MOTIONS OF TROPOMYOSIN

Crystal as Metaphor

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ABSTRACT Movements of tropomyosin play an essential role in muscle regulation. This fibrous protein is a two-chain α-helical coiled coil that bonds head to tail to form cables wound in the two long grooves of the actin helix. The regulatory switch consists of tropomyosin and a "globular" Ca^{2+}-sensitive protein complex called troponin. The structure of the tropomyosin filaments has now been determined by x-ray crystallography to ~15 Å resolution. The complete sequence of α-tropomyosin is known; by using mercury markers on the cysteine residues the ends of the molecules in the filaments have been identified. Details of the coiled-coil structure have also been visualized by refinement of models against the diffraction data. The average pitch of the coiled coil is ~137 Å, so that each tropomyosin molecule can make similar contacts with seven actin monomers. The electron density map also indicates that departures from the α-helical coiled coil occur in a few localized regions of the molecule, especially at the overlapping ends. Motions of tropomyosin in the crystal lattice are displaced by the character of the Bragg reflections and the strong diffuse scatter. These effects depend markedly on temperature. It appears that the molecular filaments fluctuate freely in a direction perpendicular to their axes. Moreover, the C-terminal half of the molecule "unfolds" to some degree at less than physiological temperatures. Crystallographic results on co-crystals of tropomyosin and a component of troponin (TnT) suggest that this subunit consists of structurally distinct domains, so that the troponin complex is not in fact simply "globular". The interactions of the extended α-helical region of TnT may "stiffen" tropomyosin and influence its motions. We picture the tropomyosin/troponin switch in muscle as a restless cable, perpetually making and breaking bonds as it vibrates on the thin filament. These movements of tropomyosin probably depend on two aspects of its design: the regular pattern of coiled-coil linkages with actin; and the aperiodic features that allow flexibility and motion.

IDEOLOGY AND STRUCTURE

Science and art are curiously connected. Each inspires the other; together they describe human experience. Both are shaped by a common principle: the human mind seeks order and is satisfied only as it wrests design from complexity or apparent chaos.

Over half a century ago, Geoffrey Scott, a little-known English aesthete, saw deeply into this problem (1). He showed how, in the field of architecture, prejudices color perception, and how design can be corrupted by the influence of false analogy. He considered that ideal styles of building, as in the Renaissance, were authentic expressions of the basic human need for coherence. "Order which in nature is hidden and implicit, architecture makes patent to the eye."

Molecular models reflect, in a similar way, the scientist's attempt to translate inference from images and experiments into conceptions of structure. Here too the canons of good architecture apply: "Well-building hath three conditions: commodity, firmness, and delight" (2). Yet the scientist must also overcome historic prejudices and unexamined beliefs, in particular the deeply rooted need for order, to interpret what is before him. E. H. Gombrich has recently noted that "We have a tendency to probe both the real world and its

representations with a hypothesis of regularity which is not abandoned until it is refuted" (3). The history of protein structure illustrates this idea. Once proteins were conceived as molecules, scientists began searching for regularities. They began, indeed, to impose regularities: hence, notions of numerology were applied; geometric fabrics were conceived for the folds of globular proteins; and x-ray diffraction patterns were interpreted in terms of groups of rods, hoped-for α-helices. With the astonishing reality of myoglobin in 1957, and the many structures that soon followed, a new level of understanding was reached. The folding of the chains in globular proteins was seen to display a hitherto unimagined complexity. Yet even now the search for local order, for domains of regularity, the search for pattern, is a felt necessity (4).

A related prejudice has been the notion that proteins are static structures. This is perhaps associated in the mind with the principle of stability, or in Vitruvius' term, "firmness". Yet the quality of strength, as Scott emphasized, does not mean the absence of movement: "For there exist in baroque architecture rhythm and direction and stress, but no repose — discord, even — till the eye comes to rest in the broad unity of the scheme." In recent years, protein crystallographers have begun to hear what physical chemists, or "solution" chemists as they are sometimes called, have been proclaiming for decades: that side chains flip rapidly, that atoms move, that binding pockets open and close; in short, that proteins are dynamic molecules. We are now beginning to arrive at what is, perhaps, a more authentic picture of protein structure.

Motion in protein is nowhere better expressed than in muscle. Here too ideas conform to fashion. Once myosin and actin were identified, they were assumed to undergo large reversible changes in folding, so that the external shortening displayed by muscle was considered to be a direct reflection of the internal shortening of these protein molecules. The discovery of the sliding filament theory of contraction in the 1950's, together with advances in protein crystallography, led to an opposing view: that shortening was due to the relative movement of assemblies (or filaments) of myosin and actin, and that the structures of these proteins were not changing. The broad truth of this conception obscured a basic error.

We now recognize that parts of both myosin and actin filaments change conformation during contraction: the enzymatic heads of the myosin molecules (so-called cross-bridges) "reach out" and interact with the actin filaments, while undergoing a rapid cycle of changes. The precise motions of the cross-bridges are not yet defined, but the process has been compared to a kind of rowing mechanism, where the actin filaments are pulled into the array of myosin filaments by the repetitive action of the cross-bridges. As we shall see, the actin filaments generally have regulatory proteins associated with them, whose motion determines whether or not contraction can take place. Muscle thus exemplifies an organized system of proteins designed for directed motion. Both aspects of protein structure, a stable framework for constraint and variable linkages for change, operate together to produce movement (5).

In this paper we show how the design of a key protein, tropomyosin, encompasses both "impulse and restraint" (6) required for its role in muscle regulation.

TROPOMYOSIN MOVES IN MUSCLE

Tropomyosin is a protein rich in paradox. Some thirty years ago, Kenneth Bailey discovered tropomyosin in muscle; its function was unknown. The wide-angle x-ray pattern of tropomyosin was like that from myosin and keratin. This simple but enigmatic α-diagram showed that the chains in this fibrous protein had a regular fold (7), but the structure turned out to be more complex a than single α-helix. Crick recognized that the α-diagram was given by a

coiled coil of α-helices, two chains wind round one another in a slow spiral, with side chains interlocking ("knobs-into-holes" packing) to stabilize the molecule (8). Tropomyosin is often considered, in fact, to be the archetype of the α-helical coiled coil. Yet this remarkably stable, remarkably regular structure shows, as we shall see, some intriguing aspects of irregularity. And these aperiodic features seem closely related to its movement in muscle.

