Changes in the ATPase Activity of Insect Fibrillar Flight Muscle during Calcium and Strain Activation Probed by Phosphate-Water Oxygen Exchange*

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During ATP hydrolysis by Ca\(^{2+}\)-activated chemically skinned fibers from the flight muscle of the giant waterbug Lethocerus indicus, there is extensive phosphate-water oxygen exchange. For unstrained fibers the pattern of exchange shows that there is more than one pathway for hydrolysis, due to the ATPase activity of cross-bridges. Multiple pathways are an established property of both vertebrate actomyosin and fibers. The pattern of exchange can be fitted by two pathways: one with low exchange because the step(s) controlling P\(_i\) release are rapid, the other with high exchange and slow P, release. The high-exchange pathway is responsible for most of the increase in ATPase activity on Ca\(^{2+}\) activation. On strain activation, only the high-exchange pathway is present, accounting for all the ATPase increase and responsible for force generation. In fully activated fibers, the cross-bridges which hydrolyze ATP and generate force behave uniformly with respect to oxygen exchange. The exchange pattern shows that the rate of P, release changes dramatically over a very narrow strain increase. Step(s) controlling P, release are at least partially rate-limiting for the overall ATPase reaction. The results are discussed in relation to models for strain activation and the identity of force-generating states.

This paper describes the measurement of phosphate-water oxygen exchange to examine rate constants in the ATPase cycle of skinned fibers from insect fibrillar flight muscle at different degrees of activation. This approach gives insight into which steps in the ATPase mechanism are activated and how these steps are coupled to force generation. The timing of contraction in most skeletal muscles is controlled by the nerve input to that muscle, which triggers the release of calcium from the sarcoplasmic reticulum. The result is an increase in [Ca\(^{2+}\)] in the myofibrillar space acts as the initial switch for contraction. However, the flight muscles of many species of insect and the tymbal muscles of some species of cicada are different as the nerve input and the muscle contraction are asynchronous, although the presence of Ca\(^{2+}\) is still necessary. Such muscles are described as fibrillar or myogenic (Pringle, 1967, 1978). The dorsal longitudinal muscle of the giant waterbug Lethocerus is the most studied of this muscle type; in this paper we shall use the term flight muscle to mean fibrillar flight muscle from Lethocerus indicus.

Insect flight muscles have a special property called strain activation; the ATPase activity and the tension (contractile force) of the calcium-activated muscle are enhanced several-fold by a small strain (extension) of a few percent (Ruegg and Tregear, 1966). Thus calcium alone is not sufficient to fully activate insect flight muscle. The mechanism responsible for strain activation seems likely to be the insect flight muscle to oscillate and perform work, resulting in maintained wing movement and flight (Thorson and White, 1969, 1983). At present, no one model explains all the characteristics of strain activation in fibers. The models are of two extreme types. One invokes the recruitment of cross-bridges on strain activation; more myosin heads can interact with actin, increasing both the tension and ATPase rate (Thorson and White, 1969, 1983; Wray, 1979). The other model explains strain activation by changes in rate constants within the cross-bridge cycle (Thorson and White, 1969, 1983). Because the oxygen exchange technique can measure changes in rate constants in muscle fibers the approach has the potential to test to what extent such extreme models explain the muscle behavior.

Much of our information about the ATPase mechanism has been derived from solution studies on isolated actomyosin from rabbit skeletal muscle (Taylor, 1979; Eisenberg and Greene, 1980; Sleep and Hutton, 1980; Hibberd and Trentham, 1986). For the isolated proteins there is a close relationship between the rabbit skeletal mechanism and that for insect (White et al., 1986). Scheme 1 shows the main features of this mechanism that are currently thought to apply to insect flight muscle. A and M represent actin and myosin, respectively. The steps are numbered so that step i has forward and reverse rate constants \(k_+\) and \(k_-\), and an equilibrium constant \(k_+ / k_- = K_c\).

