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# Orientation and rotational mobility of spin-labelled myosin heads in insect flight muscle in rigor

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## Summary

We have performed electron paramagnetic resonance (e.p.r.) experiments on spin-labelled myosin heads in glycerinated insect flight muscle fibres and myofibrils in rigor. Conventional e.p.r. was used to determine the orientation distribution of spin labels relative to the fibre axis, and saturation transfer e.p.r. was used to determine the submillisecond rotational mobility. An iodoacetamide spin label has previously been shown to react selectively with a reactive SH group on the myosin heads of rabbit skeletal fibres, and this label appeared to react with a similar group in insect fibres. Although selective labelling of this group was achieved in insect fibres and myofibrils, the reaction proceeded more slowly than in skeletal muscle, making it more difficult to label myosin heads selectively in insect muscle. The fraction of spin labels bound to myosin was $0.88 \pm 0.07$ in insect fibres and $0.97 \pm 0.05$ in rabbit. The fraction of myosin heads labelled was $0.65 \pm 0.15$ for insect and $0.81 \pm 0.10$ for rabbit. Both conventional and saturation transfer e.p.r. spectra of insect myosin, myofibrils and fibres were very similar to those of rabbit. The orientation distribution of spin labels relative to the fibre axis in rigor was narrow (16° for rabbit, 22° for insect), and the centre of the angular distribution was essentially the same for insect as for rabbit (68°–69°). This high degree of orientation was accompanied by strong immobilization of the probe on the microsecond time scale. The same immobilization was observed for rigor myofibrils as for purified myosin in the presence of excess actin, but considerable microsecond rotational motion was observed in myosin filaments free of actin. Thus, in insect as well as rabbit, assuming that the labelled heads are representative of all heads, more than 80% of the myosin heads appear to bind to actin in rigor, and the actomyosin bonds are rotationally rigid and oriented within a narrow angular range with respect to the fibre axis.

## Introduction

An understanding of the molecular mechanism of force generation in muscle requires detailed information about the structure of the protein filament lattice in the muscle

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fibre. Attention has been focused on the myosin heads, which form interfilament crossbridges by binding to actin. It is thought that these crossbridges generate force in a cyclic interaction which involves their rotation while attached to actin (Huxley & Simmons, 1971). Thus studies of the flexibility, orientation and motion of the myosin heads are crucial to an understanding of this hypothesis (Tregear & Marston, 1979). We have recently shown that spin labels can provide considerable information on the orientation and motion of myosin heads in rabbit skeletal muscle (Thomas & Cooke, 1980; Thomas *et al.*, 1980). The probes can be selectively and rigidly attached to a single reactive SH group on the myosin head. The conventional electron paramagnetic resonance (e.p.r.) spectra of the probes reveal their angular distribution relative to the axis of the fibre (Thomas & Cooke, 1980), and saturation transfer e.p.r. spectra are used to measure the motions of the probes over a wide range of correlation times, $10^{-8}$–$10^{-3}$ s. These data have provided considerable information on the orientation and dynamics of myosin in heads in vertebrate skeletal muscle.

Insect flight muscle is highly specialized for producing high-frequency oscillations of small amplitude. Its filament array has a higher degree of order than that of other muscles, and thus it has been the object of numerous structural studies (Reedy *et al.*, 1965; Reedy, 1967, 1968; Miller & Tregear, 1972). In particular, these studies have concentrated on the arrangement of the myosin crossbridges in rigor muscle, i.e. in the absence of ATP, a condition in which myosin heads form strong bonds with actin (Holmes *et al.*, 1980). Data obtained using electron microscopy and X-ray diffraction on insect flight muscle in rigor have led some investigators to conclude that a large fraction of the myosin heads would be unable to bind to actin for steric reasons (Haselgrove & Reedy, 1978; Offer & Elliot, 1978; Offer *et al.*, 1981). However, in previous work on spin-labelled rabbit skeletal muscle, it was found that virtually all (>90%) of the myosin heads are attached to actin, immobilized, and uniformly oriented in rigor (Thomas *et al.*, 1980; Thomas & Cooke, 1980). These results were in agreement with less direct estimates obtained from enzymatic activity (Cooke & Franks, 1980) and from tryptic digestion patterns (Lovell & Harrington, 1981). Due to the detailed information that has been obtained on the filament array of insect flight muscle, measurements on crossbridge configurations and motions should be more clearly interpretable in insect than in vertebrate muscle. For this reason, in the present study we have extended our previous studies of vertebrate muscle to insect flight muscle. We report here parallel studies of the two muscle types, probing both the orientation and mobility in rigor of spin labels attached selectively to myosin heads.

