EPR SPECTRAL OBSERVATIONS ON THE BINDING OF ATP AND F-ACTIN TO SPIN-LABELED MYOSIN

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SUMMARY. Myosin, sulfhydryl groups of which were spin-labeled with N-2,2,6,6 - tetramethylpiperidine nitroxide iodoacetamide (2 moles / mole of myosin), was used to examine the interaction of myosin, actin and ATP. F-actin decreased the mobility of spin labels bound to myosin, whereas ATP increased it. The maximum change in the electronparamagnetic resonance spectrum produced by F-actin was obtained when the solution contained 2 moles of actin monomer per mole of myosin. When F-actin was added to the myosin labeled with spin label, then with an analog of ATP, 6-mercapto-9β-ribofranosyl purine 5'-triphosphate, significant changes in the spectrum were not observed, suggesting that the myosin labeled with this analog does not interact with F-actin.

There are many physical indications of actin-myosin association, but largely they sense properties of the whole complex, e.g., its mass, radius of gyration, flow birefringence, etc. For certain questions [see, for example, Tokiwa and Morales (1)] it would be advantageous to have a method which directly "counts" the local contacts of individual myosin molecules with F-actin. [The recently reported methods of Cooke and Morales (2) count individual contacts, but only indirectly by counting G-actins incorporated into F-actin due to contact with myosin moieties.] Here we present observations which we feel may be of use in developing such a method. We have used the spin labeling technique (3) to examine the interaction of myosin, actin and ATP. The strategy of these experiments was to label the specific thiol groups located in the vicinity of the actin- and ATP-binding sites of the myosin molecule with "SH-directed" spin label in order to probe for possible changes in the structure of myosin induced by actin.

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EXPERIMENTAL: F-actin and myosin were prepared by the methods of Cohen (4) and Tonomura et al (5), respectively. Spin labeling of the sulfhydryl groups of myosin was performed according to the method of Seidel et al (6). Myosin was allowed to react with N-2,2,6,6 -tetramethylpiperidine nitroxide iodoacetamide (spin label, 2 moles / mole of myosin; MW of myosin, 4.8×10^5) in 0.6 M KCl - 0.04 M Tris (pH 8.0) on ice for 40 - 60 min. The unbound spin labels were removed by dialysis against 0.6 M KCl - 1 mM N-tris (Hydroxymethyl) methyl 2-aminoethane Sulphonic acid (TES) (pH 7.0) at Ca. 4° C for 48 hr. Labeling of myosin with 6-mercapto-9β-ribofranosyl purine 5'-triphosphate (SH-ATP) was as previously described (1). Electronparamagnetic resonance (EPR) spectra were obtained with a Varian Associates type E-3 spectrometer. The field strength was set at 3390G, with a scan range of ±50 gauss. During scanning the time constant of detection was 10 sec, and the scan time was 30 min. The modulation frequency was 100 KHz, and the amplitude, 2 or 4 G. The microwave frequency was 9.530 - 9.535 GHz, and the power, 50 mW. The receiver gain used was in the range of 10^5 to 10^6. To avoid spectral changes due to microwave heating effect, cooling air was circulated through the cavity. ATPase activity was determined by measuring the released inorganic phosphate according to Fiske and Subbarow (7). Protein concentration was determined by a variant of the Folin-Ciocalteau method (8). Spin label was prepared according to instruction provided by Dr. MacConnell. SH-ATP was synthesized as described previously (9), and was a gift from Dr. Joseph A. Duke. ATP was purchased from Sigma, and other reagents were commercial products of analytical grade.

RESULTS AND DISCUSSION: Myosin was labeled with iodoacetamide-like spin label as described above. This spin labeling enhanced the Ca^{++} mediated ATPase activity of myosin 3.5 fold. Figure-1 shows the effect of F-actin and of ATP on the EPR spectrum of spin labels bound to myosin molecules in 0.6 M KCl - 1 mM TES (pH 7.0). The

