Myosin II Folding Is Mediated by a Molecular Chaperonin*

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The folding pathway of the heavy meromyosin subfragment (HMM) of a skeletal muscle myosin has been investigated by in vitro synthesis of the myosin heavy and light chains in a coupled transcription and translation assay. Analysis of the nascent translation products for folding intermediates has identified a major intermediate that contains all three myosin subunits in a complex with the eukaryotic cytosolic chaperonin. Partially folded HMM is released from this complex in an ATP-dependent manner. However, biochemical and functional assays reveal incomplete folding of the myosin motor domain. Dimerization of myosin heavy chains and association of heavy and light chains are accomplished early in the folding pathway. To test for other factors necessary for the complete folding of myosin, a cytoplasmic extract was prepared from myotubes produced by a mouse myogenic cell line. This extract dramatically enhanced the folding of HMM, suggesting a role for muscle-specific factors in the folding pathway. We conclude that the molecular assembly of myosin is mediated by the eukaryotic cytosolic chaperonin with folding of the motor domain as the slow step in the pathway.

Muscle myosin, the prototype of the myosin II family, is a large asymmetric, multidomain protein composed of six polypeptides: two heavy chains of molecular mass 220 kDa and two pairs of non-identical light chains of molecular mass 17–23 kDa. The amino-terminal ~845 residues of each heavy chain associates with two different light chains to form an elongated, globular domain (S1) that contains the sites for ATP hydrolysis and actin binding (1). This domain is sufficient for powering the movement of actin filaments. The carboxyl-terminal ~1100 residues of the myosin heavy chains associate to form an α-helical coiled-coil rod essential for myosin filament assembly. Crystallographic determination of the S1 structure and electron microscopic studies have contributed a great deal to our understanding of the structure of the molecule and mapping of the functional sites (2, 3). The myosin catalytic domain is a compact structure formed by the amino-terminal ~765 residues of the heavy chain. The ATP-binding pocket in this domain is formed by a structural motif that is shared with the kinesin family of microtubule-based motors and with G-proteins that are involved in signal transduction (4). The core motif is a seven-strand mostly parallel β-sheet that is flanked by three α-helices on each side. A large insertion into the core motif forms the actin binding surface and distinguishes the myosin structure from kinesin and the G-proteins. Extending from the myosin catalytic domain is an 85-A-long α-helix that is stabilized by the binding of two myosin light chains. This domain is believed to act as a lever arm to amplify and transmit structural changes in the catalytic domain into relative motion of actin and myosin (5). The light chain binding helix terminates in a proline that starts the long rod domain.

Despite the extensive knowledge about the structure of the molecule, our understanding of the folding pathway is limited. It must involve the folding of catalytic domain, association of the light chains, and dimerization of the long COOH-terminal α-helical segment of the two heavy chains. Myosin light chains and various rod subfragments can be synthesized in bacteria and fold properly, suggesting an autonomous folding pathway (6–8). However, myosin head fragments of various lengths expressed in bacteria are functionally inactive, suggesting that eukaryotic factors acting post-translationally may be required for folding (9).

It is now well established that, although the folding of a protein is largely directed by its amino acid sequence, this process is often mediated by accessory proteins known as molecular chaperones (10, 11). Chaperonins are a class of molecular chaperones that consist of multisubunit toroidal ring structures and are believed to mediate folding by providing a sequestered environment within the ring (12). The chaperonin containing TCP-1 (CCT) present in the eukaryotic cytosol, is a relatively new member of this class (13). It has been implicated in the folding of actin; α, β, and γ tubulins; and an actin-related protein (14–18). The folding pathways of actin and tubulin are now understood in great detail due to the discovery that the folding of these proteins is mediated by CCT in the eukaryotic cytosol (19).

Several recent studies have helped to elucidate the genetic, structural and mechanistic aspects of this chaperonin (12, 13, 20, 21). One intriguing anomaly of CCT-mediated folding is the apparently limited target range in comparison to the bacterial analog GroEL (22). However, a more general role for CCT in protein folding is slowly emerging. In addition to the substrates already mentioned, CCT has recently been shown to mediate the folding of the G-protein, Gα-transducin (21). CCT is also thought to be involved in the organization of the neuronal cytoskeleton and in hepatitis virus capsid assembly (13).

Here we present an analysis of the folding of the heavy meromyosin subfragment (HMM) of myosin synthesized in a
rabbit reticulocyte lysate and show that it is mediated by CCT. Dimerization of heavy chains and the association of heavy and light chains are accomplished efficiently. However, HMM synthesized in this system lacks a properly folded motor domain, and is defective in its actin binding properties. Thus, in addition to the protein folding machinery available in the reticulocyte lysates, myosin may require additional folding factors. The expression systems that are widely used for non-muscle myosin (5, 23) and smooth (24), and, to a more limited extent, cardiac muscle myosin (25) have not proven useful for the expression of fast skeletal muscle myosin. On the other hand, the mouse myogenic cell line C2C12 has been successfully used for the expression of this myosin isoform (26). Based on this, we hypothesized that the folding of skeletal muscle myosin may require specific folding factors present in muscle cells. Folding assays performed in the presence of C2C12 cytoplasmic extracts, yield significantly more “native-like” protein. This molecular form of HMM displays enhanced folding of the motor domain, improved actin binding properties, and stabilization of the coiled-coil α-helix.