## Methods

Glycerinated rabbit psoas muscle fibres (Cooke & Bialek, 1979) and rabbit myofibrils (Thomas *et al.*, 1980) were prepared as described previously. Insect flight muscles (both fibres and myofibrils) from dung beetles (*Heliocopris japetus*) were kindly provided by Dr Richard Tregear, glycerinated in 75% glycerol, 100 mM KCl, 20 mM $KP_i$ (pH 7.0). Myofibrils were prepared by briefly homogenizing fibres, washing by centrifugation in 100 mM KCl, 10 mM $KP_i$ (pH 7.0), 1 mM $MgCl_2$, 1 mM EGTA,

1 mM $NaN_3$, 0.5% Triton X-100, then washing clear of Triton in three further spins, and finally adding 75% glycerol as above and held at 0° C for 10 days. The fibres and myofibrils were stored in 75% glycerol at −80° C for up to two years without significant changes in functional or spectroscopic properties (Clarke & Tregear, 1980). Fibre bundles (0.5 mm in diameter) of myofibrils were labelled with an iodoacetamide spin label (IASL): *N*-(1-oxyl-2,2,6,6-tetramethyl-4 piperidinyl)-iodoacetamide, purchased from SYVA Co., Palo Alto, California, U.S.A. The labelling was carried out at 0° C in rigor buffer (0.12 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM $NaN_3$, 25 mM MOPS, pH 7.0) plus 2 mM pyrophosphate ($PP_i$). Rabbit fibres were labelled for one hour at an IASL concentration of 0.5 mM and insect fibres were labelled for 2 h at an IASL concentration of 1 mM. Unreacted label was removed by washing first with rigor buffer plus 2 mM $PP_i$ followed by rigor buffer. The labelled fibres were then treated with 25 mM $K_3Fe(CN)_6$, in the presence of 5 mM $MgCl_2$, 1 mM EGTA, 25 mM MOPS (pH 7.0). Rabbit fibres were treated for 2 h, insect fibres for 4 h. The fibres were washed with rigor buffer and stored in rigor buffer plus 50% glycerol at 20° C until needed (less than one month).

Myosin was extracted from labelled rabbit myofibrils as described previously (Thomas *et al.*, 1980). The extraction of myosin from labelled insect muscle was carried out essentially as described by Bullard & Reedy (1973). Insect myofibrils or fibres were soaked for 10 min in a solution containing 1 M KCl, 10 mM sodium pyrophosphate, 1 mM $MgCl_2$ and 20 mM potassium phosphate (pH 6.5) at 0° C. Following centrifugation at 150 000 ***g*** for 2 h the supernatant was dialysed against the desired solution.

Actomyosin was prepared by mixing myosin and F-actin such that the ratio of actin monomers to myosin was 5:1. Concentrated KCl was then added to raise the KCl concentration to 0.6 M. The resulting solution was mixed thoroughly, then dialysed exhaustively against rigor buffer at 0° C.

The degree and specificity of spin labelling were determined as follows. Either myosin or myofibrils were dissolved in 4 M urea, 2% SDS, 20 mM sodium phosphate (pH 7.0) at 20° C. These solutions were used for measurements of protein and spin-label concentrations and for electrophoretic analysis.

