SATURATION TRANSFER EPR SPECTROSCOPY ON SPIN-LABELED MUSCLE FIBERS USING A LOOP-GAP RESONATOR

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ABSTRACT Previously, saturation transfer (ST-EPR) studies of biomolecular dynamics have involved the use of a resonant cavity and the V'_2 display (absorption, second harmonic, out of phase). In the present study, we replaced the resonant cavity with a loop-gap resonator and used the U'_1 display (dispersion, first harmonic, out of phase) to study spin-labeled muscle fibers. The new resonator and display showed several advantages over those previously used. It produced virtually noiseless U'_1 spectra on a 0.4 μl sample using a 4 min scan; previous U'_1 experiments on spin-labeled muscle, using a conventional rectangular cavity, resulted in an unacceptably low signal-to-noise ratio. The high filling factor of the resonator facilitated the study of these extremely small fiber bundles and permitted high microwave field intensities to be achieved at much lower incident microwave power levels, thus greatly enhancing the signal-to-noise ratio in U'_1 experiments. This reduction in the noise level made it possible to benefit from the other advantages of U'_1 over V'_2, such as stronger signals, simpler line shapes, and simpler data analysis. For these muscle fiber samples, the resulting sensitivity (signal/noise/sample volume) of the U'_1 signals was >100 times that of V'_2 signals obtained in a conventional cavity. Another advantage of the U'_1 display is that signals from weakly immobilized probes, i.e., probes that have nanosecond rotational mobility relative to the labeled protein (myosin), are greatly suppressed relative to strongly immobilized probes. This reduces the ambiguity of spectral analysis, and eliminates the need for chemical treatments [e.g., using $K_3Fe(CN)_6$] that were previously required in muscle fibers and other systems. Further suppression of this weakly immobilized component was achieved in U'_1 spectra by increasing the microwave power and decreasing the field modulation frequency.

INTRODUCTION

Saturation transfer EPR (ST-EPR) has greatly extended the applicability of nitroxide EPR to the study of slow (microsecond and millisecond) biomolecular motions, particularly in studies of muscle protein dynamics (Thomas et al., 1975, 1976, 1980; Hyde and Thomas, 1980; Thomas, 1982). Previous ST-EPR studies involved the use of the V'_2 display (absorption, second harmonic, out of phase) and a standard rectangular resonant cavity. In the present study we have used spin-labeled muscle fibers to demonstrate some of the advantages of using the U'_1 display (dispersion, first harmonic, out of phase) and a loop-gap resonator (Froncisz and Hyde, 1982; Hyde et al., 1982).

Dispersion EPR has several potential advantages over the more widely used absorption EPR (Thomas et al, 1976). U'_1 signals are 3–10 times stronger than absorption ST-EPR (V'_2) signals, and since U'_1 does not saturate as easily as V'_2, an even greater advantage can be achieved at higher microwave powers. Lastly, the U'_1 line shape is much simpler than that of V'_2, and spectral simulations require less computer time, facilitating the quantitative analysis of data. Despite these advantages, dispersion ST-EPR has not often been used in previous biophysical studies, mostly due to the very poor signal-to-noise ratios obtained when using a standard resonant cavity. The source of this noise is the high sensitivity of dispersion to klystrom FM noise at the high microwave power levels needed to saturate nitroxide spin labels. The introduction of the loop-gap resonator has alleviated this problem; because of the very high filling factor in the loop-gap

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resonator, a microwave field amplitude, H_1, of 0.25 G (gauss) can be achieved with 1 mW of incident microwave power, a power setting 80 times lower than in a resonant cavity. Klystrom FM noise is negligible at this low power level. The high filling factor also allows high-quality signals to be obtained from very small samples. Small sample size can be very important to the application of ST-EPR to biological systems.