MATERIALS AND METHODS

Coupled Transcription/Translation—The skeletal muscle heavy meromyosin expression plasmid was constructed from a full-length chimeric cDNA corresponding to the epitope tagged embryonic chicken myosin cDNA previously described (28). The HMM cDNA encodes amino acids 1–1293 of myosin and was inserted between the EcoRI and XbaI cloning sites of pGEM4 (Promega Corp., Madison, WI) with the 5’ end of the cDNA adjacent to the plasmid Sp6 promoter (p4HMM). The chicken fast skeletal muscle myosin light chain 2 cDNA (GenBank accession no. M11030) was provided by Dr. Fernando Reinach (27), and LC3 cDNA was provided by Dr. Bruce Patterson (GenBank accession no. J80858). The LC2 and LC3 cDNAs were cloned adjacent to an SP6 promoter into plasmids pEM3 and p73 (Promega Corp.), respectively (p3mLC2 and p73mLC3). The myosin coding regions of the expression constructs were confirmed by DNA sequencing.

The expression plasmids for the HMM heavy chain and the essential light chains (LC3) and regulatory (LC2) light chains were expressed by coupled transcription and translation in the TNT lysate system (Promega Corp.). The reactions were performed as per manufacturer’s protocol, in the presence of 40 μCi/ml of incorporation of [35S]methionine (1175 Ci/mmol; NEN Life Science Products). All translations were carried out at 30 °C for 2 h. The molar ratio of the three myosin expression plasmids was adjusted such that approximately equimolar amounts of myosin heavy and light chains are produced.

Chromatographic Analysis—Translation reactions (200 μl) containing approximately 50 μg HMM and light chains were depleted of ATP and exchanged into column buffer by rapid gel filtration on a Superdex G-50 spin column (Amersham Pharmacia Biotech) equilibrated with 20 mM MES (pH 6.9), 100 mM KCl, 1 mM MgCl2, 0.5 mM EGTA, 2.5% glycerol, 1 mM dithiothreitol (14). The effluent was diluted 5-fold with this buffer and applied to a HR 5/5 Mono-Q column (Amersham Pharmacia Biotech). The bound proteins were eluted with a linear gradient of 20–500 mM MgCl2 over 14 min at a flow rate of 1 ml/min. Column fractions were collected and analyzed by scintillation counting and SDS-PAGE. The myosin heavy and light chain subunits were analyzed by phosphorimaging of the SDS gels.

Fractions from the gel filtration and anion-exchange columns were subjected to SDS-PAGE. The protein gel patterns were transferred to nitrocellulose using a semi-dry blotting apparatus (29), and the nitrocellulose replicas were probed with mAb 91A, a rat monoclonal antibody against TCP-1α subunit (30) (Stressgen Biotechnologies), or anti-S1 mAb 4H7.6 (31). Incubations with primary antibodies were carried out for 4–6 h on ice, followed by 2–4 h of incubation with rabbit anti-rat IgG or mouse IgG secondary antibody (Sigma). The secondary antibody was used in 2-fold excess over the primary antibody.

Native and Denaturing Immunoprecipitation—Native immunoprecipitates were done with either mAb 23C or H2O (control). The immunocomplexes were then incubated with Immunoprecipitin for an additional 15 min. The lysates were clarified by centrifugation and used in coupled transcription/translation assays as described above.

Immunoprecipitation of denatured translation products with anti-S1 mAb 4H7.6 and anti-S2 mAb 10F12.3 was done with translation reaction aliquots equilibrated with 150 mM NaCl, 10 mM MgCl2, 20 mM HEPES (pH 7.5), then denatured by diluting with an equal volume of buffer containing 2% SDS and 12 mM β-mercaptoethanol and boiling for 10 min. After clarification with a brief microcentrifuge spin, samples were diluted with one volume of H2O and eight volumes of buffer containing 1% Triton X-100. Aliquots of the diluted sample were incubated either with buffer or 5 μg of the appropriate antibody. The subsequent processing steps were identical to native immunoprecipitation.

Preparation of Myotube S30 Fraction—Growth and fusion of C2C12 cells has been previously described in detail (26). Well differentiated myotubes, 3–4 days after fusion, were used to prepare an S30 fraction according to a protocol developed by Moldave and Fischer for Chinese hamster ovary cells (32). Myotubes growing on 100-mm dishes were washed with 0.015 M HEPES-NaCl (pH 7.5), 0.12 mM KCl, 5 mM magnesium acetate, and 6 mM β-mercaptoethanol. The postmitochondrial fraction was obtained by centrifugation at 20,000 × g for 20 min at 4 °C.

