Myosin cross-bridge orientation in rigor and in the presence of nucleotide studied by electron spin resonance

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ABSTRACT The tilt series electron spin resonance (ESR) spectrum from muscle fibers decorated with spin labeled myosin subfragment 1 (S1) was measured from fibers in rigor and in the presence of MgADP. ESR spectra were measured at low amplitude modulation of the static magnetic field to insure that a minimum of spectral lineshape distortion occurs. Ten tilt series ESR data sets were fitted simultaneously by the model-independent methodology described in the accompanying paper (Burghardt, T. P., and A. R. French, 1989. *Biophys. J.* 56:525–534). By this method the average and standard error in the mean of order parameters for the probe angular distribution were calculated for the two states of the fiber investigated. The average order parameters were used to reconstruct the probe angular distribution in two dimensions, one angular dimension corresponding to a polar angle measured relative to the fiber axis, and the other a torsional angular degree of freedom of the probe. We find that the probe angular distributions for the rigor and MgADP states of the fiber differ such that the rigor distribution is broader and shifted relative to the distribution in the presence of MgADP. The shape of the rigor distribution suggests the presence of two probe orientations, one similar to that in the presence of MgADP, and another at a different orientation. The shape of the distribution in the presence of MgADP suggests that the binding of the nucleotide to the rigor cross-bridge shifts the spin population into a more homogeneous one by causing a cross-bridge rotation.

INTRODUCTION

The possibility that the cyclical interaction of myosin and actin during muscle contraction produces the work for muscle shortening is suggested by the observation that the specific actomyosin affinity in a fiber varies over several orders of magnitude depending on the substrate intermediates that occupy the myosin ATPase site. A class of models for the molecular mechanism of muscle contraction focus on the movement of the myosin cross-bridge and propose that the cross-bridge rotates while attached to the actin filament to produce muscle shortening (Huxley, 1969; Huxley and Simmons, 1971; Huxley and Kress, 1985). Consistent with this model is the proposed ability to alter the orientation of the cross-bridge by changing the substrate in the myosin ATP binding site or by applying mechanical stress to the cross-bridge by altering the length of the fiber. There have been many investigations of the cross-bridge orientation as a function of chemical and mechanical perturbations that have led to somewhat contradictory interpretations (for a review see Burghardt and Ajtai, 1989*a*).

The orientation of the cross-bridge has been clearly demonstrated to be influenced by the binding of nucleotide to the nucleotide binding site on S1 and by the application of mechanical stress. Fluorescent probe studies of cross-bridge orientation, using myosin sulfhydryl one (SH1) and SH2 bound probes, indicate that the cross-bridge can maintain various orientations relative to the actin filament. Iodoacetamide tetramethylrhodamine (IATR) and 5-[2-(iodoacetyl)aminoethyl]aminonapthalene-1-sulfonic acid (1,5-IAEDANS) specifically modifying SH1 indicated that the cross-bridge domain containing SH1 rotated upon the binding of the nucleotide MgADP (Borejdo et al., 1982; Ajtai and Burghardt, 1986; Ajtai and Burghardt, 1987). The IATR probe was also used to investigate the orientation of the cross-bridges, in active isometric fibers (Burghardt et al., 1983), as a function of temperature (Ajtai and Burghardt, 1986), and as a function of mechanical stress (Burghardt and Ajtai, 1989*b*). These studies again showed that the cross-bridge domain containing SH1 could maintain more than one orientation when bound to the actin filament The probe, 4-(*N*-(iodoacetoxy)ethyl-*N*-methyl) amino-7-nitrobenz-2-oxa-1,3,diazol (IANBD), was used to modify myosin SH2 in fibers and showed that although the binding of MgADP did not alter the orientation of the cross-bridge domain containing SH2, this domain was rotated relative to the rigor orientation when the fibers were in isometric contraction (Ajtai and Burghardt, 1989).