Protein concentrations (w/v) were determined by a Biuret assay, using bovine serum albumin as a standard. Spin-label concentrations were determined by double integration of conventional e.p.r. spectra, using free spin label as a standard. The molar myosin concentrations were determined by assuming a molecular weight of 480 000; the fraction of myofibrillar protein corresponding to myosin was 54% for rabbit and 57% for dung beetle, as determined from gel scans.

E.p.r. spectra were obtained using Varian E-3 and E-109 spectrometers equipped with temperature controllers (Varian Associates, Palo Alto, California, U.S.A.). The E-109 spectrometer was interfaced to a Northstar computer for digital analysis of data. Fibres were contained in a specially designed flat cell made with Kel-F, in which the fibre bundles were aligned either parallel or perpendicular to the magnetic field (Thomas & Cooke, 1980). Protein solutions and myofibril suspensions were contained in a standard variable temperature aqueous cell inside the temperature control dewar (Varian Associates). Saturation transfer e.p.r. spectra were obtained as described previously (Thomas *et al.*, 1980).

Analysis of conventional e.p.r. spectra on oriented muscle fibres was carried out by theoretical simulation, as described previously (Thomas & Cooke, 1980). These spectra are determined almost completely by the orientation distribution $P(\theta)$, where $\theta$ is the angle between the magnetic field (usually the fibre axis) and the principal axis of the probe. Conversely, each spectrum corresponds to a virtually unique orientation distribution $P(\theta)$. Since the probe axis corresponds roughly to the long axis of the myosin head in rabbit (Thomas *et al.*, 1975), the spectra yield the orientation distribution of the myosin heads relative to the fibre axis. Previous theoretical analysis of oriented rabbit fibres, labelled with IASL, showed that the orientation distribution in rigor corresponds closely to a spherically weighted Gaussian distribution:

$$P(\theta) = \sin\theta \exp\left[-4 \ln 2\left(\frac{\theta - \theta_0}{\Delta\theta}\right)^2\right] \quad (1)$$

where $\theta_0$ is the centre and $\Delta\theta$ is the full width at half maximum of the Gaussian factor. When a good fit to the experimental data is obtained, $\theta_0$ and $\Delta\theta$ can be determined independently, with an uncertainty of the order of 1° (Thomas *et al.*, 1982).

Analysis of saturation transfer e.p.r. (ST-e.p.r.) spectra was carried out by comparison with reference spectra obtained from theoretical simulations and from experimental model systems (Thomas *et al.*, 1976, 1980).

## Results

*Characterization of spin-labelled myofibrils and fibres*

Myofibrils were prepared and spin-labelled as described in the Methods. Polyacrylamide gel electrophoresis showed that the extracted myosin was at least 90% pure. E.p.r. analysis of the myofibrils and the extracted myosin showed that most of the spin labels were bound to myosin for both insect (88 ± 7%) and rabbit (97 ± 5%). This confirms the previous results for rabbit (Thomas *et al.*, 1980), in which it was further shown that myosin is labelled exclusively in the head region (S-1). However, significant differences in the labelling results were observed for the two muscle types. In addition to the slightly lower specificity of labelling in insect, the degree of labelling was less in insect, despite the use of a higher spin-label concentration. 81 ± 10% of the rabbit myosin heads and 65 ± 15% of the insect myosin heads were labelled. Under identical labelling conditions, the rate constant for labelling was about four times less in insect than in rabbit. Treatment with 1 mM DTT before labelling had no effect on either the rate or extent of reaction. The mechanical properties of labelled fibres under isometric conditions appeared to be normal. In particular, isometric tension was greatly reduced by removal of $Ca^{2+}$.

