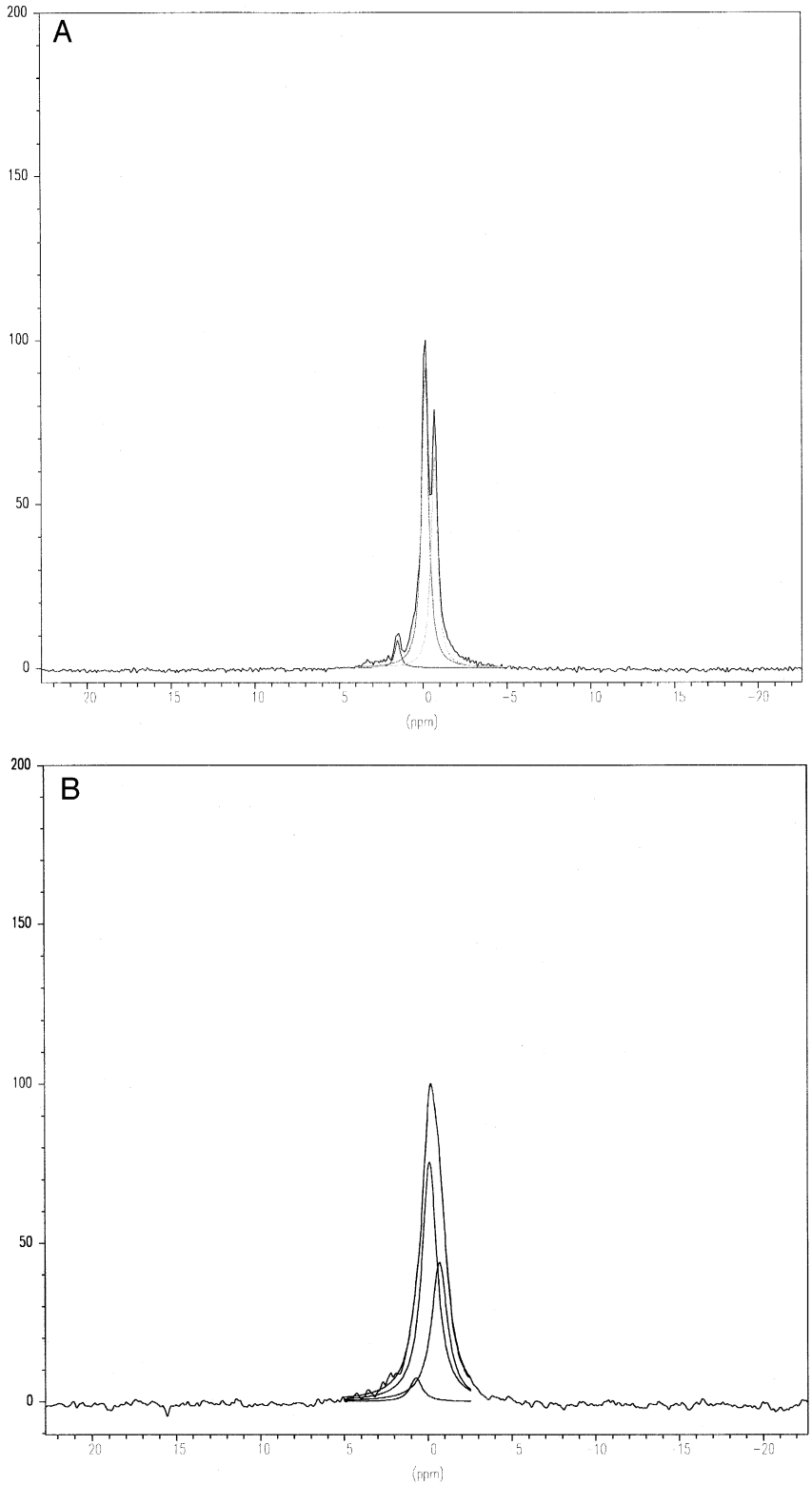

Fig. 2. MAS ^{31}P-NMR spectra of ROS disks containing phosphorylated rhodopsin. (A) phosphorylated, unbleached ROS disks, with the spectral simulation superimposed; (B) phosphorylated, spin labeled, unbleached ROS disks with the spectral simulation superimposed.

tense resonance (just downfield from the phosphatidylcholine) comes from phosphatidylethanolamine. Resonances from phosphatidylserine and phosphatidylinositol, also found in the disk membrane, are much less intense, and fall between the resonance positions of the other two phospholipids.

Downfield of all the phospholipids is seen a resonance of much less intensity, which was previously assigned to phosphoserine residue(s) on rhodopsin [26]. Controls in that previous work demonstrated unequivocally that this resonance arose from phosphate covalently attached to the protein and did not arise from small phosphorus containing compounds in the preparation or from phospholipids, and that it specifically appeared only upon light-stimulated phosphorylation of rhodopsin. Furthermore, as described above, the phosphorylation procedure produces rhodopsin labeled with two phosphates, on average, and previous work established the position of these phosphorylated serines [25]. This ^{31}P-NMR resonance thus corresponds to the phosphorus nuclear spin label on ser 343 and ser 338 of rhodopsin in disk membranes by reaction of light-stimulated rhodopsin with rhodopsin kinase and ATP.