Phosphate-water oxygen exchange occurs during ATP hydrolysis under a wide variety of conditions, both for isolated actomyosin (reviewed by Webb and Trentham, 1983) and for skinned rabbit skeletal fibers (Hibberd et al., 1985b). Scheme 2 shows how the exchange arises; for simplicity, hydrolysis is shown only for the attached cross-bridge state. To measure this exchange, the solvent water is labeled with \(^{18}\)O, represented here by filled circles. When ATP is cleaved in \(^{18}\)O water, one water oxygen is incorporated into the product P, as in the top line of Scheme 2. Because the P, can rotate in the catalytic site and the cleavage step is reversible, oxygen exchange can occur between the P, and water, due to multiple reversals of the cleavage step, and more than one solvent oxygen is incorporated into the P, prior to its release. In this simple model for intermediate exchange the extent of ex-
The fibers were rinsed in two successive baths of 60 µl of relaxing solution in [3H]water to remove unlabeled water prior to the experimental incubations and then immersed in 30 µl of the relevant solution in a small temperature-controlled Perspex bath. A layer of dry silicone oil was used to prevent the entry of atmospheric unlabeled water during the course of all these intermediate exchange experiments. Procedures followed for these oxygen exchange experiments are given in detail in Hibberd et al. (1985b). The enrichment of the [3H]water of each solution was assayed immediately before the fibers were placed into that solution: 5 µl of each sample was reacted with dry PCln in a sealed vial, and the P1 product (as triethyl phosphate) was analyzed on a gas chromatograph-mass spectrometer (Hewlett-Packard 5995B). The concentration and enrichment of the product P1 can then be corrected for the lack of isotopic purity of the solvent, due to the presence of unlabeled water. Incubation times with fibers were between 15 and 40 min, and there was a minimal decrease in the enrichment of the [3H]water during these relatively short incubation periods.

After approximately 10% ATP hydrolysis for each sample solution the fibers were removed from the sample solution to stop further hydrolysis. The extent of hydrolysis was monitored by high performance liquid chromatography (Gilson model 302, 802, and HBM bolocrome system) of a 1-µl sample on a strong anion exchange column (Waters Associates, 10 × 0.8-cm diameter). The nucleotides were eluted with 0.5 M (NH4)2HPO4, at pH 4.0. The kapp value of the ATPase was calculated assuming 8 pmol of myosin heads/cm of fiber (Chaplain and Tregear, 1966). After the addition of 30 nmol of unlabeled carrier Pi, the solution was removed, made to 1 ml with unlabeled water (so that any subsequent breakdown of ATP will not form labeled Pi), and stored at −86°C. The proportion of P1 molecules containing 1, 2, 3, or 4 3H atoms was measured, corrected for lack of isotopic purity in the labeled water, and theoretical distributions were fitted to the experimental values as described by Hibberd et al. (1985b).

For each value of R, a theoretical distribution of exchanged oxygens is calculated using the following equations for the fractions (F1, F2) of P1 containing 1–4 solvent oxygens (Webb and Trentham, 1981).

\[ F_1 = \frac{R}{3/4 + R} \]
\[ F_2 = \frac{R}{3/4 + R} \times \frac{3/4}{1/2 + R} \]
\[ F_3 = \frac{R}{3/4 + R} \times \frac{3/4}{1/2 + R} \times \frac{3/4}{1/2 + R} \]
\[ F_4 = \frac{R}{3/4 + R} \times \frac{3/4}{1/2 + R} \times \frac{3/4}{1/2 + R} \times \frac{3/4}{1/2 + R} \]

The best fit was determined by minimizing the sum of the deviations of the theoretical distributions from the experimental distribution. This procedure, a range of theoretical distributions can be calculated for the percentage P, and values of R, which are within the standard errors of the mass spectral measurements (typically 1% or less of the total intensity). Under “Results,” the best fit theoretical distributions are given. The range of values is given for one result (see legend to Fig. 1) to provide an example of how sensitive the data are to errors. As discussed under “Results,” this sensitivity is a problem only at extreme values of R.

