TIME-RESOLVED STRUCTURAL STUDIES ON INSECT FLIGHT MUSCLE AFTER PHOTOLYSIS OF CAGED-ATP

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ABSTRACT The time course of structural changes occurring on ATP-induced relaxation of glycerinated insect flight muscle from the rigor state has been investigated using synchrotron radiation as a source of high intensity x rays and photolysis of caged-ATP to produce a rapid rise in ATP concentration. Temporal resolutions of 1 ms for the strongest equatorial reflections and 5 ms for the 14.5 nm meridional reflection are attainable from single events (i.e., without averaging over several cycles). The equatorial intensity changes completely, the meridional intensity partially, towards their respective relaxed values on a much faster time scale than relaxation of tension. The results suggest that actively cycling bridges present shortly after ATP-release are either too few in number to be detected in the equatorial diffraction pattern or that their structure is different from that of rigor bridges.

INTRODUCTION

The recent introduction of photolabile "caged" nucleotides from which rapid step increases of nucleotide concentration can be generated has introduced a number of novel experimental approaches to the investigation of the mechanism of muscle contraction (1–4). One such approach is the measurement of the time course of the structural changes resulting from binding of ATP to the contractile proteins. We report here the first results of time-resolved low-angle x-ray diffraction measurements following ATP release within glycerol-extracted insect flight muscle fibers using synchrotron radiation as a high intensity x-ray source.

MATERIALS AND METHODS

Fibrillar flight muscle from *Lethocerus indicus* was glycerinated as previously described (5) and stored at −80°C in a rigor buffer containing 75% glycerol. Caged-ATP was prepared by condensation of caged-phosphate (1) with ADP after activation with diphenylphosphorochloridate, as described for other nucleotide anhydrides by Michelson (6). ATP production from caged-ATP was assayed by HPLC on a Polyol Si 60 RP-18 column (4.5 × 62.5 mm; Serva, Heidelberg, Federal Republic of Germany) using an acetonitrile gradient in 50 mM phosphate buffer (pH 6.0).

For the time-resolved experiments, bundles of 15–25 fibers were mounted between a tension transducer hook and a fixed pin ~4 mm apart. The fibers were wrapped around the tension transducer hook and both ends were glued to the fixed pin, so that 30–50 fibers were exposed to the x-ray beam. This system was mounted on optical bench X-33 at the EMBL facility, DESY, Hamburg. A point source xenon flash tube (Chadwick-Helmuth, El-Monte, CA) was used as a light source for photolysis of caged-ATP. The power supply for the lamp consisted of a bank of condensors (capacity 2,250 μF, charged to 600 V, corresponding to 404 J), which produced a pulse of ~120 mJ in the 300–400 nm range. The quartz optical system consisted of a condensor (f = 16 mm, aperture = $f/0.8$) and a compound focusing lens (f = 50 mm, aperture = $f/1.0$). Due to lack of availability of cell windows with optimal properties in both the UV and x-ray regions, the cell was lowered to expose the fibers immediately before illumination. A single flash produced ~700 μM ATP from 12.5 mM caged-ATP with $t_{1/2}$ = 2 ms (calculated from reference 1) under the conditions used. The amount of ATP released was determined in trial experiments by immersing the fibers immediately after the flash in 0.1 M HCl at 0°C for 10 min followed by neutralization and HPLC analysis as described above.

RESULTS AND DISCUSSION

An important requirement for time-resolved x-ray diffraction experiments from weakly diffracting specimens such as muscle fibers is that the reflections in the scattering pattern can be easily recognized above the background. After optimizing the settings of the low-angle diffraction bench for the small size of the samples, equatorial and meridional diffraction data of the quality shown in Fig. 1 were obtained, allowing estimation of the peak intensities in very short time frames. Insect flight muscle is particularly suitable in this respect due to the small widths of the reflections.