Fig. 2A also shows a spectral simulation of this spectrum. Included in the simulation are the resonances from the phosphorylated protein, from the phosphatidylethanolamine, and from the phosphatidylcholine. Table 1 summarizes the chemical shifts and the linewidths from this analysis.

The relative intensity of the ^{31}P-NMR resonances was determined from the spectral simulation. The delay between acquisitions was nearly enough to produce full relaxation of all resonances. The acquisition was a normal Bloch decay, and there was no significant nuclear Overhauser effect under these conditions, so that the resonance intensity was a reasonable measure of the relative populations of the various species. By comparing the resonance intensities from the spectral simulations and taking into account the ratio between protein and phospholipids in the disk membrane, one can conclude that, on average, about two phosphates are found on each rhodopsin. (While some phosphorylation also occurs on peripherin under these conditions, in absolute concentration that phosphorylation is much less than the phosphorylation that occurs on rhodopsin.) As described in Methods, experimental conditions were optimized for the most limited phosphorylation as described [17], corresponding two phosphates per rhodopsin. This is consistent with the ^{31}P-NMR analysis.

Table 1

Resonance	Chemical shift	Linewidth
control	1.53 ppm	69 Hz
spin label (simulation)	0.8 ppm	114 Hz
spin label (partially relaxed spectrum)	0.9 ppm	130 Hz

4.3. Paramagnetic enhancement of the transverse relaxation of the MAS ^{31}P-NMR resonances of phosphorylated, spin labeled rhodopsin

MAS ^{31}P-NMR spectra were obtained in the dark from disk membranes that had been phosphorylated and specifically spin labeled at cysteine 140 as described above, and regenerated with 11-cis retinal. Fig. 2B shows this spectrum. The resonance from the phosphorylated serine is not readily observed in this spectrum. This observation indicates that the ^{31}P-resonance from the phosphorylated protein has been significantly broadened by the presence of the paramagnetic spin label. From this one can deduce that the spin label and the phosphorus of the serine phosphate must be relatively close to each other, since that broadening diminishes with distance by $\mathbf{r}^{-6}$.

To determine the extent of the paramagnetic line broadening, two methods were used. The first employed spectral simulation. Fig. 2B also shows these simulations, which include the resonances from the protein, from the phosphatidylethanolamine and from the phosphatidylcholine, the major contributors to the spectrum. Table 1 shows the chemical shifts and the linewidths obtained from this analysis.

To determine the linewidth of the resonance from the phosphorylated rhodopsin in the presence of the spin label by another means, a partially relaxed MAS ^{31}P-NMR spectrum was obtained. This experiment was designed on the premise that the T_1 of the phosphoserine resonance was likely different from the T_1 of the phospholipids. The T_1 of the ^{31}P resonances from the phosphoprotein and from the membrane phospholipids were measured in the ab-

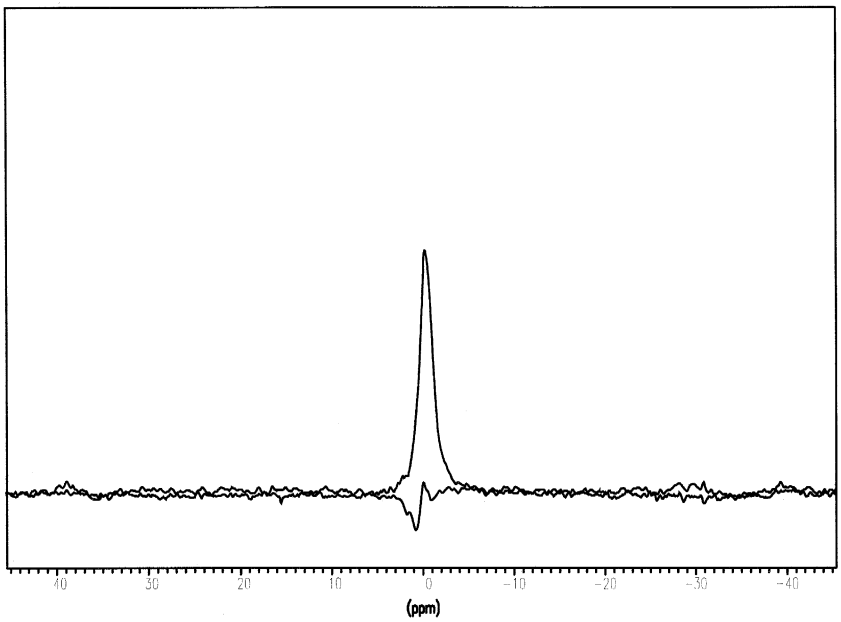

Fig. 3. Partially relaxed MAS ^{31}P-NMR spectra of ROS disks containing phosphorylated, spin labeled rhodopsin ($\tau = 80$ ms).