Renewed interest in tropomyosin sprang from Ebashi's discovery that his protein was a part of the switch-controlling contraction (9). Whether or not interaction between myosin "heads" and actin occurs is determined by regulatory proteins and calcium ions. In most muscles the regulatory switch consists of tropomyosin (M.W. 66,000) and a globular protein complex called troponin (of comparable molecular weight) associated with the actin helix. Ebashi formulated a model where tropomyosin filaments wind in the two grooves of the actin helix, positioning the troponin complex every 385 Å (10). (See Fig. 7.) At low levels of Ca^{2+}, the complex would prevent myosin heads from interacting with actin; above a critical level of calcium, this conformation would change and the muscle would be switched on. Tropomyosin acts as a kind of amplifier of troponin, in that only one complex is required to control the availability of myosin binding sites on seven actin monomers (11).

Further structural aspects of this mechanism are emerging. X-ray diffraction of whole muscle and three-dimensional reconstruction of electron micrographs of isolated proteins have led to the so-called "steric blocking model" (12–14). At rest, tropomyosin is thought to be situated at the edge of the actin groove, fixed in this position by the troponin complex. This location of tropomyosin physically blocks attachment of myosin heads to actin, preventing contraction. Binding of calcium causes a conformational change in troponin that allows the tropomyosin to move deeper into the actin groove, exposing the site where myosin can attach. The extent of this movement is ~10–15 Å. Contraction then occurs until the calcium level is lowered, resulting in relaxation. This plausible model accounts for a number of aspects of regulation, although controversial aspects remain.

The amino acid sequence of tropomyosin is known (15), and a completely α-helical, perfectly regular coiled coil has been used to model the molecule (16). Yet a number of features of the structure are not established. The two chains in the molecule appear to run in the same direction and are closely aligned. That a disulfide bridge can be formed between the single -SH groups of each chain has been taken as evidence that there is no stagger between the chains (17–20). The precise relationships between the two chains along the length of the molecule have not, however, been established. A further point of contention has been the estimate of helix content. Small, nonhelical domains may be present and have been postulated on the basis of secondary structure predictions (21; also footnote 1). Additional aspects of its structure relate to interactions with actin. Since one tropomyosin molecule binds seven actin monomers, the molecule was first thought to consist of seven identical domains. The complete sequence revealed, in fact, quasi-equivalent repeats, in particular a fourteenfold pattern of nonpolar and acidic residues. This periodicity has been related to regulation in muscle. McLachlan and Stewart (22) have interpreted the 14 quasi-equivalent zones on tropomyosin as two alternative sets of binding sites for actin. When tropomyosin is bound to the actin filament it would make seven half-twists in one molecular length, so that pseudoequivalent faces of the coiled coil would link to seven identical actin monomers in the "off" position. When the muscle is switched "on" the tropomyosin molecule would make a quarter-turn, and the seven other sites on the molecule would interact with a second set of seven sites on actin.

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[1]Argos, P. Personal communication.

These speculations assume a fully α-helical tropomyosin molecule making highly regular symmetrical interactions.

TROPOMYOSIN CRYSTAL STRUCTURE

The tropomyosin crystal, like the molecule itself, displays its overall structure rather plainly in the diffraction patterns. But a clue was necessary to decipher these diagrams: this was the recognition of the close correspondence between the image of the crystal seen in electron micrographs and the x-ray pattern of the crystal viewed in a particular direction (23). The micrograph shows strands of molecular size forming an open, kite-shaped mesh with long and short "arms" defined by cross connections (Fig. 1 *a*). Occasionally, the strands appear to split into two filaments. X-ray diagrams of this projection of the crystal show two strong spikes of intensity (Fig. 1 *b*): these are in fact diffraction arising from the crossed sets of strands, which

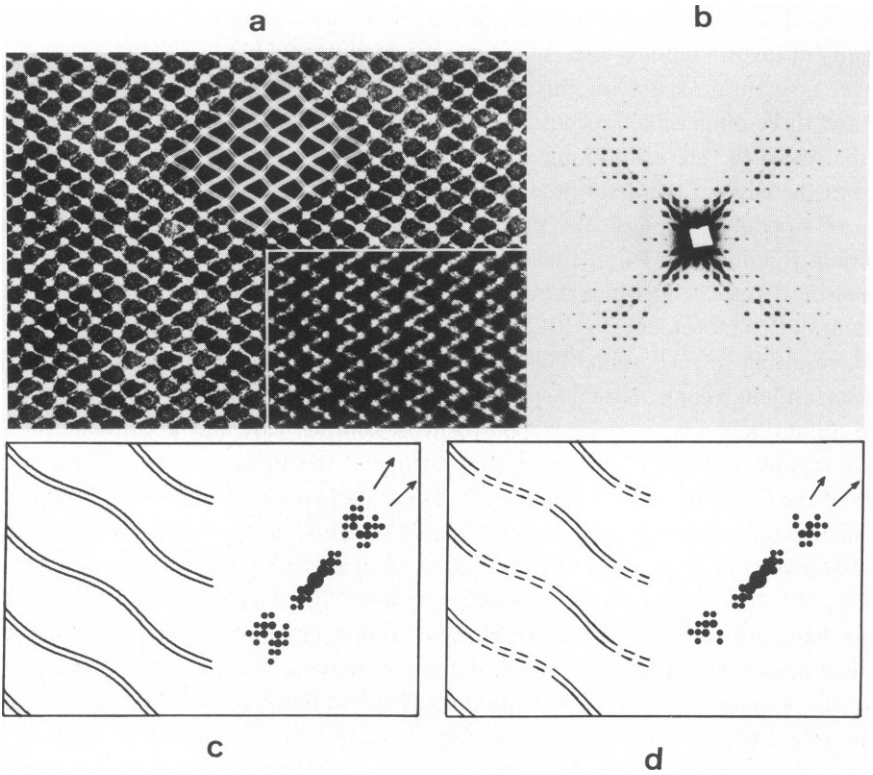

Figure 1 (*a*) Electron micrograph of negatively stained tropomyosin crystal. The micrograph is a two-dimensional projection of the three-dimensional lattice of cross-connected molecular strands. The strands are wavy, so that the mesh has a distinctive "kite" shape. The repeat along a strand (a long arm and a short arm) is ~400 Å. Some of the strands are seen to split into two filaments. Top inset: schematic diagram showing individual filaments. Lower right inset: electron micrograph of a co-crystal of tropomyosin and troponin. The troponin is seen in the middle of the long arm. (*b*) Precession x-ray diagram of tropomyosin crystal, [100] projection. The photograph shows reflections to 15 Å resolution. This pattern could not be readily interpreted without the electron micrograph of the crystal shown in Fig. 1 *a*. (*c*) The x-ray pattern is related to the crystal mesh as shown here. A spike of reflections is contributed by an array of wavy double filaments oriented in one direction. A similar spike is produced by the transversely oriented filament array. The spike is composed of two components, indicated by arrows, one derived from the long arm and the other from the short arm of the filaments. (*d*) At higher temperatures, the long arms no longer diffract strongly.

are made up of closely spaced pairs of filaments (Fig. 1 *c*). The intensity distribution of the spikes gives information about the bending of the filaments (Fig. 1 *d*). If the filaments were sharply bent into the long and short arms, the diffraction would show discrete spikes of intensity perpendicular to each of the arms. The patterns indicate, instead, that the filaments are bent into gentle sinusoids which give rise to a characteristic overlapping of the diffraction from the long and short arms at small angles. The mean direction of the respective spikes is, however, unchanged.