Fig. 2 shows the changes in the ratio of the integrated intensities of the equatorial reflections following ATP release. In the experiment shown, intensity data were collected in 5 ms time frames. It can be seen that there is a

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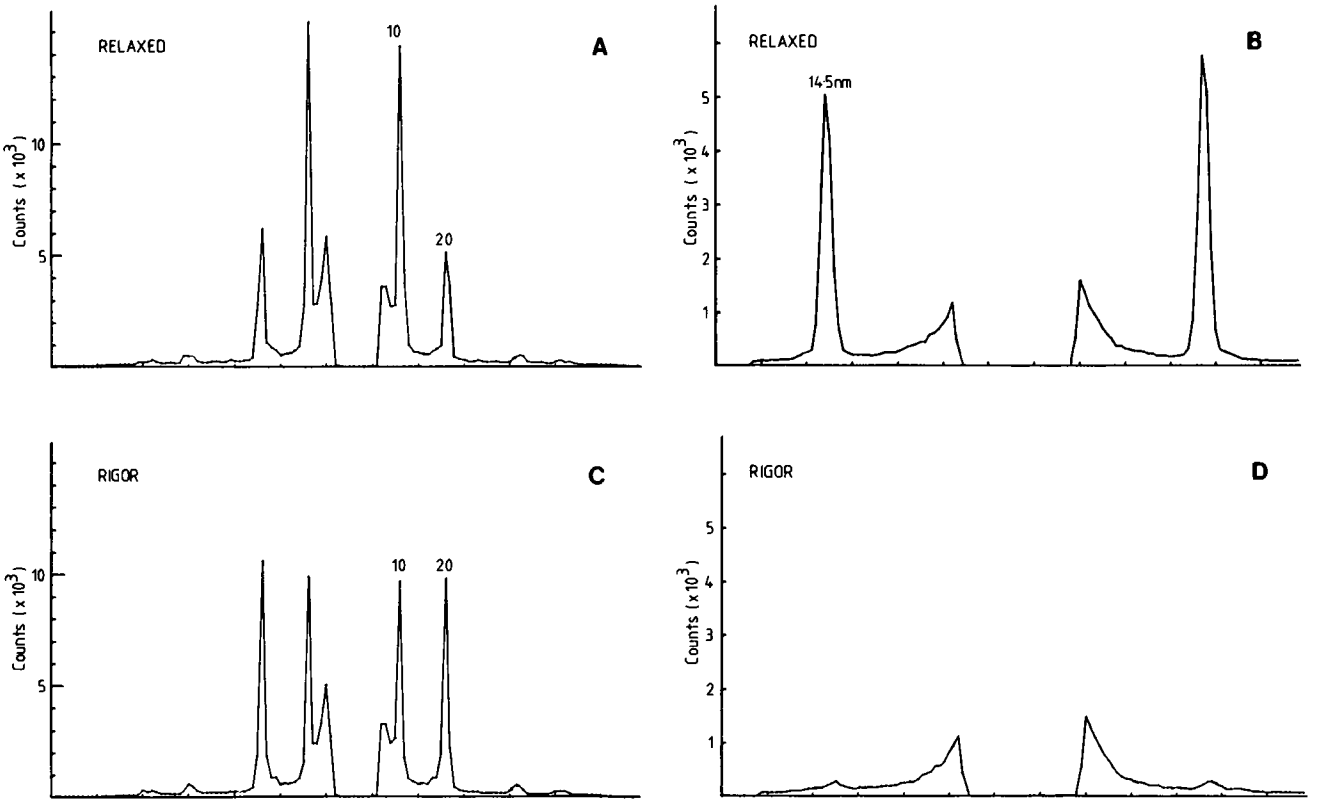

FIGURE 1 Low angle x-ray scattering from insect flight muscle. One dimensional patterns were recorded using a multiwire detector (1 mm spacing, 128 wires; reference 7) placed 3.5 m from the specimen. (*a*) and (*c*): Detector along the equator (*b*) and (*d*): Detector along the meridian. Patterns are from 1 s exposures of ~20 fibers in a cell 1 mm thick with 18 μm mylar windows containing for (*a*) and (*b*) relaxing solution (10 mM ATP, 10 mM $MgCl_2$, 5 mM EGTA, 45 mM KCl, 20 mM MOPS, pH 6.8) or for (*c*) and (*d*) rigor solution (as relaxing but without ATP).