The final step of the original protocol was modified to use 0.02 μM HEPES-KOH (pH 7.3), 0.015 mM KCl, 0.0015 mM magnesium acetate, 6 mM β-mercaptoethanol. The cells were homogenized by 30–40 strokes in a Dounce glass homogenizer and adjusted to 0.20 μM HEPES-KOH (pH 7.3), 0.12 mM KCl, 5 mM magnesium acetate, and 6 mM β-mercaptoethanol. The postmitochondrial fraction was obtained by centrifugation at 30,000 × g for 20 min at 4 °C. In step involving homogenization of myosin heavy chain in our translations. The gel-filtered S30 fraction was dispensed into 100-μl aliquots, quick-frozen, and stored in liquid nitrogen. Typically, myotubes from 16–20 dishes yielded approximately 2 ml of S30 fraction having an absorbance of 10–12 at 260 units/ml. Removal of endogenous mRNA from the S30 fraction is unnecessary since translation in the TNT assay without added exogenous plasmid resulted in background levels of [35S]methionine incorporation.

Limited Proteolysis of the Folding Reactions—Folding reactions were supplemented with native HMM at 2.5 mg/ml and subjected to limited proteolysis with 25 μg/ml bovine pancreas trypsin (Sigma) at 25 °C. Aliquots were withdrawn at various times, diluted 10-fold into SDS
sample buffer and boiled immediately, then subjected to SDS-PAGE analysis followed by immunoblotting with anti-myc mAb 4H7.6, F59, 1H2.2, and 8G12.5 (31). The proteolytic fragments of nascent HMM were visualized by autoradiography of the immunoblots.

Folding reactions were supplemented with the S30 fraction of a C2C12 myotube extract or an equal volume of S30 buffer and incubated for an additional 60 min at 30 °C to continue folding. Samples were then supplemented with 2.5 mg/ml native HMM, digested with trypsin, and analyzed as already described. For immunoprecipitation analysis, samples from a 30-min time point were diluted 8-fold into SDS sample buffer and boiled for 10 min. After clarification in a microcentrifuge, the samples were diluted 10-fold with S30 buffer containing 1% Triton X-100. Aliquots of the diluted sample were incubated with either PBS or 10 μg of an anti-myc mAb (4H7.6, F59, 1H2.2, or 8G12.5) and then analyzed as described above.

**Actin Binding Assay—**Translations (50 μl) were performed either in rabbit reticulocyte lysate alone or in lysate supplemented with C2C12 extract, in a 1:10 ratio. The samples were eluted on Sephadex G-50 and analyzed by SDS-PAGE and autoradiography, for the free and actin-bound states by 20 min. Typically, a 2-h synthesis results in an incorporation of 10–15% of the added [35S]methionine into protein. It is now recognized that, although the folding of proteins is directed by their primary sequence, in many instances this process is aided by accessory proteins called molecular chaperones. Chaperonin-mediated folding of proteins involves rapid formation of a binary complex of nascent polypeptides with the chaperonin and a slow Mg2+ ATP-dependent release of fully or partially folded protein from this complex (10). Partially folded polypeptides repeat this cycle until folding is complete. In the absence of Mg2+ ATP, polypeptides associated with a molecular chaperone often remain arrested in that form. To investigate if a similar mechanism underlies myosin folding, we depleted Mg2+ ATP from translations containing nascent HMM subunits and analyzed the products by gel filtration chromatography (Fig. 2). The chromatogram showed a major radioactive peak eluting at 11.25–12.25 ml. Native HMM elutes as a sharp peak at 9.25–9.75 ml with an apparent molecular mass that is higher (>2000 kDa) than the calculated mass (340 kDa) due to its highly asymmetric shape. The elution position of nascent HMM on the gel filtration column suggested a more compact shape or the association of HMM with a reticulocyte lysate protein (Fig. 2a). This molecular form, designated as peak I (pI), accounts for approximately a third of the total radioactivity. Two additional peaks were also observed, one of which corresponds to free light chains, and the other to unincorporated [35S]methionine and [35S]methionyl-tRNA complex (data not shown).

The effect of Mg2+ ATP on the folding intermediates was shown by incubating an aliquot with 5 mM Mg2+ ATP prior to gel filtration. There is a dramatic conversion of pI into two new forms, peak II (pII) and peak III (pIII), after incubation with Mg2+ ATP (Fig. 2b). The elution position of pII corresponds to that of the native HMM, whereas pIII elutes in the void volume of the column. Quantitation of heavy and light chains in the different molecular forms showed equimolar ratios of these subunits in pI and pII, whereas the ratio in pIII is skewed toward the heavy chain.