*Conventional e.p.r.: orientation in rigor*

Fig. 1a shows conventional ($V_1$) e.p.r. spectra of spin-labelled glycerinated muscle fibres. For both rabbit and insect, the spectra obtained with the fibres parallel to the field (Fig. 1a) are very different from those oriented randomly with respect to the field, implying a high degree of orientational order of the probes relative to the muscle fibre axis. The spectra obtained with the fibres parallel to the field can be analysed to determine the orientation distribution of probes relative to the fibre axis. As shown previously, spectra like those in Fig. 1a correspond closely to a narrow Gaussian distribution about a single angle $\theta_0$, with a narrow distribution width of $\Delta\theta$. Over a wide range of $\Delta\theta$ values, $\theta_0$ can be determined independently of $\Delta\theta$ (Thomas *et al.*, 1982). Thus relatively unambiguous estimates of $\theta_0$ and $\Delta\theta$ can be made. The dashed curves in Fig. 1a are the best fits to the data: $\theta_0 = 68.2 \pm 1°$ and $\Delta\theta = 16 \pm 2°$ for rabbit (Fig. 1a, left); $\theta_0 = 68.6 \pm 1°$ and $\Delta\theta = 22 \pm 2°$ for insect. From five preparations, the mean ± S.E. for $\theta_0$ are 68.1 ± 0.3° for rabbit and 68.6 ± 0.4° for insect. Thus, although it is possible that rabbit and insect have

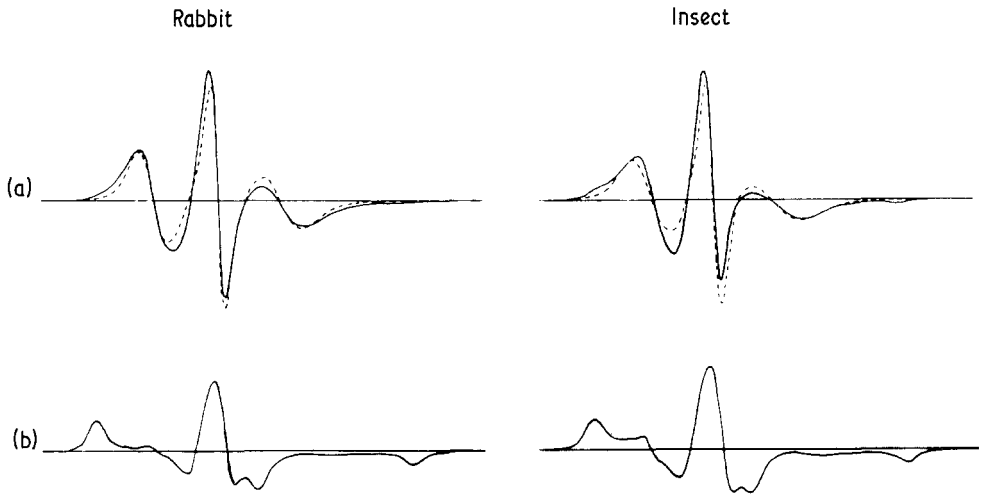


**Fig. 1.** Conventional e.p.r. spectra of spin-labelled muscle fibres in rigor solution at 20° C. (a) Fibres oriented parallel to the DC magnetic field. The solid curves show experimental spectra. The dashed curves show the best theoretical fits. (b) Randomly oriented fibres. The baselines in these and following spectra are 100 G ($10^{-2}$T) wide.

different $\theta_0$ values, this difference is no more than 1°. The only significant difference is in $\Delta\theta$: $\Delta\theta$ is greater for insect, implying more disorder.

Each of the spectra of disoriented fibres in Fig. 1b is virtually identical to that predicted for a random orientation distribution (Thomas & Cooke, 1980), as observed for rabbit myosin in solution (Thomas *et al.*, 1980) or for fibres that have been relaxed or stretched to zero overlap. This disoriented spectral component, which corresponds in rabbit to free myosin heads (not attached to actin), is virtually absent from the spectra of oriented fibres (Fig. 2a) for both rabbit and insect. Digital subtraction of the bottom spectra from those at the top shows that this disordered component corresponds to no more than 5% of the probes for rabbit in rigor and no more than 20% of the probes for insect in rigor.