**Calcium-activation Experiments—**Two solutions were made up in H218O: (i) relaxing solution (pCa > 7) was 10 mM MgCl2, 5 mM EGTA, 15 mM KCl, 30 mM histidine, 10 mM Na2ATP, 1 mM NaN3, 10 mM sodium diiodoacetamide, 0.5 mM quercetin, and 1 µl ml−1 oligomycin, adjusted to pH 7.0 with KOH; and (ii) activating solution (pCa = 4.7) was the same but with 5 mM CaEGTA in place of 5 mM EGTA. The solutions were prepared by the method of Hibberd et al. (1985b) using [18O]water of 98% isotopic enrichment (Amersham International P.L.C., United Kingdom). The solutions were made up in unlabeled water, lyophilized, and then lyophilized twice following the addition of a small amount of [18O]water. [18O]Water was then added to give the stated solution.

Intermediate calcium concentrations were obtained by mixing the relaxing and activating solutions. A computer program, based on the algorithm of Perrin and Saye (1967), was used to estimate the

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**Experimental Procedures**

Fibers from the longitudinal indirect flight muscle of the giant waterbug, *Lethocerus indicus*, were chemically skinned with glycerol, following the procedure given by White (1983), stored at −2°C, and were used within 4 weeks of extraction. The mechanical apparatus is that described by White (1983). The fibers were mounted between two hooks. One hook was attached to an electromagnetic vibrator (Ling Dynamics, type 101), by means of which the length of the muscle was varied; the other hook was attached to a silicon strain gauge tension transducer (Akers, type 801).
concentration of free calcium using dissociation constants for the various complexes present given by White and Thorson (1972).

A bundle of 20 fibers was incubated at 21 °C in 30 μl of the relevant solution. The fibers were at zero tension (negative strain) throughout the experiment to prevent strain activation at higher levels of calcium. Control experiments showed that the extent of shortening under these conditions was small (~6% to -10% strain) even at the highest calcium ion concentrations, because at negative strain the thick filaments contact the Z line in insect flight muscle fibers, preventing further shortening. The first incubation was in a relaxing solution, and the fibers were transferred to baths at successively greater calcium concentrations. The values of R and the ATPase activities of these fiber bundles were in the range found with fewer fibers/bundle (5 or less) in the absence of mechanical activation (e.g. in the strain activation experiment given here), suggesting that diffusion control does not have a major influence on these results.

Strain-activation Experiments—Two to five fibers were incubated at 21 °C in 30-40 μl of activating solution and given successively increasing or decreasing strains. Maximum tensions generated by the fibers for the strain-activation experiments varied between 50 and 350 μN/fiber, depending on the preparation. This variation is not due to variable cross-sectional areas of the fibers, which are uniform, with a diameter of 70×10^-4 m (Jewell and Ruegg, 1966).

Zero percent strain is defined as the fiber length which just gives zero tension in relaxing solution. This length is a well defined property of insect flight muscle, due to its high relaxed stiffness (Machin and Frangie, 1980; White, 1983), a property not shown by vertebrate skeletal fibers. Stiffness is defined as the ratio of the tension change to the strain change. The high relaxed stiffness is thought to arise from material connecting the thick filaments to the Z line (Auber and Couteaux, 1963), rather than the presence of attached cross-bridges in the absence of calcium (White, 1983). Because "zero percent strain" is defined for the fiber when relaxed, the tension in activating solution at zero strain is well above zero.

Previous work on strain activation (Ruegg and Tregear, 1966; Ruegg and Stumpf, 1969; Pybus and Tregear, 1975; Loxdale and Tregear, 1985) shows graphs with the activation occurring at positive values of strain of a few percent. Except for the 1965 paper, these papers do not say how zero percent strain was defined, but 'Tregear informs us' that it was defined as the length at which the tension was zero in activating solution. Loxdale and Tregear (1985) defined zero percent strain as the length at which the tension was zero in rigor solution. These differences in definition account for the apparent discrepancy in our data with some previously published data on this point.