Recent evidence indicates that the folding of actin, tubulin and related proteins in the eukaryotic cytosol is mediated by CCT (14, 16, 17). To investigate if this chaperonin is also involved in myosin folding, the peak fractions were probed with mAb 91A (30), an antibody against TCP-1 α subunit (Fig. 2c). This antibody detected a 60-kDa polypeptide in the fractions corresponding to pI, suggesting that pI may be a complex of HMM with CCT. In the presence of Mg2+ ATP, release of HMM into pII and pIII was accompanied by a decrease of TCP-1 immunoreactivity in pI. In addition, TCP-1 was also detected in pIII, but not pII. In both the cases, CCT subunits were also found in lower molecular mass fractions (120–60 kDa), suggesting disassembly of the CCT complex (36, 37).

It is possible that the co-elution of TCP-1 with HMM in the gel filtration experiment is a consequence of similarities in their size, rather than a true association. To examine this
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Chromatography before translation showed a decrease in the amount of TCP-1 when Mg²⁺ was depleted before translation. When Mg²⁺ was depleted before translation, there is a reduction in the amount of TCP-1 incubated with Mg²⁺. Analysis of these subunits in the peak fractions showed a decrease in TCP-1 in the pI fractions. There is an increase in TCP-1 in the absence of Mg²⁺. The co-elution of HMM with TCP-1 peaks in the pI intermediate as compared with the control (Fig. 5a). This result strongly argues that pI is a complex of HMM with CCT. The overall recovery of nascent HMM from the depleted lysates was very poor. The likely explanation for poor recovery is that, in the absence of CCT, nascent polypeptides become “sticky” as a consequence of exposure to hydrophobic sites; such polypeptides are easily lost during processing by binding irretrievably to plastic and glassware. One might expect that nascent HMM would be trapped as a complex with other lysate factors in the absence of CCT, and would appear as new peaks in the gel filtration assay. Lack of such new intermediates implies that HMM-CCT complex is an essential and early precursor in the folding process and that removal of CCT cannot be compensated by other lysate factors.

CCT requires hydrolysis of ATP. Thus, in the presence of ATPγS, a non-hydrolyzable analogue of ATP, polypeptides should be arrested in the complex. In control experiments performed with reticulocyte lysates in the absence of HMM, immunoprecipitation of CCT by mAb 23C was found to be most effective in the presence of Mg²⁺ ATPγS and least effective in the complete absence of Mg²⁺ ATP (data not shown). Therefore, our native immunoprecipitations were performed in the presence of Mg²⁺ ATPγS. Nascent HMM co-immunoprecipitated by mAb 23C under these conditions, although the efficiency of immunoprecipitation was not as dramatic as with an anti-S1 antibody (Fig. 4). Quantitation by scintillation counting showed that the amount of HMM present in the mAb 23C immunocomplexes was approximately 3 times above the control, and the increase was consistent in independent experiments. Thus, three lines of evidence point to a transient association of nascent HMM with CCT.

The requirement of CCT for HMM folding was addressed by an immunodepletion experiment. Translations were done in lysates that were partially depleted of CCT by immunodepletion, and the translation products were analyzed by gel filtration chromatography for the presence of a binary complex. To affect this analysis, aliquots of reticulocyte lysate were incubated with mAb 23C or with H₂O (control) and the immune complexes removed with Immunoprecipitin. Western blot analysis with mAb 91A showed significantly lower levels of CCT in the lysates treated with 23C, in comparison to the control lysates (Fig. 5a). The overall level of protein synthesis was not affected by the removal of CCT, as judged by the concentration of HMM in unfractionated translation reactions. However, lysates depleted of CCT yielded significantly lower levels of the pI intermediate as compared with the control (Fig. 5b) or untreated lysates (Fig. 2a). This result strongly argues that pl is a complex of HMM with CCT. The overall recovery of nascent HMM from the depleted lysates was very poor. The likely explanation for poor recovery is that, in the absence of CCT, nascent polypeptides become “sticky” as a consequence of exposure to hydrophobic sites; such polypeptides are easily lost during processing by binding irretrievably to plastic and glassware. One might expect that nascent HMM would be trapped as a complex with other lysate factors in the absence of CCT, and would appear as new peaks in the gel filtration assay. Lack of such new intermediates implies that HMM-CCT complex is an essential and early precursor in the folding process and that removal of CCT cannot be compensated by other lysate factors.

FIG. 2. Gel filtration chromatography of nascent HMM. a, Mg²⁺ ATP was depleted before the translation mixture was separated on a Superose 6 gel filtration column. A major radioactive peak (pI) elutes at ~900 kDa (dotted line). SDS-PAGE and phosphoimager analysis of myosin subunits HMM (●), LC2 (●), and LC3 (●) show association of these subunits in the peak fractions. b, when the reaction is incubated with Mg²⁺ ATP before chromatography, there is a decrease in pI radioactivity and the appearance of two new peaks, pII and pIII. c, immunoblotting of column fractions with an anti-TCP-1 mAb 91A detects the 60-kDa TCP-1 subunit in the fractions corresponding to pI in the absence of Mg²⁺ ATP. When samples are incubated with Mg²⁺ ATP before chromatography, there is a reduction in the amount of TCP-1α in the pI fractions. There is an increase in TCP-1α eluting at ~15 ml corresponding to disassembled oligomers.