*Saturation transfer e.p.r. spectra: rotational motion in rigor*

For both rabbit and insect we have recorded conventional and saturation transfer e.p.r. spectra of IASL attached to myosin heads in myofibrils, purified myosin and actomyosin. Since the proteins in these suspensions and solutions have a random orientation distribution relative to the externally applied magnetic field, no information can be obtained about the orientation of spin labels. However, even in the absence of orientation, e.p.r. spectra are sensitive to rotational motion over a wide range of correlation times, from $10^{-10}$ to $10^{-3}$ s (Thomas, 1978). Thus the objective of these experiments is to compare the mobility of heads under various conditions, complementing the information on orientation that was obtained from conventional e.p.r.

For both rabbit and insect, the conventional e.p.r. spectra (not shown) of IASL bound to monomeric myosin, myosin filaments, actomyosin and myofibrils are all virtually

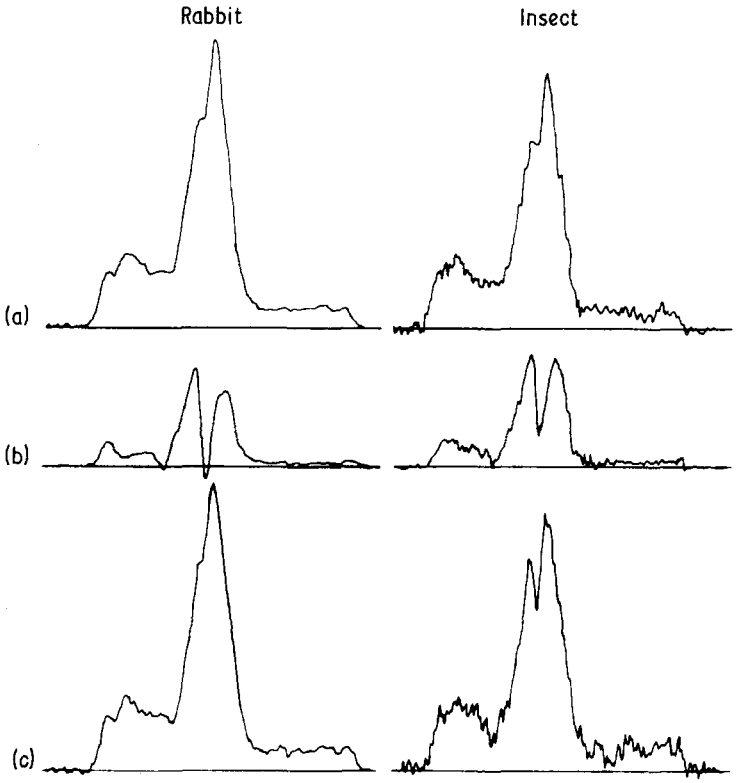


**Fig. 2.** Saturation transfer e.p.r. spectra of spin-labelled myofibrils and myosin purified from spin-labelled myofibrils, at 20° C. (a) Myofibrils in rigor solution. (b) Myosin filaments in rigor solution. (c) Myosin plus 5 mol rabbit actin per mol myosin, in rigor solution.

identical to the spectra in Fig. 1b, showing little or no evidence of orientation or rotational motion. Since conventional e.p.r. spectra are sensitive only to correlation times $\tau_2 \leq 10^{-7}$ s, these data only indicate that the probes, and hence the heads, are essentially immobile on the ns time scale. These results confirm previous results for rabbit (Thomas *et al.*, 1975, 1980). To study the motion in the $\mu$s range, it is necessary to perform saturation transfer experiments. In contrast to conventional e.p.r., the saturation transfer e.p.r. spectra of myofibrils, extracted myosin and actomyosin show large differences, implying large differences in rotational motion in the $\mu$s range (Fig. 2). The sensitivity of the spectra to the sample composition is similar for rabbit and insect.