The ATPase activity of the strain-activated fibers here in the presence of 10 mM ATP is about 1.5 s^-1. Since the maximum ATPase activity reported with 5 mM ATP and with similar numbers of fibers in a bundle is also about 1.5 s^-1 (Loxdale and Tregear, 1985), it seems unlikely that the fibers are strongly diffusion controlled here. The mechanical performance showed no evidence of the "high tension" state (Jewell and Ruegg, 1965), characteristic of diffusion-limited fibers. Note that in the incubations the P_i concentration varies from zero up to about 1 mM, which may slightly modify the performance of the fibers (White and Thorson, 1972).

RESULTS

Calcium Activation—When ATP is hydrolyzed by untensioned skinned fibers (at negative strains) in the presence of calcium, there is extensive exchange (Fig. 1A), showing significant reversibility of the cleavage step. The fibers were held at negative strains to prevent strain activation, which occurs at around zero percent strain in our hands. The best distribution that can be calculated using the exchange mechanism in Scheme 2 and a single value of R = 0.10 fits the data poorly (Fig. 1B). R is the ratio of the rate constant for the breakdown of the ADP-P_i complex leading to P_i release to the rate constant for ATP formation from this complex. An almost exact fit can be obtained (Fig. 1C) by dividing the P_i into two populations and assuming that each arises with a different value of R, corresponding to the low-exchange (R = 1.2) and high-exchange pathways (R = 0.048). This implies

\[ R \text{, T. Tregear, personal communication.} \]

FIG. 1. Oxygen exchange distribution due to untensioned fibers in the presence of Ca^{2+}. A, analysis of the ^{18}O-content of P_i released from ATP by a bundle of fibers incubated in activating solution at zero tension. The four bars represent the percentages of P_i released with 1, 2, 3, and 4 solvent oxygens. B, best fit of the experimental data using the exchange mechanism of Scheme 2, with a single value of R (R = 0.10). C, best fit using two values of R (high-exchange pathway, R_h = 0.048, 71% of P_i; low-exchange pathway, R_l = 1.8, 29% of P_i). The contributions from the two pathways are indicated by the subdivision of the histograms, the heavily shaded upper parts being the contribution from the low-exchange pathway. The range of distributions that fit the data within a standard error of 1% of the total intensity is as follows. The percentage of the low-exchange pathway (R_l = 1.35-2.5) is 26-33%. The high-exchange pathway could have R_h = 0.040-0.054, 67-74% of P_i.

that there is more than one mechanism of ATP hydrolysis, differing in at least one rate constant affecting R. For simplicity, only two pathways for exchange are assumed, although the data here do not exclude the presence of more than two pathways.

In order to probe how these two pathways arise, the exchange during ATP hydrolysis was measured at different calcium concentrations between no activation and full activation. An important feature of these series of incubations, and those described below, is that each series was done with the same fiber bundle, incubated sequentially for each condition. This circumvents the problem of fiber-to-fiber differences present in experiments with rabbit fibers, which are much less robust and so unable to survive this protocol. At all levels of calcium, analysis of the distribution of solvent oxygen in the released P_i showed that two pathways are required; one pathway has extensive exchange, and the other has very little exchange. Fig. 2 shows how the ATPase activity for each pathway varies with calcium. The pathway with high exchange accounts for most of the overall calcium activation of the ATPase. Fig. 3 shows the correlation between the value of R and the ATPase activity for the two pathways. There is a high correlation for the high-exchange pathway, but no positive correlation for the low-exchange pathway. This may reflect the difficulty of assessing the magnitude of R accurately for this low-exchange pathway, since all values of R above ~1.5 give a reasonable fit. Thus, the high-exchange pathway, which predominates at high [Ca^{2+}] is calcium-activated, and this activation is reflected in both its ATPase activity and in rate constant(s) controlling R.

Strain Activation—In a series of incubations, strain was increased from low (negative) to high strain. The distribution of oxygen isotopes changed from that requiring two values of R (at low strains, Fig. 4, A and B) to that requiring just a single value of R for the strain-activated fibers (at higher strains, Fig. 4, C and D). This change in distribution was dramatic and was always obtained over a very narrow range of changes in strain (<0.5%, Figs. 4 and 5A). The change in distribution coincides with a sharp increase in both the ATP-
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**FIG. 2.** ATPase activity as a function of $[Ca^{2+}]$ measured in a bundle of fibers at zero tension. The oxygen-exchange distributions were analyzed as shown in Fig. 1 which is the data obtained at $pCa$ 4. $T$ (■) is the total ATPase activity, $H$ (△) the ATPase activity through the high-exchange pathway, and $L$ (□) the ATPase activity through the low-exchange pathway. The ATPase activity due to each pathway was found by multiplication of the percentage of $P$ for that pathway by the total ATPase activity. *Rel*, values measured in relaxing solution.