FIG. 3. Anion exchange chromatography of nascent HMM. A translation mix depleted of Mg²⁺ ATP was applied to a Mono Q column and eluted with a linear gradient of 20–300 mM MgCl₂. The profile shows a major radioactive peak eluting at ~180 mM MgCl₂. SDS-PAGE and phosphoimager analysis (inset) of HMM (●), LC2 (●), and LC3 (●) show the association of HMM with LCs. Immunoblotting of the column fractions (inset) with mAb 91A shows the co-elution of TCP-1α with nascent HMM. The MgCl₂ concentration and the elution position of native HMM are indicated along the top of the figure.
negative and positive controls, respectively. By immunoabsorption with anti-TCP-1 mAb, 23C, and com-
 pared buffer-treated control lysate. The samples incubated with PBS (No 1°) and anti-S1 mAb (anti-S1; 4H7.6) serve as negative and positive controls, respectively.

![Image](98x254 to 248x495)

**FIG. 4.** Immunoprecipitation of HMM-CCT complex with anti-TCP-1 mAb. A translation mix depleted of Mg²⁺ ATP was incubated with 5 mM Mg²⁺ ATP-S and mAb 23C (anti-TCP-1) under native conditions. The immune complexes were isolated with immunoprecipitin, and the supernatant (s) and precipitate (p) fractions were analyzed by SDS-PAGE and autoradiography to detect HMM. The samples incubated with PBS (No 1°) and anti-S1 mAb (anti-S1; 4H7.6) serve as negative and positive controls, respectively.

![Image](311x631 to 550x729)

** FIG. 5.** Aliquots of reticulocyte lysates were depleted of CCT by immunoabsorption with anti-TCP-1α mAb, 23C, and compared with buffer-treated control lysate. a. Western blots reveal a significant depletion of CCT in the antibody-treated lysate (Ab) versus untreated (u) and buffer-treated (B) lysates. Samples were resolved on an 8% gel and probed with mAb 91A. The untreated lysate was loaded five times in excess of the antibody-treated (Ab) and buffer-treated (B) lysates. Autoradiography of the translation products resolved on SDS-PAGE shows that the levels of HMM synthesis in immunoabsorbed and buffer-treated lysates are comparable. b. The gel filtration elution profile of the lysate depleted of CCT (C) compared with the control buffer-treated (B) lysate shows a remarkable decrease in the pl intermediate. There is also a significant decrease in the recovery of radioactivity from the CCT-depleted lysate despite comparable levels of total synthesis.

![Image](317x577)

We conclude from these observations that folding of HMM in rabbit reticulocyte lysate occurs via the formation of a complex with CCT (pII); it is released from this complex into two different conformational states (pII and pIII) in an ATP-dependent manner. One of the intermediates, pII, behaves as a native protein in the gel filtration assay and does not reassociate with CCT. The second intermediate, pIII, elutes as a high molecular mass intermediate and is recaptured by CCT. The association of heavy and light chain subunits is accomplished early in the folding pathway, since light chains are found in all the intermediates.

**Characterization of the Folding Intermediates—Resistance to proteolytic digestion** is a hallmark of properly folded proteins, so limited proteolysis is often used to investigate the presence of folded domains. The myosin motor domain has been studied extensively using proteolysis. Limited proteolysis of S1 by trypsin produces three distinct fragments of molecular mass 25, 50, and 20 kDa (38). The molecular basis of this cleavage pattern is clear from the structure of myosin S1 (3). The cleavage occurs in two extended, flexible loops that are sensitive to a wide range of proteases. The formation of the fragments produced by cleavage of the loops can be considered a measure of myosin folding.

The digestion patterns of native and nascent HMM were compared by limited proteolysis of a mixture of these two proteins (Fig. 6). Native HMM produced the characteristic 50-, 25-, and 20-kDa fragments from the motor domain and a 52-kDa fragment corresponding to S2. These fragments were identified against the background of reticulocyte lysate proteins by probing Western blots of the digest with mAbs that recognize these fragments. Autoradiography of the blots revealed that nascent HMM in the same digestion mix generated fragments of 68, 55, 45, and 30 kDa. Thus, nascent HMM displays discrete protease-resistant fragments, indicative of folding; however, the lack of overlap between the nascent and native fragments indicates a significant difference in the extent of folding of the nascent HMM.