In contrast to these findings, electron spin resonance (ESR) spectra from spin labels modifying SH1 in muscle fibers were interpreted to suggest that the cross-bridge domain containing SH1 had an orientation in the presence of MgADP that was identical to that in rigor

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(Cooke, 1986). ESR studies of muscle fibers in isometric contraction were also interpreted to suggest that those cross-bridges bound to actin maintained an orientation identical to that in rigor (Cooke et al., 1982). The interpretation of the ESR data presented in these earlier studies clearly conflicts with the fluorescence data and until now an acceptable explanation has not been offered. We investigated the cause for these discrepancies using newly developed and specialized analytical techniques for the interpretation of ESR data (Burghardt and French, 1989) and show below that ambiguity in the interpretation of the ESR data is responsible for the conflicting findings.

The model-independent methodology for determining the spin probe angular distribution from a tilt series of ESR spectra is described in the accompanying paper (Burghardt and French, 1989). In these types of experiments a spin probe specifically modifies an immobile or slowly moving element of a biological assembly. The probes report the angular arrangement of the elements to which they are attached. The model-independent methodology treats the angular order of the biological assembly as a distribution of reference frames, with one frame fixed in each element. The angular distribution function is expanded in terms of complete, orthogonal angular functions, and the order parameters are the expansion coefficients. The order parameters are also related to the observed signal. These two relations allow the inference of the order parameters from the data. The order parameters uniquely determine the angular distribution of the elements. By the simultaneous analysis of a series of ESR spectra, that differ only in that the muscle fiber axis is rotated relative to the Zeeman field, ambiguities in determining the correct fit by varying the free spectral parameters are apparently removed. With this method we determined a reliable picture of the spin probe angular distribution.

We applied the model-independent formalism to spectra from muscle fibers decorated with spin labeled myosin subfragment 1 (S1). The S1 was specifically labeled at SH1 with the maleimide based spin label 4-maleimido-(2,2,6,6,tetramethyl-1-piperidinyloxy) (MSL) and the nonspecific probe was selectively destroyed using potassium ferricyanide (Graceffa and Seidel, 1980; Seidel, 1982). The tilt series spectra were measured from the decorated muscle fibers in rigor and in the presence of MgADP, and the spin probe angular distributions for the two states of the muscle were compared. We find that the spin probe angular distribution for fibers in rigor differs from that in the presence of MgADP in agreement with the fluorescent probe data. The probe angular distribution in rigor is broader and shifted relative to that in the presence of MgADP suggesting there is more than one cross-bridge orientation in rigor. These data suggest that the binding of the nucleotide to the rigor cross-bridge causes a cross-bridge rotation.

MATERIALS AND METHODS

Chemicals

ATP, ADP, and P^1,P^5-di(adenosine-5')-pentaphosphate (Ap_5A) were from Sigma Chemical Co. (St. Louis, MO). The spin label 4-maleimido-(2,2,6,6,tetramethyl-1-piperidinyloxy) (MSL) was from Molecular Probes (Eugene, OR). All chemicals were analytical grade.

Solutions

Rigor solution was 80 mM potassium chloride, 5 mM magnesium chloride, 2 mM ethylene glycol bis(*b*-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 20 mM TES at pH 7.0. In ADP solution, 4 mM ADP was added to the rigor solution, together with 100 μM Ap_5A to inhibit myofibrillar myokinase from converting ADP to ATP. Relaxing solution was the same as rigor with 0.1 mM dithiolthreitol (DTT) and 4 mM ATP added and 5 mM potassium phosphate replacing TES. Skinning solution was relaxing solution with 0.5% Triton X-100 added.

Muscle fibers

Rabbit psoas muscle fibers were obtained as previously described (Borejdo et al., 1979) and kept in a relaxing solution containing 50% glycerol (volume-to-volume) at -15°C for up to several weeks. Before labeling, bundles of ~50 glycerinated fibers each were transferred to skinning solution for 30 min, to remove cell membranes that may inhibit diffusion of S1 through the fiber bundles, then thoroughly washed in rigor solution for 1 h. These fiber bundles were incubated in rigor buffer containing spin labeled S1 at a concentration of 4–6 mg/ml for a minimum of 2 h in the dark. The labeled fiber bundles were washed in either rigor or MgADP solution for ~5 min to remove the unbound S1, cut to a uniform length of 6 mm, and arranged with parallel fiber axes in a quartz flat tissue cell (Wilmad Glass, Buena, NJ) for the ESR studies.