**FIG. 3.** Calcium activation of the ATPase activity and values of $R$ for the two pathways. A, ATPase activity via the high-exchange pathway as a function of the value of $R$ for that pathway. B, ATPase activity via the low-exchange pathway as a function of the value of $R$ for that pathway. Results from two separate experiments. The solid symbols are the experiment shown also in Fig. 2.

**FIG. 4.** Analysis of $^{18}O$-content of $P_i$ released from ATP during incubation of fibers in activating solution at various strains. The histograms show the experimental data and give the nmol $P_i$ produced min$^{-1}$ cm fiber$^{-1}$ with 1, 2, 3, or 4 solvent oxygens. These values were found by multiplication of the percentage $P_i$ for each labeled population by the total ATPase activity. The data of A (−2% strain) and B (−0.5% strain) require two pathways for a satisfactory fit; the contributions from the high- and low-exchange pathways are indicated by the light and heavy shadings on those histograms. The combined pathways fit the experimental data almost perfectly. For A, the values of $R$ are: $R_h = 0.033, 54\%$ of $P_i$; $R_l = 1.11, 46\%$ of $P_i$. For B, the values of $R$ are: $R_h = 0.024, 46\%$ of $P_i$; $R_l = 0.912, 54\%$ of $P_i$. The data of C (0% strain) and D (0.5% strain) are fitted by a single pathway (100% of $P_i$) with values of $R$ of 0.196 and 0.211; the triangles indicate the best theoretical fit to the data. Although the fit for D is not good, a single pathway fits better than two pathways with different values of $R$. The data are taken from the same experiment as those of Fig. 5.

**FIG. 5.** The variation in value of $R_h$ and the ATPase activity for the two pathways as a function of strain. A, value of $R$ for the high-exchange pathway as a function of strain. The corresponding values of $R$ for the low-exchange pathway are: "free" fibers, $R_l = 1.12, 53\%$ of $P_i$; −2% strain, $R_l = 1.11, 46\%$ of $P_i$; and −0.5% strain, $R_l = 0.91, 54\%$ of $P_i$. B, $T$ (●), total ATPase activity as a function of strain; $H$ (○), ATPase activity via the high-exchange pathway; $L$ (□), ATPase activity via the low-exchange pathway. *Free*, values measured with the fibers at zero tension and free to shorten.
Different preparations of fibers all gave a sharp change in distribution over a narrow strain change, but the single values of 0 found with strain-activated fibers ranged in magnitude from 0.11 to 0.21, corresponding to the high-exchange pathway. During increasing strain activation major changes occurred both in the value of R for the high-exchange pathway (Fig. 5A) and in the ATPase activity through each pathway (Fig. 5B). The low-exchange pathway ATPase activity disappears when the value of R (Fig. 5A) and the ATPase activity for the high-exchange pathway jump to the maximum value (Fig. 5B). This result with strain activation is unlike the smooth changes in the R values and their pathway ATPase activities seen during calcium activation. Thus the high-exchange pathway is responsible for all of the increase in the ATPase activity on strain activation. We have identified the sole pathway at high strain with the high-exchange pathway because this assumption produces a good correlation between the values of R and the change in ATPase activity for this pathway, with Ca2+ activation. Therefore, the increase in this value of R correlates with the ATPase enhancement seen when calcium-activated insect flight muscles are strained. From both calcium and strain activation it is clear that one hydrolysis pathway for the ATPase is responsible for most, if not all, of the overall ATPase activation observed.