The interpretation of this one projection also gives a good qualitative understanding of the three-dimensional structure of the crystal at low resolution. Continuous filaments of the coiled-coil α-helical tropomyosin molecules wind as supercoils in the cross-connected lattice. To be sure, this is a kind of idealized picture where the molecules are regarded as regularly bent uniform wires. The next stage of the analysis carried to a higher resolution yields a more realistic view of the structure.

The crystal structure has now been solved to a resolution of ~15 Å (24, 25). The uniquely high water content of the crystal ~95%) limits strong diffraction to about this resolution; the fact that so much of the unit cell must be a flat, uniform region places a powerful constraint on the phases. Because of the continuity of the filaments and this large solvent content, three centro-symmetric projections (space group $P2_12_12$) are sufficient to define the three-dimensional path of the filaments in the lattice. The filaments wind along a line parallel to the body diagonal of the unit cell and trace out a path which is not, in fact, helical but has the form of an ellipse when projected down the axis of the molecule. Knowing this path allows for an accurate determination of molecular length: this turns out to be 410.3 ± 1.4 Å for the molecule in the filament. Since a fully α-helical tropomyosin molecule would be 423 Å long, there must be an overlap of ~8 or 9 residues in the head-to-tail bonding between molecules.

At this resolution, we can also determine where the molecules begin and end in the filaments. Cardiac tropomyosin consists of two identical chains (α-subunits), each of which has one -SH group (26, 27). The position of these groups on the filaments has been detected by mercury markers in difference Fourier maps. This result still leaves an ambiguity in the direction of the filaments and the location of the molecular ends. An argument based on

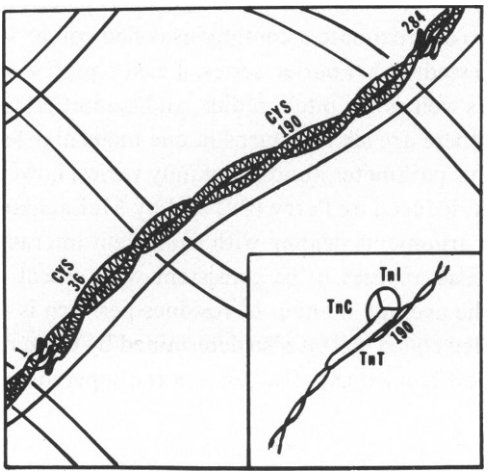

Figure 2 Schematic illustration of a tropomyosin molecule in the crystal lattice. The amino acid sequence has been related to the x-ray structure by locating Hg markers attached to cysteines 36 and 190. This result defines the location of the molecular ends.

matching predicted and observed electron density profiles along the filaments gives a clear "best fit" with one solution. With this polarity for the molecules, the head-to-tail overlap would occur near a crossover region in the short arm of the filament. Inspection of the electron density map shows, in fact, a relatively dense peak precisely in this region.

This choice for the molecular direction has recently been confirmed by a crystallographic study of rabbit fetal skeletal tropomyosin, which contains a high proportion (70%) of β-subunits. The sequence of β-tropomyosin is virtually identical with cardiac tropomyosin, but there is an -SH group at position 36 in addition to that at 190.[2] Difference maps of mercury substituted and native fetal tropomyosin crystals show a strong peak at the site predicted by the argument given above. This result establishes the basis for relating the entire sequence to the detailed features of the electron density map (Fig. 2).

The molecular filaments follow a relatively smooth path, without abrupt bends or kinks. At the region we have identified with head-to-tail overlap, the filament shows a slight "swelling" in all directions. The shape of this feature does not appear to be consistent with a simple overlap of regular coiled coils (16). A plausible alternative model is one in which short regions of chain at the molecular ends interact to form a compact globular domain. Such a notion is consistent with conformational predictions applied to the tropomyosin sequence; these indicate that neither end of the molecule is α-helical (e.g., [21]). Tropomyosin is generally considered the archetype of the α-helical coiled-coil structure, but the requirement of filament formation appears to have produced a specialized structure for linking the molecular ends.

There are other regions along the filament as well where departures from a regular coiled coil seem likely. These are found in the long arms and show that, even at 4°C, there may be irregularities in the molecule. At a resolution of 15 Å, then, we are beginning to detect small departures from a perfect coiled coil.

An additional feature seen at this resolution is the pitch of the coiled coil. The pattern of three-dimensional interactions that tropomyosin can make is determined both by the physical repeat of the coiled coil, expressed in the pitch, and the chemical repeat, expressed in the amino acid sequence. Based on knobs-into-holes packing, a two-chain structure with 3.60 residues per turn would be predicted to have a pitch of 186 Å (8). The pitch and the radius of the coiled coil of tropomyosin have been determined by refinement of models against the diffraction data. The models used consist of point scatterers placed at small enough intervals (5 Å) along the filament to approximate a continuous coiled coil at 15 Å resolution. The path of the filament was expressed as a Fourier series. Least squares refinement of the models yielded path parameters as well as the pitch, radius, and azimuthal origin of the coiled coil.[3]

The results show that there are six half-turns in one molecular length so that the average pitch is 137 Å (Fig. 2). This parameter almost certainly varies, however, along the coiled coil. This figure is, in fact, that deduced by Parry (28) and by McLachlan and Stewart (22) using quite different theoretical arguments dealing with equivalent interactions of tropomyosin and actin. A pitch of 137 Å also appears to be consistent with recent studies of the α-helix in globular proteins where the average number of residues per turn is $\sim$3.64.[4] The hand of the filament supercoiling (coiled coiled coil) is also determined by these results, since the α-helical coiled coil is known to be left handed (8). The sense of the supercoiling would seem to be right

[2]Smillie, L. B. Personal communication.
[3]Phillips, G. N. Unpublished observations.
[4]Chothia, C. Personal communication.

handed (i.e., of opposite handedness to the coiled coil). The implication of these findings for the interaction between tropomyosin and actin will be discussed in a later section.

The picture of the molecule that emerges from the crystallographic studies carried out thus far is an intriguing one: we see both regularity and aperiodic features. The molecular motions are determined by this complex design.