The manipulations of the fibers before placing them in the flat cell were done at 4°C. Once in the flat cell the fibers were allowed to warm up to room temperature and the ESR spectra were measured at room temperature.

We estimated the contribution of free MSL-S1 to the decorated fiber spectrum by comparing the fiber spectrum (in both the presence and absence of MgADP) to that obtained from MSL-S1 in solution. The free MSL-S1 in rigor and in the presence of MgADP had identical spectra that were characteristic of a randomly oriented, slowly moving, spin label. The decorated fiber spectrum at zero tilt showed no evidence of the low field resonance characteristic of free MSL-S1. We estimated the contribution of free MSL-S1 to the zero tilt fiber spectrum to be $\leq$5%.

The possibility that the MSL label rotated independently on the MSL-S1 was investigated previously (Thomas et al., 1980). There it was reported that the MSL remains completely immobilized with respect to the S1 in the presence and absence of nucleotides. We have also confirmed this observation (unpublished result).

Preparation of spin labeled myosin subfragment 1

Rabbit myosin was prepared by a standard method (Tonomura et al., 1966). S1 was obtained by digesting myosin filaments with α-

chymotrypsin (Weeds and Taylor, 1975). 30 to 50 μM myosin or S1 was labeled for 24 h with a 1.5-fold molar excess of MSL in 50 mM TES and with 0.5 M KCl (myosin) or without salt (S1) at pH 7.0 and 4°C. The reaction was stopped by precipitating the myosin or by the dialysis of S1 in buffer without MSL. The protein was treated for 24 h with 25 mM potassium ferricyanide, 1 mM cystein, 1 mM EDTA, and 50 mM TES at pH 7.0 to selectively destroy electron spins not linked to SH1 (Graceffa and Seidel, 1980). This treating solution was removed by the precipitation of the myosin or dialysis of the S1. Labeled S1 was prepared from the labeled myosin by digestion with α-chymotrypsin (see above).

We measured the extent of the spin labeling of the SH1's using the K^+-EDTA ATPase and CA^{2+} ATPase activities of the labeled S1 (Seidel, 1982). The Ca^{2+} and K^+-EDTA activated ATPase activity was measured by using the modified Fiske and SubbaRow phosphate determination method of Bárány et al. (1967). The K^+-EDTA ATPase activity was measured in 0.6 M KCl, 6 mM EDTA, 25 mM Tris-HCl at pH 8, and 25°C. The Ca^{2+} activated ATPase was measured in 0.6 M KCl, 5 mM $CaCl_2$, 25 mM Tris-HCl at pH 8, and 30°C. The protein concentration of the assay was 0.02–0.03 mg/ml, ATP concentration was 2.0 mM. There were four different S1 preparations. The ATPase activities for unlabeled S1 were, for K^+-EDTA 4.82 ± 0.02 μmol phosphate/mg S1.min and for Ca^{2+} 0.62 ± 0.03 μmol phosphate/mg S1.min. The K^+-EDTA ATPase of labeled S1 was inhibited by 60 to 70% and the Ca^{2+} ATPase was activated by 270 to 370% compared with control unlabeled S1 indicating specific and efficient (0.6–0.7 mol spin to mole S1) modification of the SH1 group. The prolonged ferricyanide treatment did not effect either of the ATPase activities of the S1 or spin labeled S1.

ESR measurements and preliminary analysis

ESR measurements were carried out on a Bruker series instrument model ER200 (Bruker Instruments, Inc., Billerica, MA) using a TM_{110} cylindrical cavity. This cavity has zero electric field along a plane containing the Zeeman magnetic field and the axis along which the sample is inserted into the cavity (the sample axis). The zero tilt spectrum is measured when the flat cell is in the zero electric field plane. Tilted spectra were measured by rotating the flat cell about the sample axis and relative to the Zeeman field. This procedure was the simplest method of producing the tilted spectra but had the disadvantage of placing a larger amount of the microwave electric field absorbing material from the sample and holder in a nonzero electric field region of the cavity. Tilted spectra had a lower signal-to-noise ratio than the zero tilt spectrum due to this effect. For all of the experiments mentioned here a tilt series consisted of two spectra, one at zero tilt and one at 90° from the Zeeman field. The measurement of both spectra in a tilt series was completed in ~30 min.