Strain had no significant effect on either the ATPase activity or the distribution of solvent oxygens in R, during ATP hydrolysis by relaxed fibers (Fig. 6). The changes in oxygen exchange require both Ca2+ and strain; strain alone is not associated with measurable changes in R.

**DISCUSSION**

**Assignment of the Two Hydrolysis Pathways**—The calcium and strain activation experiments show that one ATP hydrolysis pathway is responsible for most if not all of the overall ATPase activation observed. This high-exchange pathway is the only one present in strain-activated fibers and, therefore, appears to be responsible for tension generation in active insect flight muscle. The cross-bridges with ATPase activity in mechanically activated muscle behave uniformly with respect to their ATP hydrolysis pathway, as assessed by oxygen exchange.

Previous results both with rabbit actomyosin (Shukla et al., 1983) and with skinned rabbit skeletal muscle fibers (Hibberd et al., 1985b) suggest that the low-exchange pathway is also associated with myosin. We added azide and oligomycin (Cross and Boyer, 1975) to our solutions as submitochondrial ATPase inhibitors and quercetin in order to inhibit any remaining Ca2+-pump ATPase activity (Shoshan and MacLennan, 1981). The sarcoplasmic reticulum in insect flight muscle is poorly developed (Smith, 1968), and a correspondingly low Ca2+-pump ATPase might be expected. Neither quercetin nor oligomycin was found to have any noticeable effect on the low- or high-exchange pathway, which is consistent with both pathways being due to the myosin ATPase and not a contaminant ATPase. Furthermore, the 0 present due to the low-exchange pathway in unstrained fibers is apparently not present in the strain-activated fibers. It is not possible to fit the low-exchange R into the P distribution from strained fibers, so that the remaining P has a distribution that can be fitted to a single hydrolysis pathway. This is illustrated in Fig. 4, where the P distributions are shown adjusted for the relative ATPase activity for each condition. These results suggest that the low-exchange pathway is not due to a contaminant ATPase or to damaged myosin. Protein damage may be a factor in the large amount of low-exchange P, in relaxed fibers, where the overall ATPase activity is low.

**Calcium Activation**—Most of the calcium activation of the ATPase activity in fibers at zero tension (negative strain) can be explained by an increase in ATPase activity via the high-exchange pathway, which correlates with the increase in the value of R for that pathway (Fig. 3A).

R was defined in the Introduction, in terms of Scheme 1, as $k_{cat} / (k_{-2A} + k_{-3D}/K_i)$. Based on our understanding of vertebrate skeletal actomyosin we can assume that $k_{-3A}$ is approximately equal to $k_{-3D}$ (Rosenfeld and Taylor, 1984). The rate constant for reformation of bound ATP from (AM[A,DP]P) is $k_{-3} = k_{-3D} = k_{-3A}$, and the rate constant for $k_{-3}$ release is $k_{+5} = k_0K_i/(1 + K_i)$. This gives $R = k_{-3}/k_{+5}$. Assuming that $k_{-3D}$ and $k_{-3A}$ also have similar and constant values in skinned insect fibers, the changes in R are due to the rate constant for steps controlling P, release, $k_{-5}$. For schemes more complex than Scheme 1, $k_{-5}$ could be controlled by any slow steps following ATP cleavage up to the P, release step. Our data then show that this process is sensitive to the level of Ca2+ for the pathway with high exchange. The data of White et al. (1986) suggest $k_{-5D} \approx 10^{-1} \text{s}^{-1}$ at 20 °C, which may well be similar to the 15 s-1 found for vertebrate subfragment 1 in solution (Webb and Trentham, 1981). With a value of R of 0.048 (Fig. 3A) for the high-exchange pathway, and 10 s-1 for $k_{-3}$, since $R = k_{-5D}/k_{-3}$, then $k_{-5D} = 0.5 \text{s}^{-1}$ for this pathway at full Ca2+ activation. If the $k_{cat}$ for this pathway is approximately the same as the overall pathway ($k_{cat} \approx 0.025 \text{s}^{-1}$ for R = 0.048), then the similarity of $k_{cat}$ to $k_{-5D}$ suggests that P, release steps may contribute to limitation. The good correlation between the increase in ATPase activity and the value of R supports this conclusion, although Ca2+ could affect more than one step on the ATPase pathway similarly, with these other step(s) controlling rate limitation. The changes in oxygen exchange for the high-exchange pathway at different levels of activation apparently correlate with the change in stiffness. The high-frequency stiffness increase found in insect flight muscle on both calcium activation and strain activation shows a linear relationship with the fiber tension (White et al., 1979) and is most simply interpreted in terms of the number of attached cross-bridges (Huxley and Simmons, 1971; Ford et al., 1985). Therefore, step 4 (cross-bridge attachment) could control both the stiffness and the high-exchange R value. However, we cannot tell from oxygen-exchange work alone if step 4 or step 5 of Scheme 1 is sensitive to the change in [Ca2+].
For the low-exchange pathway there is no positive correlation of the value of \( R \) with the ATPase activity. With vertebrate fibers also, the low-exchange value of \( R \) shows only a poor correlation with the ATPase activity on calcium activation (Hibberd et al., 1985b). Therefore, the ATPase activity due to the low-exchange pathway in fibers may not be controlled by the steps which limit P, release, but by recruitment, for example. If \( k'_{-\alpha} \) controls \( k_{-\alpha} \) for the low-exchange pathway, the low-exchange flux could be due to at most a few percent of the total cross-bridges which hydrolyze ATP. There is also no correlation of the value of \( R \) for the low-exchange pathway with the ATPase activity for vertebrate skeletal actomyosin or acto-heavy meromyosin at varying levels of actin. It has been proposed that myosin heads following the low-exchange pathway are highly associated with actin and so are insensitive to changes in the actin concentration (Meldford, 1981; Shukla et al., 1983).