One of the most extensive biological applications of ST-EPR has been to the study of the molecular dynamics of muscle contraction. Many detailed models have been proposed to explain the large-scale motion of sliding filaments during muscle contraction, most of which involve the cross-bridges that connect the thick and thin filaments. (Huxley, 1957; Huxley and Simmons, 1971; Huxley, 1969; reviewed by Tregear and Marston, 1979). To monitor the crossbridge motion directly, ST-EPR experiments have been performed on selectively spin-labeled myosin heads in intact myofibrils during rigor and relaxation (Thomas et al., 1980). ST-EPR is optimally sensitive to rotational correlation times (τ_2) in the range of $10^{-7} < \tau_2 < 10^{-3}$ s, which is relevant to muscle dynamics (Hyde and Thomas, 1980). With the use of ST-EPR, Thomas et al. (1980) showed that the cross-bridges underwent rapid submillisecond rotational motion in the presence of ATP, but this motion was not seen in the absence of ATP. One problem with this technique is that to acquire good signal-to-noise ratios, relatively large samples (10–50 mg) are needed, preventing the study of small bundles of muscle fibers. For easier control and monitoring of the mechanical properties of muscle fibers, it is preferable to study small bundles, ideally as small as a single fiber. In the present study, we have been able to study such small sample sizes through the use of the loop-gap resonator and the U'_1 display.

Another general problem in saturation transfer spectroscopy on spin-labeled proteins is the occurrence of signals due to weakly immobilized labels; i.e., signals due to labels that have nanosecond rotational mobility relative to the protein. With this component present, correlation times in the microsecond range, expected for large-scale motions like those proposed for myosin, are difficult to interpret from absorption (V'_2) saturation transfer spectra. One method for eliminating this component is treatment with $K_3Fe(CN)_6$ (Graceffa and Seidel, 1980). However, a long treatment with a high concentration of $K_3Fe(CN)_6$ may also destroy some signal due to strongly immobilized labels (Graceffa and Seidel, 1980; Thomas et al., 1980; Thomas and Cooke, 1980). In most cases, in fact, $K_3Fe(CN)_6$ does not show sufficient selectivity for weakly immobilized labels. Thomas and Cooke (1980) found that much of the signal due to weakly immobilized spin labels in muscle fibers can be destroyed by treatment with concentrated $K_3Fe(CN)_6$ for several hours. In the present study, we report an instrumental means, out-of-phase dispersion (U'_1) ST-EPR, for suppressing this component.

METHODS

Preparation of Spin-labeled Muscle Fibers

Glycerinated rabbit psoas muscle was dissected into small bundles of 10–15 fibers and washed with rigor solution (0.06 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 25 mM MOPS, pH 7.0) at 0°C. The spin-labeling procedure was similar to that of Thomas and Cooke (1980). Fibers were transferred to the labeling solution (rigor solution, except that [KCl] = 0.12 M, [pyrophosphate] = 4 mM, and pH = 6.4) for 10 min. IASL [*N*-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl) iodoacetamide] was added to a final concentration of 0.5 mM for 1 h. This label is selective for SH-1 thiols on the S-1 subfragment of myosin (Thomas et al., 1980; Seidel et al., 1970). After labeling, the fibers were soaked in labeling solution for 15 min and then in rigor solution for 1 h. Next, half of the fibers were treated with 25 mM $K_3Fe(CN)_6$ in rigor solution without KCl for 6 h and then returned to normal rigor solution.