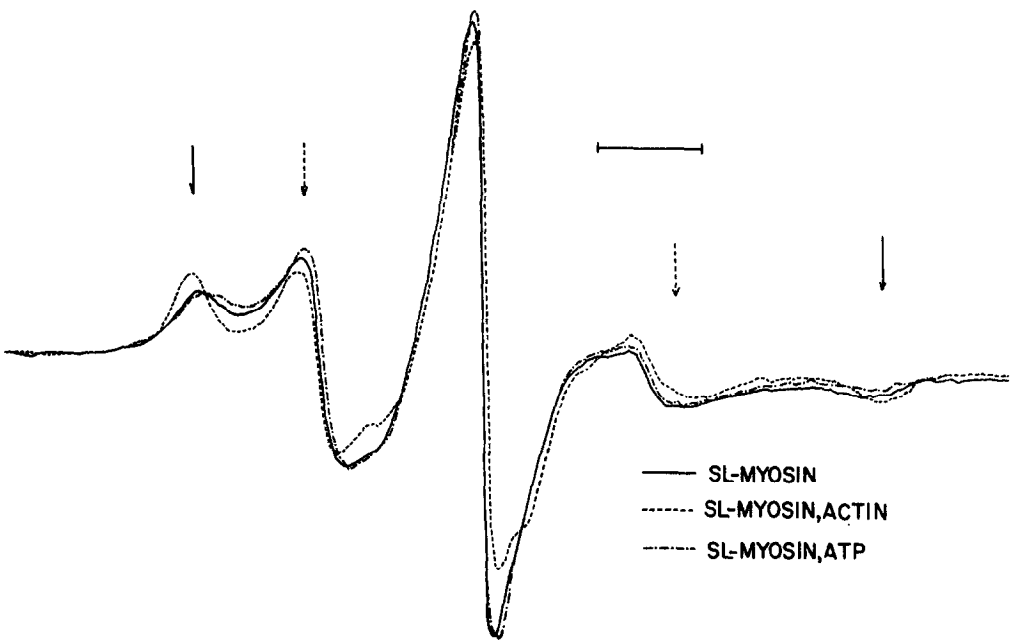

Figure-1. Effects of F-actin and of ATP on EPR spectrum of spin-labeled myosin. 4.13 mg/ml spin-labeled myosin, 0.6 M KCl, 1 mM TES (pH 7.0) at room temperature. (—) myosin alone, (----) myosin plus 1.07 mg/ml F-actin, (-·-·-) myosin plus 4 mM ATP. (↓) and (⇣) indicate a strongly immobilized signal and a weakly immobolized signal, respectively. In all figures on EPR spectrum, the scale inserted shows 10 gauss of scanning.

peaks indicated by dotted arrows arise from weakly immobilized labels, whereas the peaks indicated by full arrows arise from strongly immobilized labels. The addition of ATP increased the mobility of spin labels bound to myosin molecules; the height of weakly immobilized peaks increased, the height of strongly immobilized peaks decreased, and the distance between the first and last peaks decreased from ~64 to ~62 gauss. In contrast, when F-actin was added, the height of weakly immobilized peaks decreased, the height of strongly immobilized peaks increased, and the distances between the first and second peaks and the first and last peaks increased from ~9.1 to ~9.8 gauss and from ~64 to ~66 gauss, respectively. The changes produced by F-actin represent a decrease in the mobility of the spin labels bound to the myosin molecule. In the presence of 4 mM Mg^{++}-ATP, the spectrum of a mixture of spin-labeled myosin and F-

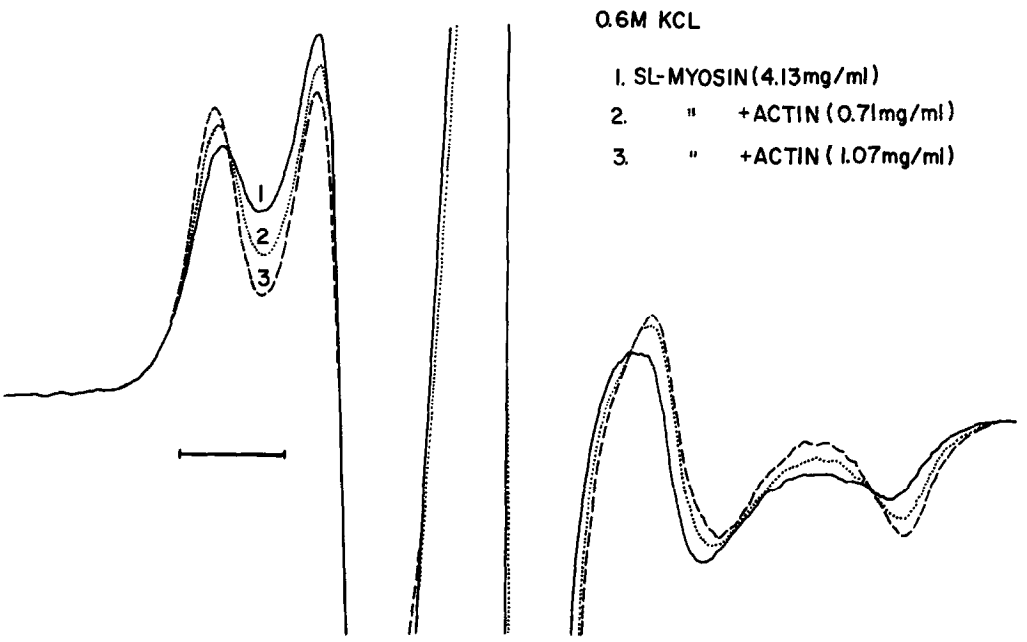

Figure-2. EPR spectrum of spin-labeled myosin in the presence of different concentrations of F-actin. 0.6 M KCl, 1 mM TES (pH 7.0), at room temperature.