Two Pathways of Hydrolysis at High Ca\(^2+\)—The habitual finding of multiple hydrolysis pathways with unstrained but fully Ca\(^2+\)-activated insect fibers (and rabbit skeletal fibers) suggests that the multiplicity of pathways is likely to be a genuine feature of the contractile mechanism. The two (or more) populations of myosin heads giving rise to the populations of P, must differ in their ATPase mechanism in at least one rate constant, presumably that controlling P, release, \( k'_{-\alpha} \). Any interconversion between pathways must be slow on the time scale of the exchange process, in order for the two populations to be discrete.

One explanation of two pathways relies on the presence of two heads on each myosin molecule (Shukla et al., 1983). However, current simulations of our results with this type of model have not proved successful when it is assumed that there is no inherent difference between myosin heads. On strain activation, the presence of a single exchange pathway requires either that both heads are identical in their exchange properties or else that one head has such a low ATPase activity that it is “silent.” A more likely way in which different heads could experience different values of \( k'_{-\alpha} \) is if this rate constant depended on the degrees of distortion of the individual attached cross-bridges. Distortion is defined here as the spatial removal of a cross-bridge from its position of minimum potential energy. The part of the cross-bridge cycle which mediates oxygen exchange could also be involved in force generation. Force-generating intermediates may have a free energy dependence on distortion (Huxley, 1957), in which case some rate constants must also have such a dependence (Hill, 1974). Cross-bridges with high distortion might have an increased free energy of activation for the transition state for \( k'_{+\alpha} \), resulting in slower P, release and giving P, with high exchange. Conversely, cross-bridges with low distortion would experience more rapid P, release and produce P, with low exchange. The model qualitatively fits the observation of one high-exchange pathway for conditions where force generation is maximal. This model also fits well with a bound ADP state being a major force-generating state for rabbit skeletal muscle (Webb et al., 1966).

Mechanism of Strain Activation—The increase in both ATPase activity and tension with strain has been explained in terms of an increase in the population of cross-bridges taking part in the cycle (recruitment) or in terms of an increased cycling rate of currently active bridges. Recruitment implies that cross-bridges are functionally divided into two populations at low strains: one population unable for some reason to bind to actin or otherwise take part in the active cycle and the other population able to do so. Published models of strain activation emphasize one or the other of these mechanisms (Thorson and White, 1969, 1983; Wray, 1979).