MOTIONS IN THE CRYSTAL

The very properties of extreme plasticity and thermolability that make tropomyosin crystals difficult specimens for data collection signal the presence of marked molecular motions. In general, a number of displacements (including vibrations, conformational substates, and lattice disorders) affect x-ray scattering (29). In the case of proteins, however, recent work has shown that the major effects are due to lattice disorders and molecular motions (30). The one involves essentially static displacements; the other, dynamic. By studying the Bragg diffraction as a function of temperature it is possible to separate lattice disorder from conformational flexibility. In protein crystals thus far examined, it appears that lattice disorder is relatively invariant with temperature (30). Further information about the flexibility of the molecule can be obtained by analyzing the diffuse scatter which arises from fluctuations within each molecule, and from coupled movements between neighboring molecules.

In the following section we describe results on the tropomyosin crystal that allow us to characterize the nature and extent of molecular motions.

Bragg Reflections

Motions of tropomyosin are displayed in several aspects of the x-ray diffraction. The first effect is a markedly anisotropic decrease in the intensity of the Bragg reflections. Such an effect is found in certain organic and inorganic crystal structures, but is rarely described for proteins. The diffraction pattern fades out at ~20 Å resolution along the a^* direction, whereas the data extend beyond 15 Å in the b^* and c^* directions. This intensity fall-off indicates a component of disorder which is greatest along the a axis (Fig. 3).

"Temperature" factors calculated from the model of tropomyosin described previously are

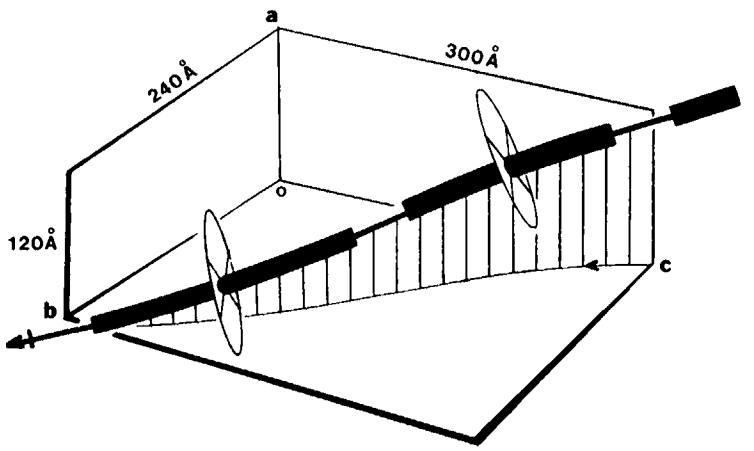

Figure 3 Path and motions of tropomyosin filaments in the unit cell of the crystal. Molecules run parallel to the body diagonal of the unit cell, with the largest component of motion perpendicular to the filament axis.

defined as follows: $F_{corrected} = F \exp\{-2\pi^2[U_{11}(h/a)^2 + U_{22}(k/b)^2 + U_{33}(1/c)^2]\}$, where F is the uncorrected structure factor for reflection *hkl*. The values obtained from the data by least squares minimization are: $U_{11} = 47$ Å^2, $U_{22} = 27$ Å^2, and $U^{33} = 14$ Å^2. These "temperature" factors depend both on disorder and on the accuracy of the model used for the structure. (The latter can be shown to make only a small contribution.) The results can be interpreted to mean that most of the disorder producing the anisotropic intensity decrease is perpendicular to the axis of the filament. Thus, the displacement components along the *a*-axis are greater than along the *b*-axis; and these in turn are greater than displacements along the *c*-axis. We must emphasize, however, that these results do depend to some extent on the nature of the model used, as well as lattice disorder, so that we cannot estimate the actual magnitude of the displacements from this study.

A more detailed picture of the properties and motions of tropomyosin filaments has been derived by studying the diffraction patterns as a function of temperature. In principle, this kind of analysis might allow us to distinguish between static and dynamic disorders. The nature of the motions might also be characterized with some precision.

The effect of temperature on the patterns is dramatic. X-ray photographs along the [100] direction, taken between 0 and 30°C (Fig. 4), show that as the temperature is increased, the "spike" from the long arm is greatly reduced relative to that from the short arm (Fig. 1 *d*). This diffraction fall-off from the long arm far exceeds the rate expected for a simple vibrational effect. It appears, in fact, that as the temperature is increased, the molecule undergoes conformational transitions and that this effect is confined to the long arm. Simply put, it could correspond to local "unfolding", to some degree, of regions in the long arm. The crystal is not damaged under these conditions, since the effect is reversible. It appears then that the long arm, i.e., the C-terminal half of the molecule, is a considerably less stable coiled coil than the N-terminal half. This diffraction result agrees with other physical chemical studies (31, 57).

Diffuse Scatter

A further aspect of the diffraction where displacements of filaments are apparent is in the "thermal diffuse" or "incoherent" scatter. Fig. 5 shows a still photograph down the [100] axis

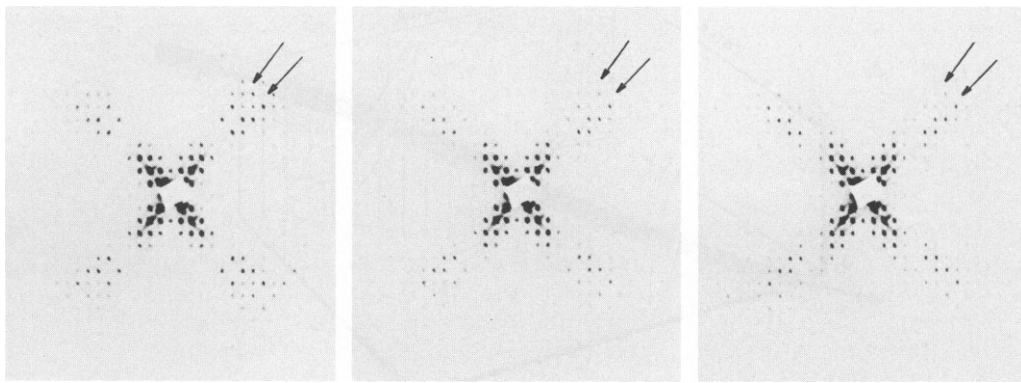

Figure 4 Precession x-ray photographs of a tropomyosin crystal, [100] projection, taken at (*a*) 0°C; (*b*) 15°C; and (*c*) 30°C. The discrete spikes of intensity arising from the long and short arms of the structure are marked by arrows. The dramatic decrease in the intensity of the spike arising from the long arm at near physiological temperature demonstrates that this portion of the molecule has exceptional conformational flexibility.