In intact myofibrils (Fig. 2a), the spectra have the shape and intensity characteristic of immobility on the ST-e.p.r. time scale, i.e. there is no submillisecond rotational mobility (Thomas *et al.*, 1976, 1980). The spectrum of insect myofibrils (Fig. 2a, right) is about 10% less intense than that of rabbit (Fig. 2a, left) indicating slightly more mobility in insect. When myosin is extracted, purified and made to form filaments, the spectral intensity decreases greatly and the shape changes (Fig. 2b) implying substantial $\mu$s mobility. This mobility is slightly less for insect than for rabbit. Finally, when excess actin is added to

this extracted myosin, the $\mu$s mobility is abolished, producing spectra like those of the original myofibrils (Fig. 2c).

Let us assume that each myofibril spectrum (Fig. 2a) is a linear combination of two components: (1) the spectrum of free myosin filaments (Fig. 2b) corresponding to heads not attached to actin, and (2) the spectrum of actomyosin (Fig. 2c) corresponding to attached heads. Based on this assumption, computer addition of spectra indicates that the contribution of the free myosin spectrum to the myofibril spectrum is less than 5% for rabbit and less than 10% for insect, on a molar basis. That is, more than 90% of the probes are as immobilized in myofibrils as they are in actomyosin, for both muscle types. An alternative, but less direct, method of analysis is to use the myosin (mobile) and actomyosin (immobile) spectra from *rabbit* to estimate the fraction of immobile probes in *insect* myofibrils. This approach indicates that at least 80% of the probes are immobile (on the $\mu$s scale) in insect myofibrils.

## Discussion

Although insect flight muscle has proved extremely important in the development of structural models of muscle function in general, there are significant functional and structural differences between vertebrate skeletal muscle and insect flight muscle. While the vertebrate muscle contracts smoothly over a wide range of lengths, the insect muscle performs high-frequency oscillations with an amplitude of about 10 nm per half sarcomere, approximately the distance of one proposed crossbridge 'powerstroke' (Pringle, 1967). Both the arrangement of the filaments in the superlattice of the myofibril and the arrangement of the subunits in the filaments are different in the two classes of muscle (Squire, 1971). Of particular interest is the fact that in vertebrate muscle there is complete mismatch between the 5.5 nm repeat along the helix axis of the actin filament and the 14.3 nm repeat of the myosin filament, while in the insect muscle they are in register every 115 nm. The arrangement of the filaments and their subunits has led to the speculation that the filament structure of insect flight muscle influences the actin–myosin interaction in such a way as to produce the observed oscillatory contraction (Wray, 1979). Thus the orientation of the myosin heads (crossbridges) in the insect muscle could differ from that found in vertebrate muscle. However, the present results show that there is great homology between the crossbridge configurations of insect and vertebrate muscles in rigor. This homology includes the reactivity of the spin labels for groups on the myosin heads, the orientation and mobility of the labels on the heads, the ability of myosin heads to interact with actin, and the effects of actin on the mobility of the probes.

A number of studies of myosin from vertebrate muscle have shown that one SH group, termed $SH_1$, is far more reactive than other SH groups (Seidel *et al.*, 1970). This SH group has provided a convenient location for the placement of probes on the myosin molecule and it has been exploited for the study of fluorescence polarization (Borejdo & Putnam, 1977) and in previous e.p.r. investigations (Thomas *et al.*, 1975, 1980; Thomas & Cooke, 1980). However, in spite of the extensive studies on $SH_1$ in vertebrate mysoin,

no previous investigation has shown that a similar group exists in insect myosin. In the present study we used a spin label which reacts predominantly with SH groups and reacts very specifically with the $SH_1$ of rabbit myosin (Seidel *et al.*, 1970). When this probe was reacted with dung beetle myofibrils and fibres under conditions similar to those previously employed in rabbit, we obtained selective labelling of a group whose properties, i.e. orientation, rotational motion and accessibility to reagents, closely resemble those of the $SH_1$ of rabbit myosin. The reactivity of this insect SH group is about four times less than the corresponding one in rabbit muscle. In addition to the experiments reported here on dung beetle flight muscle, we have spin-labelled flight muscle fibres from three other insect species, *Lethocerus indicus*, *Lethocerus cordofanas* and *Bombus*. In these other species the reactivity of myosin to the spin label was found to be even less than in the dung beetle, making the selective labelling of myosin much more difficult, even when $K_3Fe(CN)_6$ was used.