Strain activation is accompanied by a dramatic change in \( k'_{+\alpha} \) so that models are ruled out which rely solely on the recruitment of previously unavailable cross-bridges to account for the ATPase activation. This extreme form of recruitment would only alter the number of cross-bridges able to interact with actin, and so it would not change any rate constants of the active cross-bridges, including \( k'_{-\alpha} \). However, extensions of such recruitment models, e.g. involving the graded distortion of cross-bridges on recruitment, might explain the large increase in \( k'_{+\alpha} \), although our work does not indicate whether this is due to an increase in \( k'_c \) or \( k_{-\alpha} \).

The striking feature of our data with respect to strain activation is that the major change in P, release rate occurs over a very narrow change in fiber strain, implying a cooperative structural change within the fiber. None of the above models in its published form readily accounts for this finding. In insect flight muscle the strain at which this rapid change in both oxygen exchange and ATPase takes place is what we have defined as zero strain, the point at which relaxed fibers are just slack. Zero strain is the point at which the thick filament changes from being pushed to being pulled by the Z line as the sarcomere is strained. At less than zero strain the Z line might disrupt the highly regular array of the thick filaments in such a way as to allow a wider range of cross-bridge distortions at attachment, and thus a wider range of rate constants for P, release as discussed above. Alternatively, tension in either filament on strain activation might also remove geometrical irregularities in the filaments, resulting in the removal of the low-exchange pathway.

The uniform nature of the oxygen exchange in mechanically activated insect flight muscle fibers (as reflected in a single pathway for exchange) might arise from the common periodicities observed in the thick and thin filaments of this muscle (Wray, 1979). This common periodicity, which is unique to insect flight muscle, could produce uniform cross-bridge distortion on attachment to actin in the strain-activated fiber, and thus a single value of \( R \). Vertebrate muscle, in which two pathways are always required to describe the oxygen exchange of the activated fibers, has a different periodicity for the thick and thin filaments. This different periodicity could result in a range of distortions for attached cross-bridges and, therefore, a range of values of \( R \).

P, Release and Force Generation—The highest ATPase activity seen here for fibers, obtained under conditions of strain activation of the calcium-activated fibers gives a \( k_{+\alpha} \) of about 1.5 s\(^{-1}\), calculated from the ATPase activity (0.77 nmol min\(^{-1}\) cm fiber\(^{-1}\)) assuming 8 pmol of myosin heads/cm of fiber (Chaplain and Tregear, 1966). For these conditions the value of \( R \) is about 0.2 (Fig. 5A) and \( k_{-\alpha} \) is 10 s\(^{-1}\). Since \( R = k'_{-\alpha}/k_{-\alpha} \) this gives an estimate of about 2 s\(^{-1}\) for \( k_{-\alpha} \) the steps controlling P, release. The similarity of \( k'_{-\alpha} \) to the \( k_{+\alpha} \) suggests that P, release contributes to rate-limiting steps for the cross-bridge cycle in mechanically activated insect flight muscle.

There is little evidence about which intermediate states produce force in insect. It is probable that such states are a large proportion of the total attached states in fully activated muscle and so might be expected to be followed immediately by the rate-limiting step. Tension generation following a small length change occurs at \( \pm 10-14 \) s\(^{-1}\), which value we assume to be the rate constant for the formation of the major force-generating state. This rate constant is faster than P, release at 2 s\(^{-1}\), from which the simplest conclusion is that force generation precedes P, release. A plausible extension to Scheme 1 would then include two AM-ADP-P, states with the second being force-generating, so that actin does not bind
directly to give a force-generating state. Oxygen exchange shows that AM-ADP-P formation is reversible. If an AM-
ADP-P is a major force-generating state on the ATPase pathway, it follows that force generation will be reversible.
However, the ability of P, in the medium to lower the steady
state tension (Riege et al., 1971) suggests that a subsequent
state, e.g. AM-ADP also contributes to force generation in
insect, as suggested by Hibberd et al. (1985a) for vertebrate
skeletal muscle.

In summary, for insect flight muscle we find that the oxygen
exchange results make Pi release a good candidate for con-

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