The derivative of the ESR absorption spectra were digitized into 4,096 data points at equal intervals over the sweep width of the Zeeman field and temporarily stored on a floppy disk. Later the spectra were transferred to the disk of a larger computer for analysis. The ESR spectra were subjected to a linear baseline correction to set the low and high field asymptotic absorption, and total area under the curve, equal to zero. Spectra were normalized arbitrarly.

Numerical analysis of ESR spectra

Up to ten sets of tilt series spectra were simultaneously analyzed by the method described in the accompanying paper (Burghardt and French, 1989), to derive the order parameters of the probe angular distribution. In this method spectral parameters, such as the values of the elements of the g and T-tensors, were varied to minimize a fitting quality factor, Q, defined in Eq. 20 of Burghardt and French (1989). The probe angular distribution was reconstructed using the relation,

$$N(\Omega) = \sum_{j=0}^{j_{max}} \sum_{m,n=-j}^{j} a^j_{m,n} \sqrt{\frac{2j+1}{8\pi^2}} D^j_{m,n}(\Omega), \qquad (1)$$

where $\Omega = (\alpha, \beta, \gamma)$ are the Euler angles of the probe orientation, $a^j_{m,n}$ is the order parameter, $D^j_{m,n}$ is a Wigner function (Davydov, 1963), and j_{max} is the maximum rank of the included order parameters. We will also use the polar angular distribution, $n(\beta)$ derived from Eq. 1, by averaging over α and γ such that,

$$n(\beta) = \sum_{j=0}^{j_{max}} a^j_{0,0} \sqrt{\frac{2j+1}{8\pi^2}} P_j(\cos\beta), \qquad (2)$$

where $P_j(\cos\beta)$ is a Legendre polynomial (Arfken, 1970).

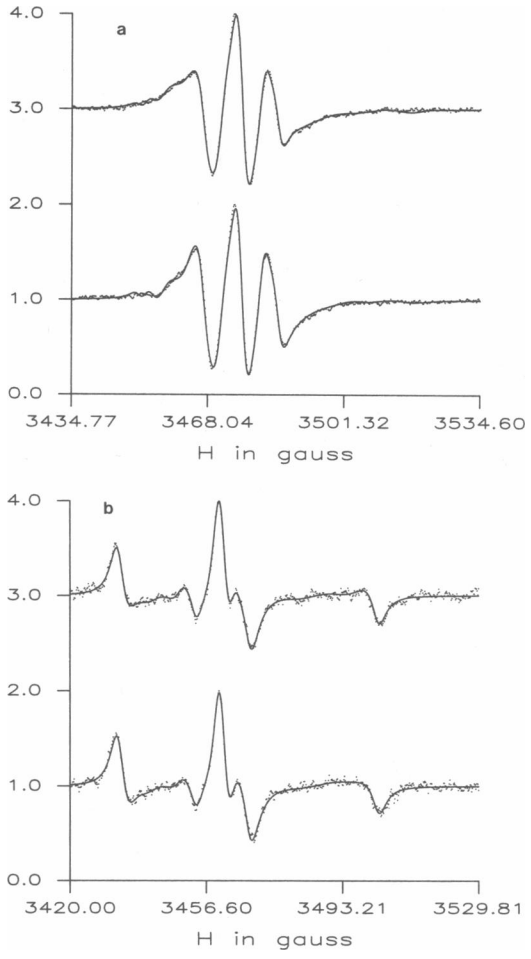

FIGURE 1 Representative tilt series ESR spectra for fibers decorated with spin labeled S1 in rigor and in the presence of MgADP. Panels *a* and *b* contain rigor (*top*) and MgADP (*bottom*) spectra with tilt angles $\psi = 0$ and 90°. Dots correspond to the data and the solid line is the best fit as judged by minimizing fitting quality factor, Q (see Eq. 20, Burghardt and French, 1989). The maximum heights of the spectra are normalized to one.

The computer program implementing the analysis formalism was written in FORTRAN. The simultaneous analysis of 10 tilt series data sets consisting of 20 ESR spectra could typically be completed within ~12 h of computer time on a VAX 8810.