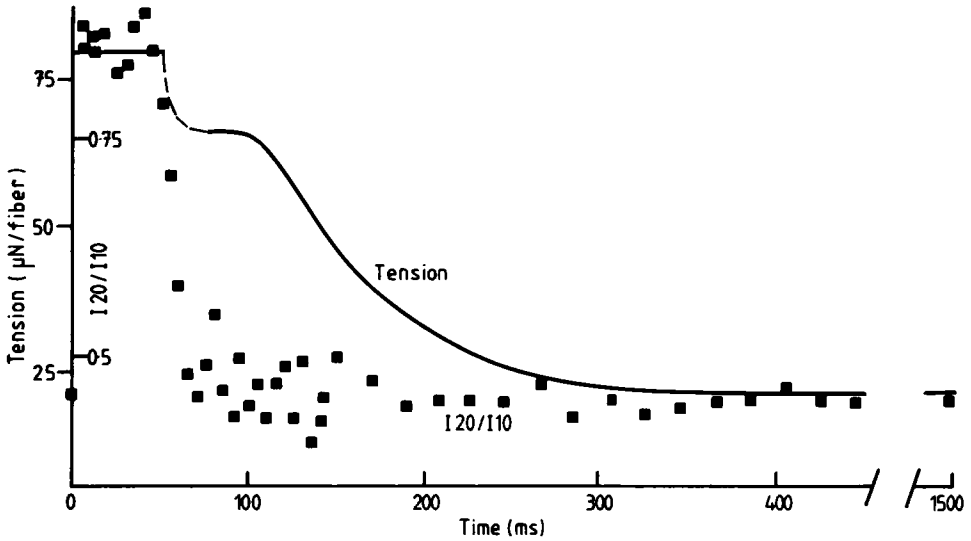

FIGURE 2 Change in the equatorial diffraction pattern on rapid release of ATP. Relaxed fibers were washed in rigor solution, and after development of rigor tension, the solution was replaced with a caged-ATP solution (as rigor + 12.5 mM caged-ATP and 12.5 mM DTE). After 5 min, the cell was lowered to expose the fibers to air, and the experiment was started within 5 s. The first 150 ms of the record were collected in 5 ms time frames, followed by a series of 20 ms frames. The intensities of the $20\bar{2}0$ and $10\bar{1}0$ reflections have been integrated (without subtraction of background), both sides of the pattern added, the intensity ratio calculated (designated here as I20/I10) and plotted (■). The point on the ordinate is the characteristic relaxed value determined on the same fiber bundle. The solid line is the tension recorded. The first 20–30 ms of this record were lost due to an artefact, but the dashed line shows the behavior as obtained from subsequent experiments performed under identical conditions. T = 26°C.

to be complete, as judged by the equatorial diffraction pattern, at a time when tension in the fibers is still relatively high. Assuming the ratio of the equatorial peaks to be a signal of cross-bridge dissociation, the expected time course can be simulated with the additional assumption of particular values of the apparent second order rate constant for ATP-induced dissociation of cross-bridges. Fig. 3 shows that the data can be reasonably well fitted using values within the range 1×10^5–5×10^5 $M^{-1}s^{-1}$. Since the curves are approximately exponential, the half-life was also calculated from a least squares fit of a single exponential to the experimental data. This leads to a value of $3–5 \times 10^5$ $M^{-1}s^{-1}$ for the apparent second order rate constant. The corresponding rate constant for the dissociation of the complex between actin and insect myosin subfragment 1 in solution has recently been determined to be 4.6×10^5 $M^{-1}s^{-1}$ at 20°C (White, D. C. S., manuscript in preparation). A total of ~40 experiments of this type have been carried out on 20 fiber bundles, but only under the best possible conditions (high current in the storage ring and optimally aligned bench) was it possible to obtain 1 ms data with an acceptable signal to noise ratio. In ~10 experiments where these conditions were fulfilled, the rate of the equatorial change was always similar to that in the example shown.

A potential problem associated with quantitative interpretations of the rates of structural changes monitored in our experiments concerns the uniformity of ATP release across the fiber bundle. Although we have no accurate information on this point, the following argument leads us to believe that this does not constitute a serious problem with bundles of the size used. The rate of mechanical relaxation was seen to be dependent on the amount of ATP released in the fibers, and when this was decreased by lowering the lamp intensity or reducing the caged-ATP concentration, there was a correspondingly slower relaxation. Such an effect was not seen on increasing the size of the fiber bundle from 5 to 25 fibers, as might be expected if there was a gradient of ATP released across the fiber bundle. In addition to this, the fraction of ATP released on photolysis of large bundles of fibers incubated in caged-ATP was not significantly lower than in small bundles.

In more recent experiments in which up to 2 mM ATP was released, the equatorial transient was even faster, as expected from solution experiments. Thus, there appears to be good agreement between the kinetics of ATP-induced dissociation of cross-bridges under the influence of ATP in muscle fibers and in isolated proteins. This conclusion has also been reached based on an interpretation of the mechanical transients resulting from the release of ATP from caged-ATP in rabbit psoas muscle (2, 3).