![Image](398x569)

**FIG. 6.** Limited proteolysis of nascent HMM to analyze the extent of folding. A mixture of native HMM and nascent HMM was digested with trypsin. Samples withdrawn at 0, 5, and 30 min were analyzed by SDS-PAGE. Native panels represent immunoblots of the gels probed with anti-25-kDa (4H7.6), anti-50-kDa (F59), and anti-20-kDa (IH2.2), and anti-S2 (8G12.5) (31, 35). The arrows indicate the positions of these fragments. The panels labeled Nascent are autoradiographs of the immunoblots and show the radioactive proteolytic fragments of nascent HMM. Nascent HMM displays 68-, 55-, 45-, and 30-kDa fragments in contrast to the characteristic 50-, 25-, and 20-kDa fragments of the native protein. The distinct digestion pattern of nascent HMM is indicative of partial folding.

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Native HMM molecules consist of dimers of the heavy chains associated through a long α-helical coiled-coil interaction. We analyzed the folding of S2 region using anti-S2 mAb 10F12.3, a conformation-sensitive antibody that preferentially binds S2 in its coiled-coil conformation (39, 40). In Western blot analysis, this antibody reacts strongly with native HMM resolved on native gels, but shows very weak recognition of the same fragment blotted from denaturing gels (data not shown). The immunoprecipitation of nascent HMM was analyzed with this antibody under native and denaturing conditions (Fig. 7). Nascent HMM formed stable immunocomplexes with mAb 10F12.3 under native conditions, suggesting that the S2 region is in the native dimer conformation. Quantitation of HMM associated with 10F12.3 under native conditions suggests that at least 30% of the nascent population exist as dimers. The absence of significant precipitation of HMM by 10F12.3 under denaturing conditions confirms that this antibody is sensitive to conforma-
Depleted of Mg\(^{2+}\) ATP and immunoprecipitated with anti-S2 mAb (anti-S2; 10F12.3) under native and denaturing conditions. The samples were analyzed by SDS-PAGE. The presence of nascent HMM in 10F12.3 immunocomplexes only under native conditions is indicative of S2 folding. Fractions for each case were analyzed by SDS-PAGE. The presence of native dimer conformation in at least a third of the nascent subunits HMM (●) and pellet (p) fractions for each case were analyzed by SDS-PAGE. The presence of nascent HMM in 10F12.3 immunocomplexes only under native conditions is indicative of S2 folding.

**Fig. 7.** Investigation of nascent S2 domain using a conformation-sensitive antibody, mAb 10F12.3. a, a translation mix was incubated with PBS (No 1°) and mAb 4H7.6 (anti-S1) serve as negative and positive controls, respectively. The supernatant (s) and pellet (p) fractions for each case were analyzed by SDS-PAGE. The presence of nascent HMM in 10F12.3 immunocomplexes only under native conditions is indicative of S2 folding.

**Fig. 8.** C2C12 myotube extracts enhance folding of nascent HMM. A translation mix depleted of Mg\(^{2+}\) ATP was incubated with an S30 fraction from C2C12 myotubes, and the products were resolved on Superose 6 column. The major radioactive peak in this case corresponds to pII of Fig. 2b. SDS-PAGE and PhosphorImager analysis of myosin subunits HMM (●), LC2 (Δ), and LC3 (▲) show association of these subunits in an approximately 1:1:1 ratio.

Reactions showed a stable 75-kDa fragment, which co-migrates with the same sized fragment derived from native HMM (Fig. 9a). In addition, the supplemented digest showed a 68-kDa fragment, similar to that seen in the unsupplemented digest (Fig. 6). Immunoprecipitation of the proteolytic fragments with anti-myosin antibodies, showed that an anti-25-kDa mAb (4H7.6) and an anti-50-kDa mAb (F59) both recognize the 75-kDa fragment in digests of the supplemented folding reaction (Fig. 9b), indicating that it is a composite of the NH\(_2\)-terminal 25- and 50-kDa fragments of the myosin motor domain. The anti-S2 mAb (8G12.5) recognized the 68-kDa fragment in both digests. This fragment probably arises from the S2 region together with the 20-kDa fragment of myosin S1. The presence of this fragment in both digest confirms our previous result regarding S2 dimerization. However, the recovery of this fragment from the supplemented digest was significantly higher, suggesting that the S30 extract further stabilizes the S2 region.

**Functional Assay**—Finally, we tested the effect of the S30 extract on the functional properties of nascent HMM. Generation of motion involves cyclic binding and release of actin filaments by myosin. In the absence of Mg\(^{2+}\) ATP myosin binds actin with high affinity; the binding of Mg\(^{2+}\) ATP to myosin induces conformational changes leading to a low affinity state of myosin for actin (41). Folding reactions were done either in lysate supplemented with the S30 extract or in reticulocyte lysate alone. Nascent HMM from these reactions was analyzed for its ability to bind actin in the absence of Mg\(^{2+}\) ATP, and release from actin in the presence of Mg\(^{2+}\) ATP. Native HMM in the presence of Mg\(^{2+}\) ATP was released from actin in an Mg\(^{2+}\) ATP-dependent manner (data not shown). These results suggest that the myotube cytoplasmic extract contains a factor or factors that enhance the folding of nascent HMM. The HMM formed in the presence of the extract exhibits improved folding of the myosin motor domain, stability of the S2 region, and actin binding.