RESULTS

Representative ESR spectra from fibers, decorated with spin labeled S1, in rigor and in the presence of MgADP are shown in Fig. 1. The spectra shown form two tilt series data sets, one for fibers in rigor, and one for fibers in the presence of MgADP. Order parameters from these and eight other tilt series data sets (for a total of five spectra from rigor fibers and five from fibers in the presence of MgADP), computed by the method outlined in the accompanying paper (Burghardt and French, 1989), are averaged and their standard error in the mean (SEM) calculated. These results are summarized in Table 1 where only the order parameters from the zero tilt spectra are shown. The order parameters listed there are considered significant because of their weighting factor (see Eq. 28, Burghardt and French, 1989).

From the tilt series data sets we ascertained the spectral parameters for decorated fibers in rigor and in the presence of MgADP. We found that the spectral parameters for these two fiber states are identical and given by $g_x = 2.0080$, $g_y = 2.0060$, $g_z = 2.0020$, $T_x = 8.8$ G, $T_y = 7.2$ G., $T_z = 34.8$ G, where g_i are elements of the g-tensor coupling the electron spin of the nitroxide to the Zeeman field and T_i are elements of the T-tensor coupling the electron spin to the nuclear spin of the nitrogen in the nitroxide.

The probe angular distribution of the zero tilt fibers is assumed to not depend on the Euler angle α. The angle α corresponds to the azimuthal angle of the probe when the z axis points along the fiber axis. The fiber axis is generally assumed to be a symmetry axis of the fiber and experimental evidence supports this assumption (Burghardt et al., 1983). This implies that for the zero tilt spectrum,

$$a^j_{m,n} = a^j_{0,n}\,\delta_{m,0}, \tag{3}$$

where $\delta_{i,j}$ is the Kronecker delta. It is reasonable to align the fiber axis with the Zeeman field for the zero tilt

TABLE 1 Order parameters from spin labeled S1 decorating muscle fibers

$a^j_{m,n}$	Rigor	SEM	MgADP	SEM
$a^0_{0,0}$	0.1125	0.0	0.1125	0.0
$a^2_{0,0}$	−0.0843	0.0015	−0.08110	0.0030
$a^2_{0,2} + a^2_{0,-2}$	0.1731	0.0140	0.1877	0.0063
$a^4_{0,0}$	0.0534	0.0020	0.0570	0.0059
$a^4_{0,2} + a^4_{0,-2}$	−0.0982	0.0043	−0.0765	0.0049
$a^4_{0,4} + a^4_{0,-4}$	−0.1247	0.0472	−0.2826	0.0361
$a^6_{0,0}$	−0.0253	0.0017	−0.0293	0.0035
$a^6_{0,2} + a^6_{0,-2}$	0.1997	0.0127	0.2266	0.0143
$a^8_{0,0}$	0.0172	0.0020	0.0192	0.0040
$a^8_{0,2} + a^8_{0,-2}$	0.1103	0.0089	0.1286	0.0035
$a^{10}_{0,0}$	−0.0122	0.0011	−0.0188	0.0019
$a^{10}_{0,2} + a^{10}_{0,-2}$	0.1751	0.0067	0.1839	0.0121
$a^{12}_{0,0}$	−0.0069	0.0014	−0.0075	0.0014
$a^{12}_{0,2} + a^{12}_{0,-2}$	0.0197	0.0024	0.0108	0.0013
$a^{14}_{0,0}$	0.0040	0.0023	0.0014	0.0008
$a^{14}_{0,2} + a^{14}_{0,-2}$	0.0425	0.0041	0.0443	0.0048
$a^{16}_{0,0}$	−0.0026	0.0008	−0.0007	0.0013

Order parameters deduced from tilt series ESR spectra from muscle fibers decorated with spin labeled S1 in rigor and in the presence of MgADP. The standard error in the mean (SEM) is calculated from five independent S1 and fiber preparations. Listed are the order parameters that are significant, as judged by their weighting factor (see Eq. 28, Burghardt and French, 1989), that appear in the calculation of the probe angular distributions in Fig. 2. The asterisk indicates order parameters that differ ≥ one SEM from rigor to MgADP.