Following the initial reaction of muscle fibres with spin labels, several classes of probe environments can be identified in the spectra, including probes with ns rotational mobility, probes with random orientations, and probes rigidly oriented at a specific angle. It has been shown that $K_3Fe(CN)_6$ can destroy some protein-bound spin labels but that labels bound to the $SH_1$ group of myosin are resistant to attack by this reagent (Graceffa & Seidel, 1980). Thus, in our previous studies on rabbit we used $K_3Fe(CN)_6$ to destroy those labels not specifically attached to $SH_1$. This procedure also worked in the insect flight muscle, showing that the labels on the reactive SH group are resistant to destruction by $K_3Fe(CN)_6$ in insect as well as in rabbit.

The most remarkable similarity between rabbit and insect muscles is that the mean orientations of the paramagnetic probes on the myosin heads are virtually identical. In both muscles the probes attached to the heads of rigor muscle have a distribution of angles that can be described by a Gaussian of narrow width. We find that the centres of the distributions for the two muscles are within 1° of each other and the width of the distribution is slightly greater in insect than in rabbit. We conclude that dung beetle flight muscle myosin contains a reactive SH group similar to the $SH_1$ group of rabbit muscle and that the protein structures in the vicinity of these two groups are strikingly similar.

Previous e.p.r. studies of rabbit muscle fibres and myofibrils came to the conclusion that in rigor all myosin heads were bound to actin (Thomas *et al.*, 1980; Thomas & Cooke, 1980), and the present data suggest a similar conclusion for insect muscle in rigor. In the rabbit muscle it was found that the probes were highly oriented only when myosin bound to actin, and that when this interaction was eliminated by stretching, or greatly weakened by relaxing the muscle, the probes assumed a random distribution (Thomas & Cooke, 1980). This data on orientation correlated very well with measurements of motion using saturation transfer e.p.r. (Thomas *et al.*, 1980), which showed that in rigor myofibrils the probes were highly immobile and in relaxed myofibrils the probes could rotate in the $\mu$s time range. It was also found that probes attached to the heads of myosin filaments in solution possessed the same ability to rotate as was

found in relaxed myofibrils and that this submillisecond rotational mobility was almost completely eliminated by addition of actin filaments. Together these data indicated that in rigor fibres the myosin heads were rigidly attached in a specific orientation to the actin filaments, and when this strong interaction was broken – by stretching, relaxation or extraction – the myosin heads could rotate through large angles in $\mu$s. Since virtually all probes were oriented in rigor fibres and virtually all heads were immobilized in rigor myofibrils, we concluded that all, i.e. >90%, of the heads were bound to actin in rabbit muscle in rigor. As shown in Figs. 1 and 2 the orientations and motions of probes attached to insect myosin, myofibrils and fibres are remarkably similar to those attached to their rabbit counterparts. In particular, more than 80% of the probes are oriented in the rigor muscle. In the absence of actin (Fig. 2b) considerable rotational freedom is observed, implying that the flexibility of insect myosin is similar to that of rabbit. This rotational freedom is greatly decreased by the addition of actin and is virtually eliminated in rigor myofibrils. Thus the data indicate that most (>80%) of the heads of rigor insect fibres have formed highly rigid bonds with the actin filaments. It should be noted that the presence of a small fraction of probes which are disordered or which show some mobility does not necessarily mean that there is a corresponding fraction of unattached myosin heads. The most likely explanation for this fraction of probes is that they are attached to sites other than $SH_1$; as described above, 10–20% of the probes are not on myosin and would be expected to display other orientations, or rotational mobility. Thus the fractions of disordered or mobile probes, 20% and 10% respectively, represent upper limits on the fraction of probes on unattached heads and there is good reason to believe that the actual fraction of unattached heads is much smaller than this.