Fig. 4 *a* shows the results of a similar experiment with the detector positioned on the 14.5 nm layer line of the diffraction pattern. The large rapid increase of intensity on releasing ATP can be easily recognized in the 3-D plot

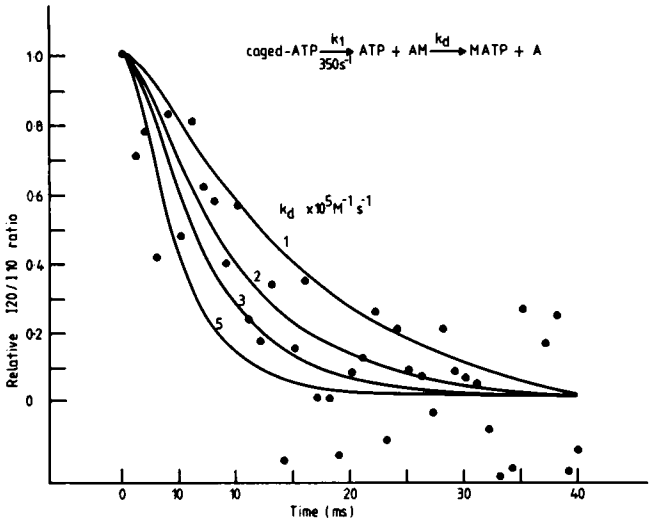

FIGURE 3 Rate of change of the equatorial pattern. The data points (•) show the 20$\bar{2}$0/10$\bar{1}$0 intensity ratio (corrected to allow for the fact that the rate of change of the ratio of the two exponentially varying peaks is faster than that of the individual peaks) in 1 ms time frames, expressed as a fraction of the total change. Solid lines are computer simulations (programme written by P. Rösch, Heidelberg) of the dissociation of actomyosin on release of 700 μM ATP at a rate of 350 s^{-1} (calculated from reference 1 for 26°C and pH 6.8) for values of 1, 2, 3 and 5×10^5 $M^{-1}s^{-1}$ for k_d in the scheme shown.

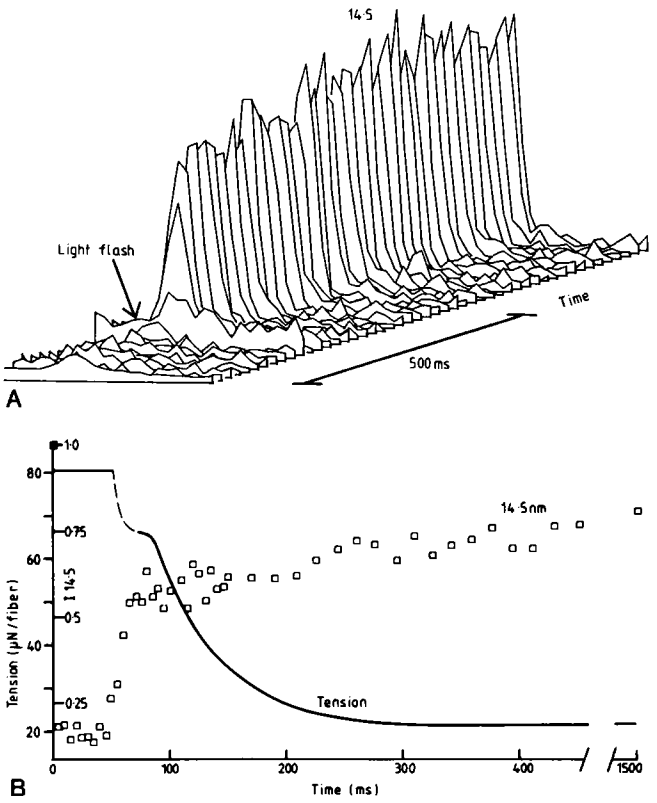

FIGURE 4 Changes in the meridional pattern on rapid ATP release. (*a*) 3-D plot of one of the two 14.5 nm reflections clearly showing the rapid increase in intensity after the flash (indicated by the arrow). (*b*) Time course of the 14.5 nm intensity change measured in 5 ms time frames. Intensity has been normalized to the value in relaxing solution.