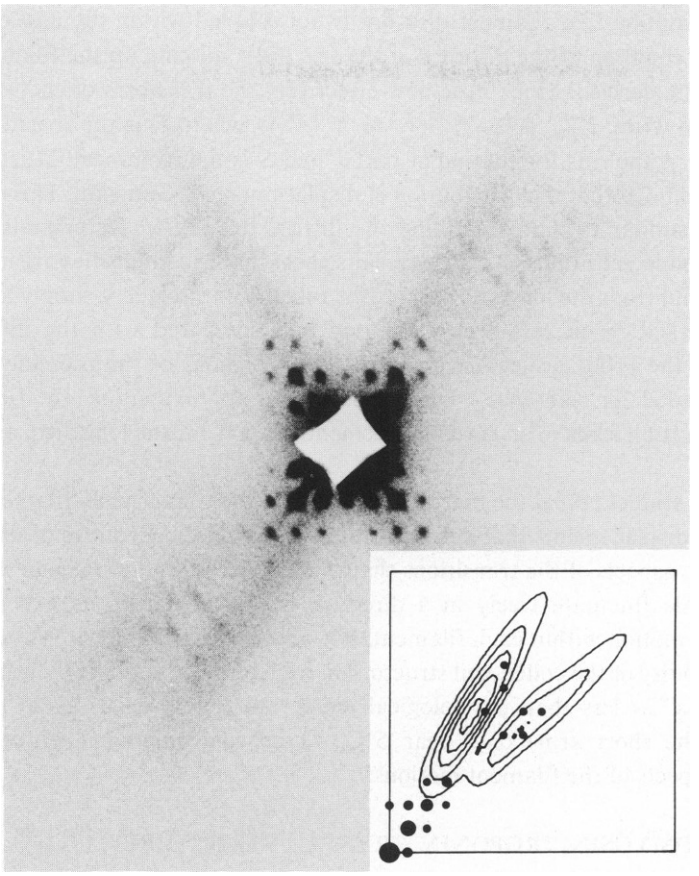

Figure 5 X-ray "still" photograph of a tropomyosin crystal at 4°C along the [100] axis. The strong diffuse streaks arise from large fluctuations of the filaments. The inset shows the diffuse scatter simulated from model structures. The blackened circles mark the locations of Bragg-associated peaks for reference to the x-ray photograph.

of the crystal. The strong streaks can be attributed to displacements of filaments from their average positions. A number of peaks are also seen surrounding reciprocal lattice points; these arise from filament disorders which are coupled from one unit cell to the next. This latter aspect is more of a lattice than an intramolecular disorder and is an expected feature from a crystal made up of filaments. The degree of cooperativity in the lattice can be estimated from this diffraction effect, but we have not yet carried out an analysis of this aspect of the scattering.

The diffuse diffraction may be characterized by extending the analysis of Guinier on imperfect crystals (32). He has shown that if the fluctuation in one unit cell are independent of all others: $I_{\text{diffuse}} = |\overline{F_n}|^2 - |\overline{F}|^2$, where I is the continuous diffuse scatter, F_n is the "perturbed" structure factor for the unit cell n, and $\overline{F}$ is the average structure factor of all unit cells.

Applying this function to the tropomyosin case, diffuse streaks would be predicted whose direction is perpendicular to each arm of the filaments. If the filaments were perturbed symmetrically, however, there would be nodes arising from the interference of pairs of filaments in the crystal. This kind of diffraction effect differs from the observations, since such nodes or minima are not observed.

On the assumption that filament disorder is not coupled within the unit cell, and indeed that there is no coupling of the disorder of the two arms making up the filament, a different expression can be derived. Since F_n is now effectively the transform of each molecule in the unit cell, we can write: $I_{\text{diffuse}} = \Sigma_j |F_j|^2 (1 - e^{-2\pi^2 |S|^2 \sigma_j^2})$, where F_j is the structure for part j of the structure, σ_j is the rms fluctuation of part j, and S is the reciprocal lattice vector. In this equation it is assumed that the distribution of displacements is Gaussian. This expression is, as expected, very similar to that describing the diffuse diffraction for crystals with perfectly independent atomic vibrations (32). The results show that the intensities arising from each of the filaments, and from the long and short arms taken separately, are simply additive.

This theoretical result has been simulated and compared with the diffuse scatter of tropomyosin in the [100] projection (Fig. 5). (The positions of the prominent Bragg peaks have been included for reference.) Using this simple approximation, the fit is remarkably good. The simulation leads to an rms displacement of ~8 Å for the long arm, and ~5 Å for the short arm.

These initial studies reveal the marked flexibility of the tropomyosin filaments. Our results have described displacements that define different conformational states of the molecule. We can now picture aspects of the transitions that must occur for these states to arise. Both arms of each molecule fluctuate freely in a direction perpendicular to its axis. There is some coupling of the motion within each filament, but not between filaments. Moreover, there is a differential stability of the coiled-coil structure in each of the arms. The C-terminal half of the molecule "melts" at less than physiological temperatures and exists as a "tight" structure (like that in the short arm) only near 5°C. These conformational states in the crystal characterize aspects of the filament motions.

TROPOMYOSIN/TROPONIN CRYSTAL STRUCTURE

Tropomyosin and troponin, together, form the regulatory switch of the thin filament. Here, too, crystallography is beginning to define states of the structure. Troponin comprises three subunits with specialized functions: TnC binds calcium; TnI binds to actin; and TnT links to tropomyosin (33). The calcium sensitivity of the tropomyosin/troponin complex resides in the specific binding of this cation to TnC, which produces large conformational changes in this subunit (34). These changes then affect the tightness of coupling to the other subunits and, it may be inferred, their structure. At low calcium concentrations, the troponin complex is linked tightly to actin. Hence, we may picture the tropomyosin molecule in its blocking position. Above the critical calcium concentration, this linkage is weakened, but the bonding between TnC and TnT is strengthened. The troponin complex is "tightened" and unlocked from actin; tropomyosin then moves deeper into the actin grooves. The myosin heads can attach to the outer portion of actin, and contraction is switched "on" (35–37).

The amino acid sequences of all of the proteins in the thin filament are now established (38–42). Both actin and TnC have in fact been crystallized (43, 44). Moreover, some aspects of the three-dimensional structure of TnC have been inferred from certain close sequence homologies to other calcium-binding proteins (45). To define the mechanism of the regulatory switch, however, this protein complex must be visualized in detail.