The conclusion drawn above, that all myosin heads bind to actin in insect muscle in rigor, is in apparent conflict with the conclusions reached by some investigators from data obtained by X-ray diffraction and electron microscopy in which the fraction of attached heads was estimated to be 50% (Offer & Elliot, 1978; Offer *et al.*, 1981), and by tryptic digestion of myofibrils, in which the estimate was 70% (Lovell *et al.*, 1981). It is thus important to explore the possible experimental problems which could influence our conclusions, and in particular to ask whether unattached myosin heads could be present in the rigor fibre but be missed by our technique. Although in rabbit muscle a high degree of labelling can be achieved (with more than 90% of all heads reacted at their $SH_1$ groups) in insect muscle only about 65% of heads are labelled, leaving the possibility that unattached heads in rigor muscle have escaped detection because they are unlabelled. However, in partially stretched rabbit fibres the unattached heads and the attached heads are labelled with approximately equal intensity by the procedures employed in this study (Barnett & Thomas, 1983). In fact, studies in solution have shown that the reactivity of the vertebrate $SH_1$ is decreased, not increased, by addition of actin (Duke *et al.*, 1976). Because of the homology between the reactive groups of insect and vertebrate myosin, it thus appears unlikely that unattached myosin heads are not labelled.

A second hypothesis, consistent with a large fraction of unattached heads, is that the

probe orientation, and hence the e.p.r. spectrum, is the same for attached and unattached heads. This possibility was eliminated for vertebrate muscle because heads not overlapped by actin were disordered, but such an approach cannot be applied to insect fibres because the insect fibres resist any extensive stretching. The hypothesis would require that, in the filament array of the rigor fibre, unattached heads are immobilized, unlike the heads of isolated filaments or of relaxed muscle. However, it also requires that the heads be immobilized with exactly the same probe orientation as those heads attached to actin. In view of the flexible nature of the myosin molecules of myosin filaments in both insect and vertebrate muscles (Fig. 2b), a flexibility which is most probably required for their function, it appears unlikely that unattached heads of insect muscle would be held rigidly in the same orientation as when they are attached to actin, so the second hypothesis also appears improbable.

There are no other obvious artefacts that would lead to selective masking of the signal from heads that are unattached to actin; we thus conclude that the most reasonable interpretation of the spectra of rigor insect muscle is that all of the myosin heads are capable of forming bonds with actin. This implies a greater degree of myosin flexibility than has been commonly accepted. Our data indicate that the attached heads in insect have a wider range of orientations ($\Delta\theta = 22^\circ$) than in rabbit ($\Delta\theta = 16^\circ$). This is consistent with the proposal that myosin heads must be in a more strained configuration if they are all to be attached in insect (Offer *et al.*, 1981).

The conclusion that all myosin heads are attached to actin suggests a rather simple and straightforward picture of the structure of insect flight muscle in rigor. Electron micrographs of cross-sections of rigor insect muscle have shown that four crossbridges originate from the myosin filament at intervals of 14.5 nm, forming a structure resembling a 'flared X' (Reedy, 1968). Recent measurements of the mass of the myosin filament have concluded that there are also four myosin molecules for each 14.5 nm of filament (Reedy *et al.*, 1981). There is some uncertainty, however, over the number of myosin heads which comprise a crossbridge, and some investigators have concluded that one head forms one crossbridge. If this conclusion is correct, then half of the heads would not be attached to actin. Since the present results show that most of the heads are attached to actin, they suggest that one crossbridge, as visualized in the electron micrographs, represents the two heads of one myosin molecule. It thus appears that in insect muscle in rigor the myosin molecules are arranged in a left-handed helical array with four molecules every 14.5 nm, with the two heads of each myosin molecule bound to the same actin filament and appearing to form one crossbridge in electron micrographs.

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