actin was identical to that of spin-labeled myosin alone. The changes in spectrum induced by F-actin depended on the concentration of F-actin added to myosin (Figure-2). The maximum change was obtained when the weight ratio of actin to myosin was 9.3×10^4 to 4.8×10^5 g, indicating that 2 moles of actin bind to 1 mole of myosin (assuming that the molecular weight of actin monomer is 4.7×10^4). At low ionic strength (0.1 M KCl), we observed a similar change produced by F-actin in the distance between the first and last peaks in the EPR spectrum of spin-labeled myosin. The significant changes in the height of peaks were difficult to detect. At low ionic strength, the concentration of proteins in the EPR cell sometimes changed during scanning, since the actin-myosin mixture is not soluble in such solutions.

Changes in the EPR spectrum produced by F-actin were small, e.g., the maximum extent of the increment of the first peak was ~15% of the height of the original peak. Nevertheless, these changes could be amplified without unduly increasing noise

levels, by using high receiver gain, slowing the scanning time, and using a large time constant of detection. Thus, the EPR technique offers a suitable method for examining directly individual molecular contacts between myosin and F-actin. The most plausible explanation of the observed changes in the EPR spectrum caused by F-actin is that local changes in conformation of the "heads" of the myosin molecule are induced by contact with F-actin.

Next, we examined whether SH-ATP labeled myosin interacts with F-actin. Spin-labeled myosin was allowed to react with SH-ATP for a week, so that both heads of the spin-labeled myosin were labeled with the analog. SH-ATP labeling caused bigger changes in the EPR spectrum of the spin-labeled myosin than that caused by ATP (Figure-3). Labeling of myosin with SH-ATP resulted in a considerable increase in the mobility of spin labels bound to myosin. When F-actin was added to the myosin labeled

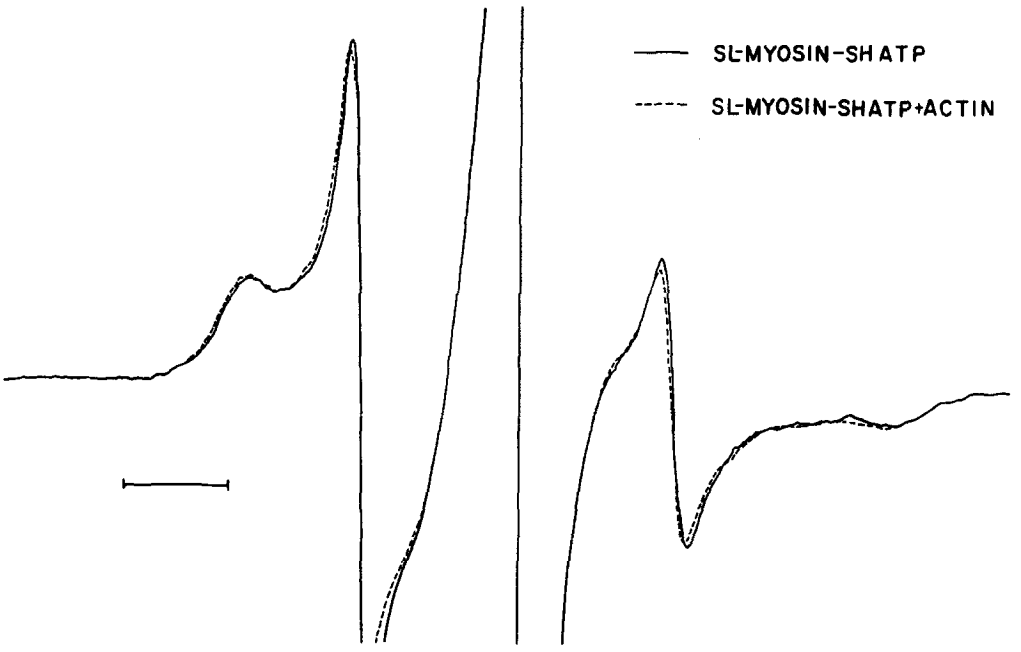

Figure-3. Effect of F-actin on EPR spectrum of myosin labeled with spin-label, then with SH-ATP. Spin-labeled myosin was incubated with 200 fold excess SH-ATP per mole for a week at 0° C, pH 8.0. 4.30 mg/ml myosin, 0.6 M KCl, 1 mM TES (pH 7.0) at room temperature. (—) myosin alone, (---) myosin plus 1.8 mg/ml F-actin.

with spin label and SH-ATP in 0.6 M KCl at pH7.0, the EPR spectrum did not change significantly, suggesting that myosin molecule totally reacted with SH-ATP does not bind to F-actin in a solution of high ionic strength.

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