The whole troponin complex can be incorporated into the open lattice of the tropomyosin crystal. TnT positions the complex on the specific binding "site" of tropomyosin. Electron microscope images of assemblies of troponin and tropomyosin seem to show a discrete location for the complex on the molecule. In paracrystals formed by precipitating tropomyosin with divalent cations (46) the ends of the molecules can be identified, and troponin seems to be

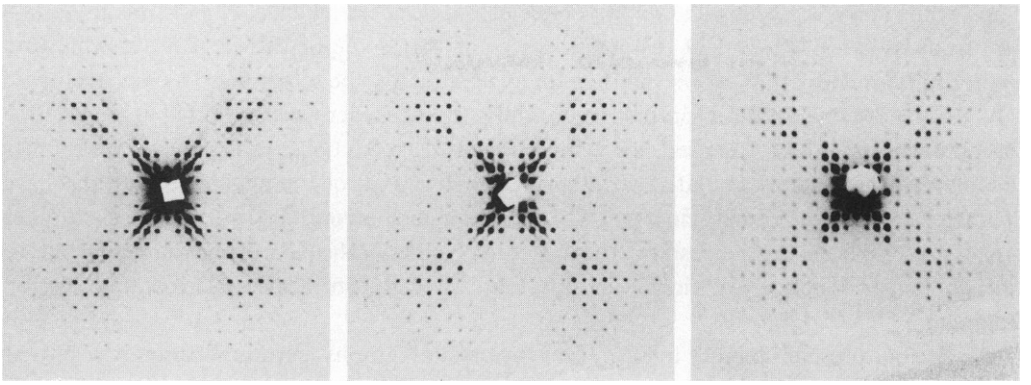

Figure 6 X-ray precession photgraphs, [100] projection, of tropomyosin crystals: (*a*) highly purified tropomyosin; (*b*) tropomyosin co-crystallized with fragment CB2 of TnT (~1/1 mole ratio); and (*c*) tropomyosin co-crystallized with a small amount of "whole" troponin (see text).

located about one-third of the way from the C-terminus (47, 48). Correspondingly, troponin appears to be located near the center of the long arms of the mesh in the crystal (Fig. 1 *a*). Since we now know where the molecules begin and end in the filaments forming the crystal as well, this site also turns out to be about one-third of the way from the C-terminus; that is, near Cys-190 (Fig. 2). We therefore expected that the x-ray diagrams of co-crystals could be readily interpreted on this basis.

This is not the case. When small amounts of troponin are bound in the crystals, the unit cell parameters change so that the cell is "squared up" (47, 49). The diagrams are difficult to phase, however, and the intensities cannot be predicted by the simple addition of a mass attached to the center of the long arm. It seems, moreover, that troponin is readily degraded during the long time it takes for such co-crystals to grow. We have therefore approached this problem by concentrating on the tropomyosin binding subunit, TnT, and its specific linkage in the filaments forming the crystal.

TnT has turned out to be far more than a "simple" globular protein. Predictions based on sequence have postulated domains of widely different conformation. The whole subunit, of molecular weight 30,000, has been pictured as forming a compact structure (50). The various interactions of TnT have been associated with discrete fragments cleaved by cyanogen bromide: thus, the small (10,000 daltons) CB2, a highly α-helical region, binds strongly to tropomyosin, and the regions following it in the sequence link to TnI and TnC (51, 52). The binding region on tropomyosin identified by chemical means appears to span a fairly long distance, including the site near Cys-190 but also considerable stretches on either side, i.e. from about residues 140 to 200 (53). Recent work places the binding site even farther toward the C-terminus.[5] These chemical studies, taken together, appear inconsistent, and they are at odds with results from electron microscopy. How do we put together these pieces of the puzzle?

The clue here lies in the x-ray analysis of co-crystals of CB2 and tropomyosin. These crystals show some "squaring up" of the lattice and intensity changes similar to those for the whole troponin complex in the tropomyosin crystal (Fig. 6). When we first began the analysis of these patterns we had hoped to consider the fragment as a very heavy "atom" with a special shape. Difference Fourier maps were calculated using phases from the native structure. The

[5]Smillie, L. B. Personal communication.

maps, while noisy, seemed to show additional electron density along a region of tropomyosin—say, from residues 125 to 160. Although we knew that some of this density was due to a change in filament path, we believed then that we might also be seeing the TnT fragment.

We have now analyzed this problem more fully. In the earlier work a limited amount of the three-dimensional data was used, involving only strong reflections whose phases were fairly reliable. We have now calculated difference maps using all of the available data and have applied weights to the coefficients to account for phase errors. The new difference maps broadly resemble our earlier result with respect to changes in the path of the filaments; but we can no longer identify additional density that we can confidently ascribe to the CB2 fragment.

Despite our present inability to locate TnT by crystallography, certain diffraction results as well as physical chemical findings prompt a speculation on the shape of this subunit and its interactions with tropomyosin. The important point is that additional density localized only in the center of the long arm (as seen in the electron micrographs) does not account for the changes in the diffraction pattern.

It is possible that TnT consists of at least two kinds of domain. One is a highly α-helical section, corresponding to the CB2 fragment. This part of the molecule could interact with tropomyosin, perhaps forming a kind of three-chain coiled-coil structure (see also 50). If, in fact, the rod region of TnT extends ~100 Å on either one or the other side of Cys-190, it would interact at the cross-over regions in the crystal lattice. Since there are such marked changes in lattice parameters when troponin is bound, this effect, may, in some part, be due to the presence of the binding subunit near the lattice cross connections.

An extended structure would also account for the extensive binding region found for tropomyosin. The rest of the TnT molecule would be more globular and would be located at one end of the helical fragment, so that it appeared in electron micrographs as a discrete mass near the middle of the long arm in the crystal mesh. The TnC and TnI subunits of whole troponin would also interact in this region. The binding site for troponin previously identified from electron microscope studies might therefore correspond to one end of this complex.

This hypothetical picture clarifies a number of hitherto contradictory results. TnT is very unstable and insoluble when isolated. It may well be that the α-helix of the CB2 fragment is stabilized when bound to tropomyosin. Staining of the thin filament with antibodies prepared against the troponin subunits has shown that an extensive region binds anti-TnT, and that this region is distinct from the narrow portion of the thin filament which binds both anti-TnC and anti-TnI. These immunological results have been interpreted to mean that the troponin complex is not globular, but rather extended in shape (54). The well-known increase of viscosity when either troponin or TnT or indeed CB2 binds to tropomyosin may now be seen to be related to the nature of the attachment of TnT to tropomyosin. It would appear that TnT does more than simply link the troponin complex to tropomyosin: this subunit may literally "stiffen" the tropomyosin molecule and thus influence the motions of the tropomyosin filaments.