sence of the spin label for unbleached membranes. These values were: phospholipid $T_1 = 0.4$ s; phosphoprotein $T_1 = 2.9$ s. Thus the relaxation of the phospholipid was significantly different that the relaxation of the phosphoprotein resonance. A π-τ-$\pi/2$ pulse sequence was used, with a relaxation delay of 10 s. Using a sample that was phosphorylated and spin labeled as described in Section 2, τ was varied until the resonance from the phospholipids was nulled. The result is shown in Fig. 3. The T_1 of the phosphoprotein resonance is longer than the T_1 of the phospholipid resonance. Because of this differential in T_1 the broadened resonances can be partially separated. From this experiment, an independent determination can be made for the paramagnetically broadened resonance from the protein (see Table 1).

4.4. Determination of the distance **r** *between the predominant phosphorylation sites and the spin label on cys 140*

The theory section provides a means to determine the distance between the phosphorylation site and the spin label site, based upon the paramagnetic enhancement of the T_2. Using the data described above this enhancement can be determined, and the distance, **r**, calculated using Eqn. (5). A distance of 18 ± 3 Å is obtained. The major error in **r** results from uncertainty in the value for T_1 of the spin label attached to rhodopsin. The difference between the values of **r** calculated from the linewidths (Table 1) in the partially relaxed spectrum and in the simulated spectrum is insignificant, due to the sixth root dependence. It should be noted that there is an underlying approximation in this approach of a static **r** on the time scale of electron relaxation. The ESR spectrum of the spin label at cys 140 is relatively rigid [20], so in that regard the approximation is valid. The phosphates may experience some local motion. However, that local motion likely involves restricted movement about an average position that produces a relatively small time-dependent perturbation on the dipolar interaction between the phosphorus nucleus and the unpaired electron.

5. Discussion

These studies exploit the previous observation [26] that the major phosphorylation sites for rhodopsin kinase on bovine rhodopsin give rise to an observable ^{31}P-NMR resonance. Under the conditions of the present experiments, phosphorylation was limited predominantly to the two most highly phosphorylated

serines on the carboxyl terminal of the protein [25]. These studies on the phosphorylation of rhodopsin have shown that ser 338 and ser 343 are the dominant phosphorylation sites by rhodopsin kinase when one or two phosphates are found on the receptor.

The distance measurements reported here, based on the paramagnetic enhancement of the transverse relaxation of the phosphorus nuclei at the rhodopsin kinase sites on rhodopsin, can be expected to be a mixture of two distances. Both ser 338 and ser 343 are likely phosphorylated, as described above, so that the resonances from both sites may contribute to the spectrum at the same chemical shift and be affected by the spin label. The distance we measure, however, is not an average of the distances to the two phosphates, as that would require rapid chemical exchange of the phosphorus between the two phosphorylation sites. Furthermore we see no evidence of two overlapping resonances, one affected differentially from the other by the spin label. The NMR data appear consistent with a single site. This may arise from at least three possibilities: one is that due to the motional characteristics at the sites, only one site gives rise to a detectable resonance in the MAS experiments; another possibility is that the resonance of one site lies underneath the resonances of the phospholipids; the third possibility is that the two sites give indistinguishable resonances in the absence of the spin label, and in the presence of the spin label, one of the sites is broadened beyond detectability. The second possibility is unlikely because work in other systems has suggested that the ^{31}P chemical shift of the phosphoprotein resonances may be characteristic of the particular kinase site [27]. Simple analysis of resonance intensity is not consistent with the latter suggestion, but that analysis is not certain due to variables in these experiments that can modulate observed resonance intensity such as T_1 values.

An additional possibility that should be considered is that a spin label on one rhodopsin might influence the relaxation of a phosphorus nucleus on a neighboring rhodopsin, or an intermolecular interaction, rather than the intramolecular interaction we have assumed in the discussion above. Calculations show that the inter-rhodopsin distance, from one rhodopsin perimeter to another rhodopsin perimeter, is in the range of 25 Å in the disk membrane. Recent structural analysis of the cytoplasmic face of rhodopsin shows that the phosphorylation sites are located well inside the perimeter [21], so that the distance from the spin label on one rhodopsin to the phosphate on a neighboring rhodopsin is in excess of 30 Å. Because of the $\mathbf{r}^{-6}$ dependence of the dipolar interaction, the intermolecular interaction will be insignificant relative to the intramolecular interaction.

This distance measurement on intact rhodopsin adds to the growing body of point-to-point distance measurements on this protein. For example, recent measurements have provided distance measurements from a site next to cys 140 to several sites on the third cytoplasmic loop [28] on rhodopsin. These measurements will be important to an effort at building a three dimensional structure of this protein.

The experimental method described here may be useful in obtaining structural parameters from other membrane protein systems. The method is powerful in that it allows a determination of inter-site distances between two sites on an intact membrane protein in its biological membrane.

Acknowledgements

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