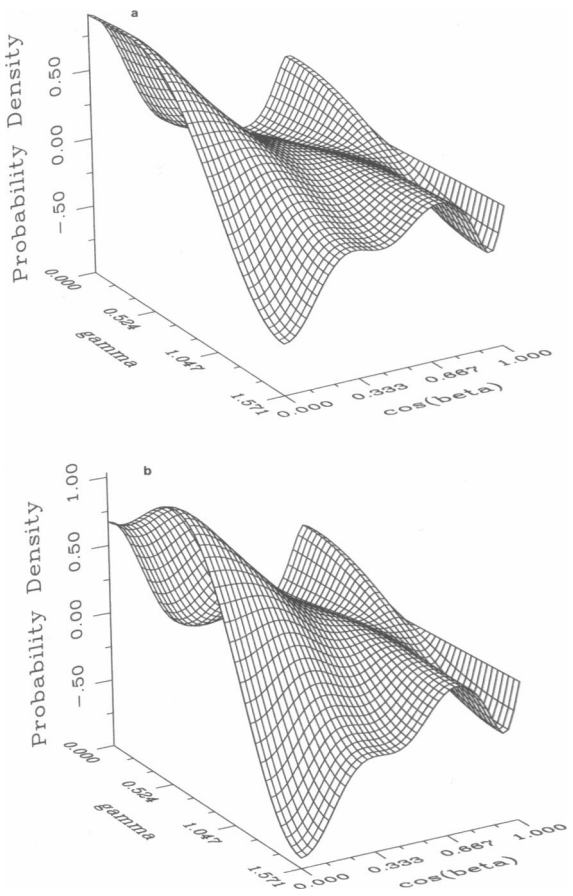

FIGURE 2 Spin probe angular distributions, N, computed from the average order parameters in Table 1 from muscle fibers decorated with spin labeled S1 for fibers in rigor (a) and in the presence of MgADP (b). N is computed from Eq. 1 in the text.

spectrum since the ESR signal is insensitive to probe distributions in α (Burghardt and Thompson, 1985). Thus, if Eq. 3 is valid, there is no loss of information from the insensitivity of the ESR signal to α.

The values of the averaged order parameters from fibers in rigor and in the presence of MgADP, shown in Table 1, indicate that there are significant differences in the probe angular distribution between the two states. The three dimensional representation of the probe angular distribution, N, of Eq. 1, is shown in Fig. 2. The Euler angles β and γ, where β corresponds to the polar angle measured relative to the fiber axis and γ to the torsional angle, are plotted on the x and y axes while the height of the distribution is plotted on the z axis. Angles β and γ define the orientation of the principal magnetic frame of the spin label where the g and T-tensors are diagonal. These plots show the rigor distribution to be broader and shifted relative to the probe distribution in the presence of MgADP. Fig. 3 shows Fig. 2 from another perspective and as constant N contours to illustrate the narrowing effect of nucleotide binding on the probe distribution.

Shown in Fig. 4 are the polar angular distributions derived by averaging the angular distributions in Fig. 2 over γ (see Eq. 2). Both distributions have a peak at β = 90° and a width of ~15°. This representation again shows, however, that the rigor distribution is broader than the distribution in the presence of MgADP although in this view, in contrast to the plots of Fig. 2, the difference is more subtle. We show this view to indicate the importance of the γ degree of rotational freedom that can be investigated with nitroxide ESR. With only the plots of Fig. 4 the narrowing effect of the addition of MgADP might be overlooked or considered insignificant.

Thomas and Cooke (1980) used a Gaussian distribution in β to model MSL labeled myosin cross-bridges in muscle fiber in rigor. There, the γ degree of freedom was ignored and they found the MSL to have an average β of ~82° and a distribution width of ~15°.

The polar plot of Fig. 4 is comparable with this previous work on the rigor cross-bridges with some stipulations. Two, effects, neglected in the previous work (Thomas and Cooke, 1980), must be considered in order to quantitatively relate the previous findings with the plot in Fig. 4. First, the effect of fiber symmetry, where opposite half sarcomeres may be related by an inversion of coordinates, would tend to broaden the polar distribution for probes near β = 90°. This effect can be understood by considering the following. If one half-sarcomere probe angular distribution is given by $n(\beta)$ then the opposite half-sarcomere polar angular distribution would be $n(\pi - \beta)$.