THE REGULATORY SWITCH IN MUSCLE

These structural results, even at low resolution, provide considerable insight into the regulatory mechanism. It is important to emphasize that neither x-ray diffraction of whole muscle, nor three-dimensional reconstruction of electron micrographs of isolated thin filaments can yield clear enough images of the native structures to define the precise nature of the conformational changes involved. The diffraction from tropomyosin and troponin in

muscle is crucial in demonstrating that tropomyosin does move, but beyond this the data are quite meager, and allow us to infer only an approximate range of motion for the tropomyosin filament and the fact that the axial repeat of the structure is maintained. Three-dimensional reconstruction of thin filaments gives information on dehydrated and negatively stained material. In addition to the intrinsic artifacts involved in this technique, the interpretation of images is by no means clearcut. The crystallographic results discussed here give us the clearest picture we now have of the structure of tropomyosin and troponin, and of the conformational states these proteins may adopt.

The structure of the tropomyosin filaments in the crystals is remarkably similar to that of the filaments wound on the actin helix in muscle (Fig. 7). The motions displayed in the crystal lattice reveal aspects of the molecular motions in muscle. The filaments are supercoiled in the lattice in virtually the same way they supercoil on actin: they have a right-handed sense, and they therefore make seven half-turns as they wind in the grooves of the actin helix. A major difference is the radius of curvature: the tropomyosin filament winds about the actin helix with a larger radius of bending than it adopts in the crystal lattice (24). Correspondingly, the projected length of the tropomyosin molecule in muscle is 385 Å, compared with 402 Å in the crystal. The end-to-end bonding involving an 8- or 9-residue overlap appears to be an invariant feature of the structure. We have inferred that this region may comprise a small globular domain, but it is important to note that the interlocking of the two molecular ends at this junction nevertheless maintains the regularity of the coiled coil. Thus at first sight, the tropomyosin filament, both in the crystal and in muscle, presents a strong aspect of regularity.

Our studies on the motions in the crystal reveal, however, a rather more chaotic and asymmetrical structure. The intensity fall-off of the Bragg reflections shows that the entire filament vibrates markedly in a direction perpendicular to the molecular axis. Moreover, the temperature dependence is such that at 30°C the long arm of the filament undergoes some "loosening" or unfolding, while the relatively compact structure of the short arm remains. Such a picture of the molecule's stability is in accord with the filament structure seen in the crystal at 4°C. The electron density maps reveal local regions of "kinking" or possible deviations from the coiled coil, chiefly in the long arm, or C-terminal portion, of the molecule. Similarly, studies of proteolytic fragments of tropomyosin show differences in melting profiles, with the C-terminal region having marginal stability (31).

The motions of tropomyosin in muscle depend on the precise structure(s) of the molecule and its interactions with both actin and troponin. If tropomyosin were fully extended when bound to the actin helix, the effective length of the molecule (410 Å) would place the filament at a large radius on the actin helix (say 40 Å) when in the blocking position at the edge of the groove, since the axial repeat of tropomyosin in muscle is 385 Å. In the "on" state,

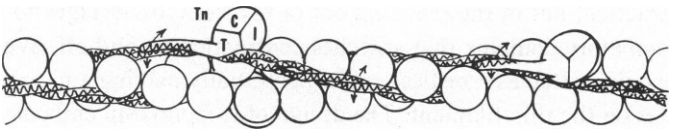

Figure 7 Schematic diagram of the thin filament. Tropomyosin molecules (coiled coils), bonded head-to-tail, wind in the two grooves of the actin helix, forming coiled-coiled-coil or supercoil filaments. Each molecule spans seven actin monomers so that the axial repeat is 385 Å. The supercoiled filaments of tropomyosin are not rigidly linked to actin, but fluctuate over a range of positions. Troponin is specifically linked to tropomyosin by α-helical region(s) of TnT. Calcium ions bind to TnC to produce conformational changes that modify the structure and motions of tropomyosin on the thin filament.

tropomyosin would move deeper into the actin groove, so that the supercoil radius would be decreased. If the molecule is tightly bound to actin, it must, therefore, bend in some way. This simple argument assumes that the filament is a perfectly uniform cable. However, experiments on the crystal indicate that at physiological temperatures, considerable distortion of a major part of the molecule is certainly occurring. Although the molecules are linked together in the crystal lattice at only a few points, whereas they have the potential for 14 binding sites on actin in muscle, it seems likely that the filament is rather loosely wound about the actin helix at some stage of the contractile cycle, perhaps held strongly at only a few regions and locally kinked at others. The switching has been pictured as involving a quarter-turn of a relatively uniform symmetrical cable making contacts with seven actin monomers at each of two types of site (22). In view of the somewhat irregular and dynamic structure that we are beginning to see, the switch seems unlikely to involve two well-defined states with all seven bonds "on" or "off". Such a picture falls prey to a kind of mechanical fallacy. Rather, we would envision a pattern of continually changing connections between tropomyosin and actin, where the net macroscopic effect is either the "on" or "off" state (55). Cooperativity is an essential aspect of such a system, but cooperativity need not imply rigidity.

TnT appears to play a critical role in the motion of tropomyosin. We have pictured this troponin subunit as having an α-helical portion that interacts with an extensive region of tropomyosin. The structural effects of TnT on tropomyosin, demonstrated by the viscosity increase produced by their association, seem considerable. It appears that just as tropomyosin enhances the stability of the actin helix, so may TnT enchance the rigidity of tropomyosin. It is of some interest in this connection that the binding region of TnT on tropomyosin extends over a considerable portion of the long arm of the filament, which disorders readily. The presence of TnT may be an additional control of the stability and motions of this part of the molecule. Thus we may picture the entire regulatory complex as a cooperative unit. In the switching between states, changes in the conformation of TnT induced by changes in the TnC subunit could have a large effect on the mechanism. At high calcium ion concentrations tropomyosin has been thought simply to be "released" from the blocking sites on actin by a tightening of the troponin complex. If this tightening led also to a change in the interactions of the helical portion of TnT with tropomyosin, it could induce a kind of "melting" of the long arm, hence a breaking of bonds to the "off" sites on actin. A shift to the bonding pattern of the "on" state might then take place. These notions about the mechanism are, of course, entirely speculative. But in vitro studies on the actin/tropomyosin association are beginning to show how strongly their interaction is determined by slight changes in external conditions, such as ionic strength; these effects seem to be particularly important at physiological temperatures (56).

Muscle is a machine for motion, but the special nature of the protein molecules in this system determine its detailed working. Movements are evident not only in the gross sliding of filaments in contraction, but in the reaching out of myosin cross-bridges to actin, and in the motions of the regulatory complex that switches contraction on and off. We now picture the tropomyosin/troponin switch as a restless cable, perpetually making and breaking bonds with actin, as it vibrates on the thin filament. The design of tropomyosin embodies two apparently contradictory aspects: regularity and aperiodic features. Together, they determine the movements of this unique and subtle protein. It is in fact our thesis that all molecular motions require this kind of structural antinomy.