**DISCUSSION**

**Chaperonin-mediated Folding of HMM**—There has been dramatic progress in the past 10 years on the structure and
function of myosin, the characterization of the motor activity, and the identification of a large number of new myosin family members (42–44). In contrast to this progress, very little is known about the myosin folding pathway. In this paper we present the first analysis of the folding of a de novo synthesized myosin subfragment, HMM. We demonstrate a folding pathway that, in a reticulocyte lysate, is mediated by CCT, yielding a dimerized molecule with associated subunits but lacking a fully folded motor domain. We further show that specific factors present in a muscle cytoplasmic extract can enhance the folding of the motor domain.

Analysis of nascent HMM by gel filtration and anion exchange chromatography and immunoprecipitation demonstrates transient association with CCT. The elution of the CCT/HMM complex on the gel filtration column is unusual considering its large mass and may suggest an interaction of the hydrophobic folding intermediate with the Superose 6 column media. The ATP-dependent release of HMM from the complex as at least two new intermediates, including a minor component with hydrodynamic properties similar to native HMM, is shown by the gel filtration analysis. The pIII intermediate behaves as an aggregate, and the association with CCT suggests that it is a partially folded species that may re-enter the folding pathway. The association of heavy and light chain subunits in all the intermediates suggests that subunit interactions are an early event in the folding pathway. Immunodepletion of CCT from the translation lysate has a dramatic effect on the recovery of the HMM heavy chain, suggesting that CCT association is a necessary step in the folding pathway.

Based on these results, we conclude that folding of HMM in rabbit reticulocyte lysate involves a cyclic reaction between HMM and CCT driven by ATP hydrolysis. In the absence of ATP, the CCT exists in a high affinity conformation for the nascent HMM molecules resulting in the formation of a CCT/HMM complex. Binding of ATP to this complex induces a low affinity conformation and releases native or partially folded HMM (21). In the analysis of the ATP-dependent release (Fig. 2), we detected disassembly of the CCT complex. Nucleotide-dependent disassembly of CCT has been reported and may represent a novel feature of this class of chaperonin (36, 37). Assembly dynamics of this type could facilitate the binding of highly asymmetric molecules like myosin by the CCT complex, thus, obviating the conceptual constraint imposed by models requiring entry of asymmetric proteins into a narrow cavity.

We investigated the role of chaperones other than CCT, in the folding of HMM. Immunoprecipitation assays showed that, in addition to CCT, nascent HMM also associates with Hsp70 (data not shown). It has been proposed that the molecular crowding inside cells necessitates a vectorial folding mechanism

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**Fig. 9. Analysis of nascent HMM produced in the presence of S30 extract.**

- **a.** Nascent HMM from folding reactions with and without the S30 extract was mixed with native HMM and digested with trypsin for various times. The fragments were analyzed by SDS-PAGE and autoradiography. Panels labeled Native show the Coomassie Blue staining of the native HMM fragments, and those labeled Nascent are autoradiographs revealing nascent fragments. In reactions containing the S30 extract (+), nascent HMM produces a stable 75-kDa fragment, which co-migrates with the same sized fragment produced by native HMM. In addition, it also shows two fragments in the size range 68–66 kDa, which corresponds to the 68-kDa fragment produced from nascent HMM even without the S30 extract (−). The proteolytic fragments from folding reactions with (+) or without (−) the S30 extract were immunoprecipitated with antibodies against the 25-, 50-, and 20-kDa fragments of myosin complexes were incubated either with apyrase to deplete ATP (−ATP) or with Mg2–ATP added (+ATP). The F-actin was collected by centrifugation, and the supernatant (s) and pellet (p) fractions were analyzed by SDS-PAGE for the free and actin-bound HMM. Total HMM produced in the translation reactions is indicated (t). Nascent HMM produced in the reticulocyte lysate alone (−) binds actin poorly irrespective of ATP. In contrast, HMM produced in the lysate supplemented with extract binds actin efficiently in the absence of ATP. Addition of ATP results in a partial release of this species from actin.
(45, 46). Small chaperones such as Hsp70 bind elongating nascent polypeptide chains in an extended conformation that then associate with a second chaperone such as CCT to complete the folding process. Given the large size of myosin and the discontinuous nature of its folding domains, it is likely that such a hierarchical mechanism is operative in myosin folding.

**Slow Steps in the Folding Pathway and Possible Role of CCT**—The domain structure of the myosin molecule raises interesting questions concerning the rates of folding of the different parts of the molecule and the nature of its interaction with CCT. Investigation of the S2 region suggests that at least 30% of the nascent population exists as dimers. Moreover, proteolysis of nascent myosin containing a full-length heavy chain produces two stable fragments corresponding to the LMM and S2 fragments of native myosin (data not shown). Given the structural stability of an α-helical coiled-coil, it is likely that the S2 region in HMM and the rod in myosin fold spontaneously in solution. This notion is supported by the observation that myosin rod fragments expressed in *Escherichia coli* adopt a native coiled-coil conformation (6, 7).