EPR Experiments

Conventional (V_1) and saturation transfer (V'_2 and U'_1) EPR experiments were performed on a Varian E109 x-band spectrometer (Varian Associates, Inc., Palo Alto, CA) equipped with a loop-gap resonator and a variable temperature controller. For a detailed discussion of the resonator see Froncisz et al. (1982). The sample, which was a bundle of 10–20 muscle fibers with a total diameter of ~0.2 mm, was loaded in a 0.5 mm capillary that had an active length of 2 mm in the resonator. Thus, within the active region of the resonator, the solution volume was ~0.4 μl, and the actual volume of the muscle fibers was <0.1 μl. For purposes of comparison with spectra taken under optimum conditions with a standard EPR cavity, absorption experiments (V_1 and V'_2) were also carried out using a rectangular TE102 cavity (Varian Associates, Inc., model E231) using a large tissue cell previously used to obtain the maximum signal-to-noise ratio from muscle fiber samples (Thomas and Cooke, 1980). The amount of muscle tissue used in this case was ~10 times the amount used in the loop-gap experiment, and >100 times the amount within the active region of the loop-gap resonator. The microwave field amplitude (H'_1) was calibrated using peroxylamine disulfonate (Thomas et al., 1976). Due to the resonator's high filling factor, the H_1 value inside the resonator was ~9 times greater than that in the conventional cavity with the same incident microwave power (Froncisz and Hyde, 1982, resonator 1). (A different conventional cavity was used for comparison in that study, resulting in a slightly different power ratio, 67.) Thus, to achieve the same H_1 value, the power setting was 80 times lower for the resonator (usually 1 mW) than for the cavity. In the conventional (V_1) experiments, the microwave power was 0.25 mW with the resonator and 20 mW with the cavity, corresponding to an H_1 value of 0.125 G in both cases; the modulation amplitude was 2 G. In ST-EPR (V'_2 and U'_1) the power setting was usually 1 mW in the resonator and 80 mW in the cavity, corresponding to H_1 = 0.25 G, and the modulation amplitude was 5 G. For V'_2 experiments, phase-sensitive detection was 90° out of phase with respect to the second harmonic of the 50 kHz modulation field. For U'_1, modulation and detection were at the same frequency, either 25 or 100 kHz, 90° out of phase. For U'_1 experiments, a Varian x-band bridge in normal configuration was used with the automatic frequency control (AFC) turned off and the reference phase shifted 90° with respect to the phase of the microwaves reflected from the resonator. V_1 spectra were obtained in 2 min using a 0.128 s time constant and saturation transfer (V'_2 and U'_1) spectra were obtained in 4 min using a 0.25 s time constant.

RESULTS AND DISCUSSION

Dispersion EPR in conjunction with a loop-gap resonator has several advantages over the more widely used absorption EPR obtained from a standard rectangular cavity. One such advantage is the capability of using a small

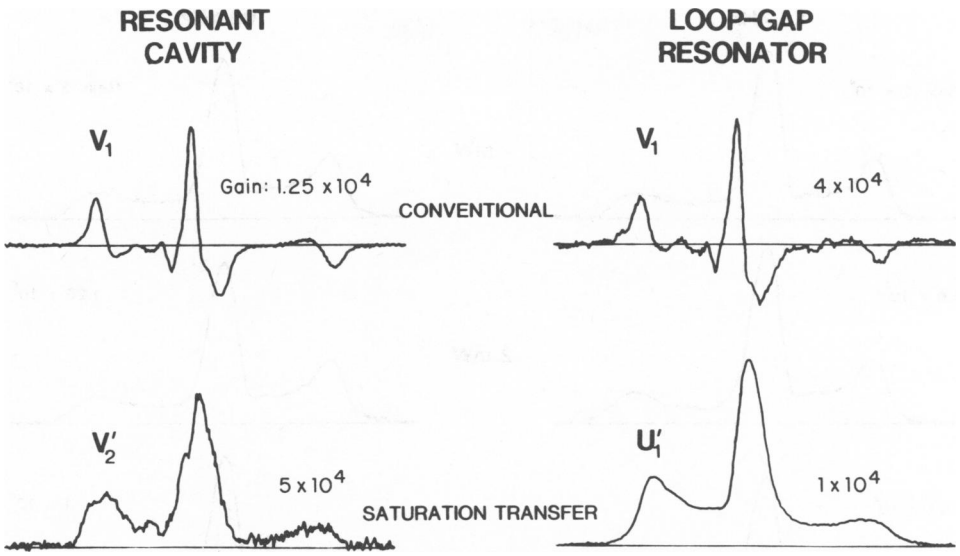

FIGURE 1 Conventional (V_1) and saturation transfer (V'_2 and U'_1) EPR from spin-labeled muscle fibers [treated with $K_3(CN)_6$] recorded under optimum conditions for high signal-to-noise ratios. All spectra in this paper were recorded in rigor solution (see Methods) at 23 ± 1°C, with the fibers aligned perpendicular to the DC magnetic field. The horizontal axis is 100 G wide. *Left*, spectra taken from a standard resonant cavity using a tissue cell (~50 mg of muscle, wet weight). *Right*, spectra taken from a loop-gap resonator (~50 mg of muscle used, but <1 mg in the active region of the resonator).