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DISCUSSION

Session Chairman: Frederic Richards *Scribe*: Andrew W. Fulmer

LEHRER: This very interesting temperature study allows us to compare the molecule in the crystal to the molecule in solution and to find out what temperature is doing in both cases. I'd like to refer to our data (1978, *J. Mol. Biol.* **118**:209 and 1978, *J. Biol. Chem.* **253**:3757), for localized chain separation and localized instability in the cys-190 region in the middle of the carboxyl half of the molecule, and then pose a question.

In circular dichroism melting profiles, we find a different melting profile for tropomyosin in which the sulfhydryls are kept reduced without a disulfide crosslink between them. Woods earlier showed a general loosening or monotonic decrease in the low temperature region up to ~40°C or below 1.0 M GuHCl. The unfolding profiles are qualitatively similar whether temperature or GuHCl is used as the denaturant. If a disulfide crosslink is introduced between the two sulfhydryl groups —if one were not careful to keep it reduced or, on purpose, by air oxidation or by a reaction

with DTNB (1975, *PNAS* **72**:3327)—there would be a completely different unfolding profile. The expected stability of the S-S crosslink is seen at high temperatures or high guanidine and an increased instability appears in the low temperature or low GuHCl region. In fact, it resembles a cooperative pretransition. The evidence indicates there's a local unfolding at physiological temperatures in the region of Cys-190. This unfolding becomes more cooperative if the molecule contains a disulfide link.

My question is, in what state is the tropomyosin in these crystal studies? I suggest that by studying these two forms of tropomyosin in the crystal one could more clearly correlate in the temperature effects in the crystal with what is known to occur in solution.

PHILLIPS: A very good point. The straightforward answer is that although we start with reduced tropomyosin we have not checked to see what the state of the sulfhydryl groups is after x-ray analysis. We plan to do this by simply running the crystals on a gel after the x-ray pattern is taken. I mentioned that our electron density maps show some deviation from a coiled-coiled structure in the C-terminal half, the region where these sulfhydryl groups are contained.

SYKES: Dr. Edwards and I, using NMR to observe tropomyosin, find it hard to keep those sulfhydryl groups reduced. Local unfolding goes back to his 153 and extends out to affect his 274. There is a big change.

KORETZ: In light of the current controversy on how tropomyosin might prevent cross-bridge binding, could you discuss your 14 quasi-equivalent sites and differential flexibility of the tropomyosin molecule?

PHILLIPS: You're referring to the controversy raised by Seymour and O'Brien (1980, *Nature* **283**:680)?

KORETZ: Yes. Specifically, does tropomyosin sterically block the crossbridges or not?

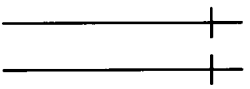
PHILLIPS: The model reviewed in the text has been referred to as the "steric blocking model." Tropomyosin is thought to lie in a position such that it can sterically block myosin heads from attaching to actin. Some results now indicate tropomyosin is on the opposite side of the actin groove relative to the side that the myosin heads attach to, so it couldn't possibly interfere with the binding of myosin to actin. I don't know if I believe those results yet. Peter Vibert of our laboratory supports the original choice of side of the actin groove for the location of tropomyosin. I think it is a question to be resolved.

KORETZ: You must have speculated about this, though.

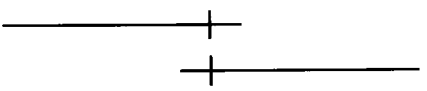
PHILLIPS: We see tropomyosin and its seven equivalent interacting regions making and breaking bonds either in the on- or off-state. We see perhaps one tropomyosin molecule, one end of it may be in the on, one end in the off. The range of motions that we see in the crystal is consistent with that type of flexibility on the thin filament. So the steric blocking model with its rigid gears and cogs moving tropomyosin from one position to the other is much too simple a view for the process.

SANDER: Just a factual question. Is it unambiguously clear from your crystal structure that the two helices are parallel in tropomyosin?

PHILLIPS: No, but taken with the chemical evidence, which is very strong, I don't think they can be antiparallel. Cross linking experiments indicate that the sulfhydryl groups in the molecule align, so that the two possibilities for the structure are like this (parallel)



or like this (antiparallel)



If it were antiparallel we would expect to see two areas where molecules overlap and indeed we see one area where molecules seem to form an end-to-end joint rather than a staggered structure.

HENDRICKSON: Back to the motion problem. Is it possible to put the crystals in a more viscous medium, for example, glucose, and ...

PHILLIPS: ...to get damping or something like that? That's possible. I have been trying to put them in ethylene glycol for another purpose, to take pictures at lower temperature. That's a good point. Glucose at the same temperature might be an interesting experiment.

HENDRICKSON: I think that the result is not completely obvious. It could be that what you have is a set of disorders rather than an actual motion.

PHILLIPS: Right. We would argue that if it is a set of disorders then you must of course go from one conformation to the other, whether or not we're actually looking at a harmonic vibration or just a set of conformational substates. We don't really have to distinguish to make inferences about the role of tropomyosin in muscle.

F. RICHARDS: You said you were trying to put in glycol, implying that it does not go easily. Are the experiments difficult?

PHILLIPS: The crystals, being 95% solvent, are very labile. They're very easy to disorder both chemically and mechanically.

F. RICHARDS: I have an anonymous referee's question here. "Did you ever attempt to follow the process of disordering of the long arm of tropomyosin in a more quantitative way? It seems to me that by scanning the photographs and plotting the total (or average) intensity of the spike due to scattering from the long arm vs temperature (using the other spike for calibration), the process could be described in a less subjective way, pointing out more accurately the transition temperature. Since at physiological temperatures the long arm will be much more disordered than the short arm, this could have potential implications for the mechanism of action. Could you comment on this point?"

PHILLIPS: We do have pictures at 5 different temperatures. I did try to plot them as a function of temperature, taking the ratio of some of the key intensities in each of the spikes. It was a fairly continuous function. I don't think the accuracy of the data allows the identification of an inflection point or a transition temperature, or of anything of that nature.

F. RICHARDS: I hope all of you are as impressed as I am by this comparison between the fiber people, who are clearly defined as a class, and the single crystal types. The fiber types have minimal data; when even this starts to disappear but reappears as diffuse scattering (which most of us throw away as background), they start to extract information from it. They've done such a successful job that they have now convinced the single crystal people that it is worth looking at the same thing. That's very impressive.