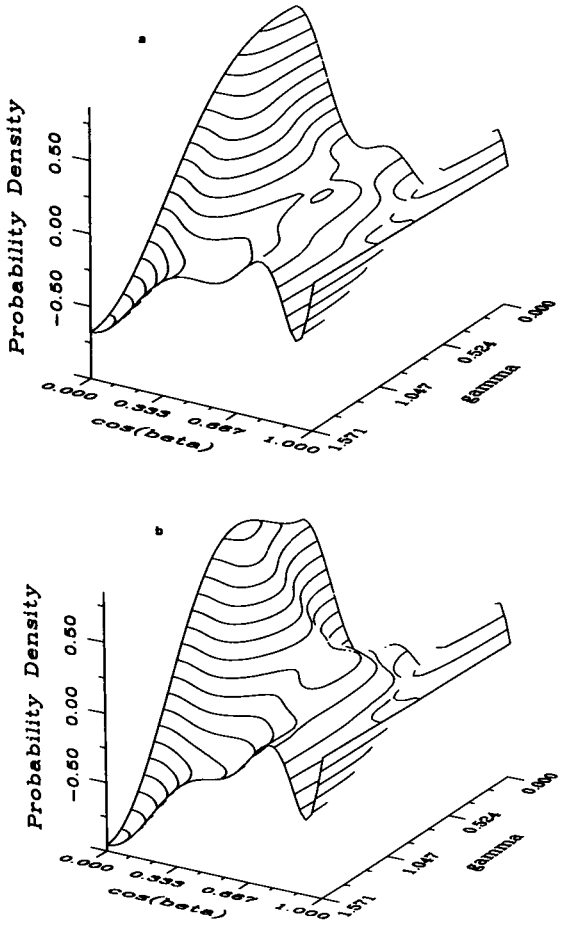

FIGURE 3 Same as in Fig. 2 with rigor (a) and MgADP (b), except that the observation point is rotated counterclockwise 60° and the data are represented by contour lines of constant N.

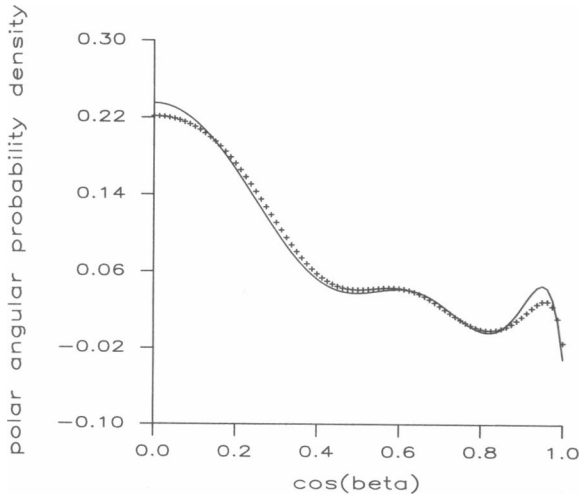

FIGURE 4 Polar probe angular distribution, $n(\beta)$, computed from order parameters in Table 1 from fibers decorated with spin labeled S1 in rigor (+++) and in the presence of MgADP (—). n is computed from Eq. 2 in the text.

The sum of these distributions is what would be observed from a labeled fiber. If n (β) peaks near enough to $\beta = 90°$, the peak of the sum $n(\beta) + n(\pi - \beta)$ is at $\beta = 90°$ due to the overlapping probe density at $\beta = 90°$. Second, even without this symmetry, an ESR spectrum is incapable of distinguishing polar angular distributions related by the transformation $\beta \rightarrow \pi - \beta$. This is equivalent to saying the ESR detects only the symmetrical part of the distribution on the interval $0 \leq \beta \leq \pi$ such that if the spin probe distribution is actually $n(\beta)$ then what is detected is $n(\beta) + n(\pi - \beta)$. Thus, any system with a spin distribution that peaks near enough to $\beta = 90°$, may be detected as a distribution with a single peak at $\beta = 90°$. When we computed the symmetrical part of the Gaussian model of Thomas and Cooke (1980), we found that this distribution peaks at $\beta = 90°$. We conclude then that our findings concerning the polar angular distribution of spin probes from rigor cross-bridges are in agreement with the earlier findings.