(Fig. 4 *a*) and Fig. 4 *b* shows the time course of the integrated peak intensity together with that of tension in such an experiment. The initial rise appears to be similar in rate to that of the equatorial peaks, as far as can be judged at this time resolution (5 ms per time frame). However, in ~20 experiments on 10 different fiber bundles, the extent of this increase varied between 40% and 90% (~50% in the example shown) of the complete change expected on relaxation, and it can be seen that a further increase occurs at a much lower rate. In the present series of experiments, it was not possible to investigate this phase in detail, and the reason for the variation in magnitude is not yet understood.

The fibers do not appear to be damaged by a small number of rigor-caged-ATP/flash-relaxation/rigor cycles, as shown by the fact that the first few mechanical and structural transients, although rarely identical (probably due to variation in the amount of ATP released), did not show a systematic tendency to change in a particular direction. In a few trial experiments, bundles began to show signs of slower relaxation after ~10 flashes. This number was reduced if the concentration of DTE in the caged-ATP solution was reduced.

An unequivocal interpretation of these results cannot yet be given largely because the exact nature of the relationship between the diffraction patterns and their respective structural states (i.e., rigor, relaxed, or active) is still to be established. Using the intensity changes as empirical signals of the state of cross-bridges, the rapid and complete change of the equatorial reflections to the relaxed value shortly after ATP release suggests that cross-bridge dissociation is not only rapid (as fast as expected from solution studies), but also complete at this point. However, since tension is still much higher than in the relaxed state at this stage, the latter point cannot be correct, assuming that cross-bridge association with thin filaments is required for tension production, as is widely accepted. Goldman et al. (3) have proposed that the similarly slow final relaxation of rabbit psoas muscle fibers after ATP release from caged-ATP is due to actively cycling bridges which have reattached after an initial rapid detachment. Our results suggest one of two things about these reattached bridges. Firstly, it is possible that their number is very small, so that they do not contribute noticeably to the equatorial diffraction, even if they do differ in structure from relaxed bridges. Alternatively, cross-bridge reattachment could have occurred in a predominant state which is structurally similar to the relaxed bridge with respect to its equatorial diffraction pattern, indicating no detectable change of position between the filaments and no angling or slewing such as is thought to be typical of rigor bridges. A combination of the two explanations is of course possible, but this also implies the existence of a bound state of myosin heads which differs structurally from that in rigor, as required by such hypotheses as the swinging cross-bridge model of Huxley (8).

The rapid but incomplete increase in intensity of the 14.5 nm meridional reflection is most easily explained by assuming that a fraction of the cross-bridges adopt their characteristic relaxed disposition very rapidly, the rest having reattached to thin filaments in what might be regarded as the active state at relatively short times after ATP release. The meridional diffraction of these bridges would have to be weaker than in the relaxed state, perhaps due primarily to a necessarily lower degree of thick-filament-dominated longitudinal order of actin-bound cross-bridges.

From the experiments described here together with information from other sources, it is not possible to decide whether the rapid structural transitions reflected by the equatorial and meridional changes in these experiments occur before or perhaps together with ATP cleavage on myosin heads. If this rate constant can be determined independently, it may be possible to make definite conclusions concerning the positioning of structural events in the enzymatic cycle.

Combining the equatorial and meridional evidence, it appears that very soon after ATP release, the mean position of cross-bridges between the filaments reaches that characteristic of the relaxed state even while tension is still relatively high, but that the longitudinal cross-bridge order is less pronounced than in complete relaxation. This suggests that cross-bridges are bound to thin filaments in a configuration that is different from that of rigor bridges, although it cannot be excluded at present that the number of active bridges is so small that they do not contribute noticeably to the equatorial diffraction pattern. It is of interest to note that the experimental results described here are in good agreement with our recent finding that after stretch activation of insect flight muscle, thought to involve a significant increase in the number of attached cross-bridges, there is a marked loss of meridional intensity at the peak of delayed tension, in agreement with results from earlier experiments on oscillating muscle (9), but no change in equatorial intensities (Rapp et al., manuscript in preparation). More detailed investigations of structural changes in insect flight muscle following sudden chemical or mechanical perturbations should help to determine which of the explanations given above is correct and provide evidence on the nature of the power stroke of the contraction cycle.

We thank Dr. P. Rösch for the kinetic simulation program and M. Isakov and R. Wojciechowsky for technical assistance.

This work was supported in part by the Science and Engineering Research Council (United Kingdom), the Deutsche Forschungsgeminschaft (grant Gü 160/6-1) and NATO grant 547/82.

Received for publication 5 November 1985 and in final form 15 April 1986.

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