Myosin light chains are also not good candidates for the target of the CCT. Light chains are readily expressed in bacteria and, once purified, can be exchanged into native myosin with full activity (8). Chimeric myosin containing native heavy chains and nascent light chains expressed in vitro similarly shows native properties in all our assays (data not shown), suggesting complete folding of the light chains. Furthermore, we have shown that myosin heavy and light chain subunits are found associated in all of the folding intermediates, suggesting that their association occurs early and is independent of complete heavy chain folding. In support of this, inactive myosin subfragments expressed in prokaryotic systems have been reported to undergo stoichiometric association of heavy and light chain subunits as well (9). The light chain binding domain of myosin constitutes an extended hydrophobic α-helix. Myosin head fragments stripped of light chains tend to aggregate around this region (47). Thus, light chain binding may be necessary to prevent aggregation of the heads during dimerization and, in a sense, light chains may be acting as chaperones.

Folding of the motor domain appears to represent the kinetic barrier in the folding pathway, whereas the S2 region and the light chains and their binding region fold efficiently. Folding analysis of the S1 subfragment, completely lacking a rod domain, shows that it also transits through intermediates similar to those of HMM (data not shown). Thus, the motor domain of HMM is the most likely target for CCT. In conjunction with this, a putative release sequence, RKAcTIF, is found at or near the COOH terminus of the CCT target proteins actin and tubulin (48, 49). A homologous sequence is found at the end of the myosin catalytic domain (R$^{708}$K$^{709}$G$^{710}$P$^{711}$) just preceding the light chain binding region. This sequence is found in all striated muscle myosin II family members at this same location. The non-muscle and smooth muscle myosin II family members also have the release sequence (RKAF), but it is located in the light chain binding helix rather than at the end of the catalytic domain. The sequence is involved in the interaction of the NH$_2$-terminal domain of the essential light chain with the myosin heavy chain, thus masking this site in non-striated myosin II family members (50). Coincidentally, these myosins have been far easier to express in heterologous expression systems (24, 50).

The core structure of the myosin catalytic domain is a nucleotide binding motif consisting of two groups of helices packed against a hydrophobic β-sheet. This core motif is shared among the myosin and kinesin families of motor proteins and a group of G-proteins including G$_a$-transducin (4). Unlike myosin, kinesin and small G-proteins (e.g. Ras) are functionally expressed in bacteria (51, 52). Myosin has large insertions extending from the core motif that distinguish it from kinesin and the small G-protein, perhaps accounting for this difference. Expression of some larger G-proteins, such as G$_a$-transducin, has been difficult in bacteria, and this has been correlated with insertions into the core motif (53). Furthermore, CCT has recently been implicated in the G$_a$-transducin folding pathway (21).

**Effect of C2C12-S30 Fraction on the Folding of HMM**—The *in vitro* analyses demonstrate that CCT is a necessary, but not sufficient factor in HMM folding. To isolate other factors necessary for the complete folding of myosin, we prepared an S30 fraction from C2C12 myotubes. Folding reactions performed in the presence of the S30 extract yield significant amounts of an intermediate that behaves as a native protein in the gel filtration assay. Analysis of this species by limited proteolysis and immunological assays reveal enhanced folding of the NH$_2$-terminal 75-kDa myosin catalytic domain and improved folding or stability of the S2 region. Significant improvement in the actin binding by nascent HMM produced in the presence of the S30 extract is also indicative of enhanced folding. Partial release of this species from actin by ATP suggests that a fraction of the population has attained a fully native conformation.

The myotube extract appears to supply factors that are missing or limiting in the reticulocyte lysate. For example, the extract may supply the right isofrom of a CCT subunit that is limiting in the reticulocyte lysate. In this context, the CCT subunit TCP-1y is developmentally regulated in muscle cells (54). All of the CCT subunits identified so far possess conserved ATPase domains and differ from each other in the putative peptide binding domains (13). Thus, it is possible that different subunits of CCT or isoforms of TCP-1 have evolved for the folding of different proteins, and perhaps even co-evolved with the target proteins. Alternatively, additional factors acting before or after CCT may be required for complete folding of myosin as has been described for the folding of α and β tubulin (55–57).

Currently, there is wide speculation regarding the range of substrates for CCT. Discovery of new targets for this chaperonin will certainly improve our understanding of the general mechanism of chaperonin-mediated protein folding *in vivo*. The unique structure of myosin also raises important questions concerning its interaction with the chaperonin, and the hierarchy of events during folding. The investigation of the folding pathway *in vivo* and the isolation and purification of the folding factors from muscle extracts are necessary for a complete description of the myosin II folding pathway.

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