The spectra in Fig. 1 were measured with an amplitude modulation of the Zeeman field, the modulation amplitude (MA), of 0.5 G peak-to-peak, i.e., a value well below the linewidth commonly assumed for nitroxide spin labels of ~2.5 G. It is a well known phenomena that a larger MA increases the distortion of the shape of the ESR spectrum (Jost and Griffith, 1976). We investigated this effect by measuring, in addition to the data summarized above, tilt series spectra from decorated fibers in rigor and in the presence of MgADP using a MA of 2.2 G peak-to-peak. We found that the larger MA distorted the lineshape enough to suppress the subtle differences between rigor and MgADP spectra evident in the spectra shown in Fig. 1 (data not shown). Numerical analysis of the data confirmed this observation. The order parameters for decorated fibers in rigor and in the presence of MgADP, derived from the high MA spectra, were not as distinctive as judged by their standard deviation although the plots of the probe angular distribution showed the same tendency as observed for the low MA spectra, i.e., that the rigor distribution was shifted and broader than the distribution in the presence of MgADP.

DISCUSSION

Probe studies of the orientation of the myosin cross-bridge in muscle fibers produced a variety of interpretations of the observations. For a time there were contradictions in the findings based on studies using fluorescent probes covalently linked to myosin SH1. In this case there was disagreement between findings using the probes 1,5-IAEDANS and IATR such that the former probe did not, while the latter probe did, sense a cross-bridge orientation change upon the binding of MgADP. It was suggested (Burghardt and Ajtai, 1985) and later confirmed experimentally (Ajtai and Burghardt, 1987) that this conflict was due to the differing orientations of the probes on the cross-bridge such that the transition dipole of IATR was in a favorable, while the dipole of 1,5-IAEDANS was in an unfavorable, orientation for detecting cross-bridge rotation. This explanation was confirmed using the technique of wavelength dependent fluorescence polarization where the transition dipole orientation of the 1,5-IAEDANS was changed, by varying the wavelength of the excitation light, and the cross-bridge rotation caused by the binding of MgADP was detected.

Recently, only the contradiction between the spin probes and the fluorescence probes remained. The spin probes were reported to detect only one cross-bridge orientation regardless of the physiological state of the muscle fiber. Here again probes connected to the SH1 thiol on the cross-bridge were reported to give conflicting results. We investigated this conflict by developing specialized analytical techniques for deducing the probe angular distributions from the ESR spectrum. We simplified the problem by considering the effect of the binding of MgADP on the orientation of S1 decorating muscle fibers. This is a simpler problem since probe specificity is considerably higher when labeling S1 as opposed to labeling the cross-bridges in fibers.

We found that with the analytical tools of the model-independent treatment of the tilt series ESR spectra we could discern a significant difference between the probe orientation in rigor and in the presence of MgADP. The rigor cross-bridges produced a probe angular distribution shifted and broadened, compared with the distribution in the presence of MgADP, suggesting the presence of more than one cross-bridge orientation in rigor (see Fig. 2). The broad probe angular distribution of the rigor cross-bridges now revealed by ESR confirms some other observations of ours using fluorescence probes. In our work with fluorescence probes we show that the rigor cross-bridge could bind to the actin filament at two different orientations, depending on the temperature under which the attachment occurred (Ajtai and Burghardt, 1986). We proposed there that the rigor cross-bridge binds to actin with at least two orientations that are in a temperature dependent equilibrium. It is reasonable to expect from this model that, with sufficient resolution, the rigor cross-bridge angular distribution would contain more than one predominant angle. Judging from Fig. 2 we are close to achieving this resolution.

The probe angular distribution in the presence of MgADP suggests that the binding of the nucleotide shifts the spin population into a more homogeneous one by causing a cross-bridge rotation. This observation is vital to the verification of the rotating cross-bridge model and is in agreement with our work with fluorescence probes. It

now appears that the probe studies of myosin SH1 orientation are in agreement.

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