sample size. Fig. 1 shows conventional and saturation transfer EPR of spin-labeled muscle fibers with their fiber axes oriented perpendicular to the DC magnetic field under optimum conditions for high signal-to-noise ratios. A tissue cell was used in the standard rectangular cavity, and in the loop-gap resonator a capillary was utilized that contained <1/10 as much muscle tissue and ~100 times less material in the active region, compared with the resonant cavity. Nevertheless, conventional (V_1) spectra from the resonant cavity and the loop-gap resonator had similar signal-to-noise ratios (Fig. 1, top). In comparing saturation transfer spectra in Fig. 1, the conventional cavity is represented by a V'_2 spectrum and the loop-gap resonator by U'_1, since these displays yield the maximum signal-to-noise ratios for the respective resonators. The loop-gap resonator (U'_1) yields four times better signal-to-noise ratio than the cavity (V'_2), despite the very small sample size used. Because the sample size is 100 times smaller in the loop-gap resonator, this represents a 400-fold increase in sensitivity (signal/noise/sample volume). When the two resonators are both used in the U'_1 mode, the results are even more striking. In the conventional cavity, the U'_1 display from this sample (not shown) yields a signal-to-noise ratio that is 25 times worse than the V'_2 display; thus in the U'_1 displays, the loop-gap resonator has a sensitivity (signal/noise/sample volume) that is 10^4 times better than the cavity.

As discussed in the Introduction, an important problem when labeling complex systems, especially muscle fibers, is the occurrence of weakly immobilized labels, whose signals interfere with signals from strongly immobilized probes. Although $K_3Fe(CN)_6$ can sometimes be used to alleviate this problem, Fig. 2 demonstrates a more general instrumental solution. We found that dispersion (U'_1) ST-EPR suppresses the weakly immobilized component relative to the strongly immobilized component compared with absorption (V_1 and V'_2) experiments (Fig. 1). In Fig. 2 the U'_1 spectra with and without $K_3Fe(CN)_6$ treatment are virtually identical, showing no sign of weakly immobilized probes, although the absorption spectra (V_1 and V'_2) clearly have components due to nanosecond motion, i.e., due to weakly immobilized labels. Because U'_1 does not saturate as easily as V'_2 and the loop-gap resonator

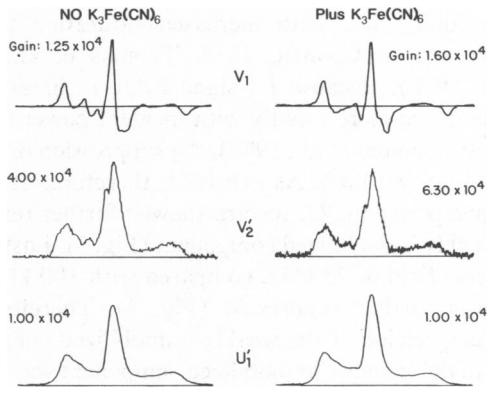

FIGURE 2 Conventional (V_1) and saturation transfer (V'_2 and U'_1) EPR spectra from spin-labeled muscle fibers. *Left*, before treatment with $K_3FE(CN)_6$. *Right*, after treatment with $K_3Fe(CN)_6$. Dispersion spectra (U'_1) were obtained using a loop-gap resonator, and absorption spectra (V_1, V'_2) were obtained using a standard rectangular cavity.

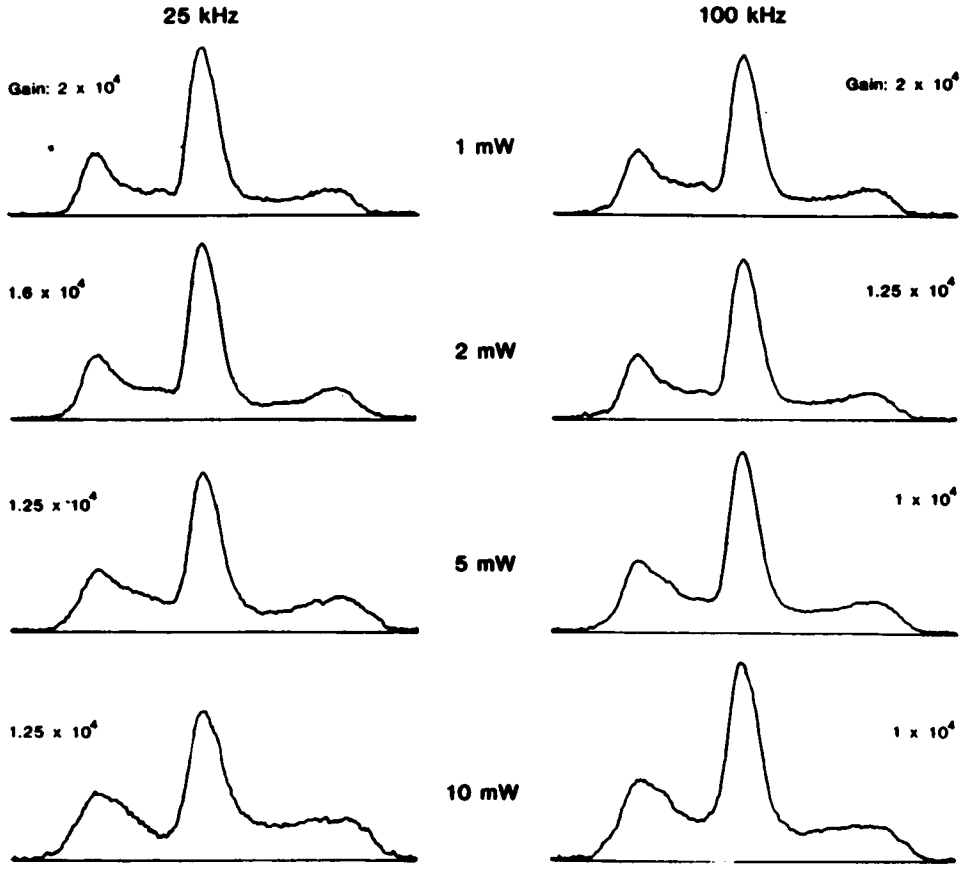

FIGURE 3 Effect of modulation frequency (25 and 100 kHz) and microwave power (1, 2, 5, 10 mW) on U'_1 spectra of spin-labeled muscle fibers with no $K_3Fe(CN)_6$ treatment.

prevented sample damage at high microwave power levels, we were able to run dispersion (U'_1) spectra at power levels up to 10 mW, equivalent to 800 mW in a standard rectangular cavity. The suppression of signals from mobile probes relative to those from immobile probes in ST-EPR is predicted theoretically, since the signal intensity increases with saturation while saturation decreases with probe mobility, i.e., with increased saturation transfer (Thomas and McConnell, 1974; Thomas et al., 1976; Thomas, 1978). Because U'_1 signals due to immobilized probes increase more rapidly with incident power than do V'_2 signals (Thomas et al., 1976), the suppression is greater with U'_1 than with V'_2. As expected, therefore, at higher microwave power the U'_1 spectra showed further reduction of the weakly immobilized component (Fig. 3). Lastly, at a modulation field of 25 kHz, compared with 100 kHz, this signal was further suppressed (Fig. 3). Therefore, the largest suppression of the weakly immobilized component relative to the strongly immobilized component was at high microwave power (10 mW) and a modulation frequency of 25 kHz.

Mechanical control and substrate saturation during EPR experiments on muscle fibers are greatly facilitated by the use of very small bundles of fibers (Thomas and Cooke, 1980; Cooke et al., 1982). Preliminary experiments indicate that acceptable signal-to-noise ratios can be obtained in V_1 and U'_1 experiments in single muscle fibers in times as short as 30 s, using the loop-gap resonator. Thus, in the near future, EPR experiments (both V_1 and U'_1) on single contracting muscle fibers should be possible.

SUMMARY

For biological studies in which it is desirable to study very small samples, such as very small bundles of muscle fibers, the loop-gap resonator provides a large advantage, with up to 100 times better sensitivity in conventional V_1 experiments than the conventional resonant cavity. In ST-EPR studies, the resonator in the U'_1 mode provides a substantial improvement in signal-to-noise ratio, even if the sample size is not limited; thus, the loop-gap resonator provides a tremendous advantage in ST-EPR studies of small biological samples such as spin-labeled muscle fibers.

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