Data from affinity chromatography, analytical ultracentrifugation, covalent cross-linking, and fluorescence anisotropy show that profilin, thymosin \( \beta_4 \) and actin form a ternary complex. In contrast, steady-state assays measuring F-actin concentration are insensitive to the formation of such a complex. Experiments using a peptide that corresponds to the N terminus of thymosin \( \beta_4 \) (residues 6–22) confirm the presence of an extensive binding surface between actin and thymosin \( \beta_4 \), and explain why thymosin \( \beta_4 \) and profilin can bind simultaneously to actin. Surprisingly, despite much lower affinity, the N-terminal thymosin \( \beta_4 \) peptide has a very slow dissociation rate constant relative to the intact protein, consistent with a catalytic effect of the C terminus on conformational change occurring at the N terminus of thymosin \( \beta_4 \). Intracellular concentrations of thymosin \( \beta_4 \) and profilin may greatly exceed the equilibrium dissociation constant of the ternary complex, inconsistent with models showing sequential formation of complexes of profilin-actin or thymosin \( \beta_4 \)-actin during dynamic remodeling of the actin cytoskeleton. The formation of a ternary complex results in a very large amplification mechanism by which profilin and thymosin \( \beta_4 \) can sequester much more actin than is possible for either protein acting alone, providing an explanation for significant sequestration even if molecular crowding results in a very low critical concentration of actin in vivo. 

The amount of unpolymerized actin in many cells is large. Several actin-monomer sequestering proteins have been identified that are responsible for maintaining this pool, and attempts have been made to account for the quantity of unpolymerized actin by calculation of the sum of sequestered actin in cells (1–3). These calculations depend not only on the concentration of each sequestering protein and its equilibrium dissociation constant for actin, but also on several other parameters, including an estimate of the critical concentration of actin (i.e. the amount of unpolymerized, unsequestered actin), the stoichiometry with which the sequestering proteins bind to actin, the potential qualitative and quantitative effects should different sequestering proteins interact simultaneously with a single actin subunit, and on assumptions regarding the effects of cytoplasmic molecular crowding on equilibrium association constants. Not surprisingly, with so many parameters to evaluate, even small quantitative errors in measurement or qualitative errors in mechanism result in a wide range of plausible estimates of total sequestered actin. In this report we investigate assumptions that have very significant effects on predictions related to the amount of sequestered actin, finding significant discrepancies with previously reported results, and discuss the expected consequences of these observations.

Based on assays measuring steady-state F-actin levels and the failure to obtain a covalently cross-linked ternary complex, thymosin \( \beta_4 \) and profilin have been reported to bind competitively to actin (4–6). These results were consistent with data that suggested that the N terminus of thymosin \( \beta_4 \) and profilin both bind to similar locations in subdomain 1 of actin (7–9). The assumption of competitive binding between thymosin \( \beta_4 \) and profilin is significant because it underlies models of actin polymerization that show profilin and thymosin \( \beta_4 \) sequentially interacting with actin (10). Also, this assumption has been used as the basis for the analysis of data concerning putative functions of profilin other than monomer sequestration (5, 6, 11). However, the recent evidence that the surface of interaction between thymosin \( \beta_4 \) and actin is extensive, involving both subdomains 1 and 2 of actin (12, 13), implies that there is no structural basis for competitive binding between these actin-binding proteins, and has lead us to re-investigate the hypothesis that profilin and thymosin \( \beta_4 \) could form a ternary complex with actin. Additional support for this hypothesis is provided by data showing that some variations in the C terminus of thymosin \( \beta_4 \) alter actin affinity and explain, in part, the differences in actin binding activity of \( \beta \)-thymosin isoforms (14, 15). Such data imply that while binding of the N terminus of thymosin \( \beta_4 \) to actin may present a steric hindrance to profilin-actin interactions, additional interactions between the C terminus of thymosin \( \beta_4 \) and actin could be independent or even augmented by profilin-actin interaction.

While thymosin \( \beta_4 \) is mostly or completely random coil in aqueous solution, much evidence suggests that the N terminus (residues 5–16) binds to actin in an \( \alpha \)-helical conformation (16–19). Trifluoroethanol stabilizes the \( \alpha \)-helical conformation and enhances actin binding affinity in a manner consistent with the postulated change in entropy (18). De La Cruz et al. (13) have recently investigated the thermodynamics of the interaction between thymosin \( \beta_4 \) and actin, and report evidence that the conformational transition may also involve changes in actin conformation. The molecular interactions of unfolded proteins, such as thymosin \( \beta_4 \) with ligand have been proposed by Wright and Dyson (20) to provide regulatory control over critical cellular functions, and indeed, in addition to its actin-binding properties, thymosin \( \beta_4 \) exhibits complex biological activities related to immunosuppression and hematopoiesis (21–23). Presumably these diverse signaling pathways require multiple binding partners, a situation made possible by the potential of thymosin \( \beta_4 \) to adopt induced structural motifs...
selective and specific for diverse ligands. In the current report, we describe observations regarding the rate and mechanism by which thymosin \( \beta_4 \) may effect these conformational transitions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit skeletal muscle Ca\(^{2+} \)-actin was prepared from frozen muscle (Pel-Freez, Rogers, AR) in buffer G (5.0 mM Tris-HCl, 0.2 mM ATP, 1.2 mM dithiothreitol, 0.1 mM CaCl\(_2\), and 0.01% sodium azide, pH 7.8), and pyrenyl-labeled actin was prepared with 0.7 to 0.95 mol of label/mol of protein using the method of Koyama and Mihashi (24). Recombinant human profilin I, rat profilin I, and rat thymosin \( \beta_4 \) were purified as previously described (25, 26). The rat profilin was mutated to have a cysteine at position 41 (C4IS) (27) and the thymosin \( \beta_4 \) contained an added C-terminal cysteine. Notably, rat and human thymosin \( \beta_4 \) contained identical amino acid sequences (28). The cysteine-modified proteins were labeled as previously described with tetrarmethy rhodamine-5'-maleimide. Previous results confirm that the labeled, mutant profilin has identical actin binding properties as wild-type profilin (27), and after depolymerization in buffer G, the concentration was determined by absorbance at 280 nm (the absorption maximum for rhodamine-labeled proteins or peptides) and 315 nm.

**Steady-state Fluorescence Assays**—A 10 or 15 \( \mu \)M stock of actin (4% pyrenyl-labeled) was converted to Mg\(^{2+} \)-actin by the addition of 125 \( \mu \)M EGTA and 50 \( \mu \)M MgCl\(_2\). In assays with capped F-actin, gelosin was added at a ratio of 1/200. After 10 min, MgCl\(_2\) and KCl were added to final concentrations of 2.0 and 40 mM, and the actin was added to 10 min to 60 mM, and the actin was then diluted to 45 \( \mu \)M for thymosin \( \beta_4 \). Excluded volume experiments included 6% PEG 8000 in the F-actin stock solution and dilution buffer. The samples were incubated for 20–24 h at 22 °C, and steady-state readings were obtained in a spectrofluorimeter with excitation 365.6 and emission 386.6 nm. Steady-state measurements of intrinsic fluorescence were done with Ca\(^{2+} \)-actin in buffer G as previously described (30) except that excitation and emission wavelengths were 284 and 315 nm.

**Affinity Chromatography Assay**—Actin, profilin, and thymosin \( \beta_4 \) were mixed in buffer G at concentrations of 29, 29, and 88 \( \mu \)M, respectively, and loaded on a polypropylene affinity column (25) equilibrated with buffer G. After elution of the unbound material, the column was washed, and the bound profilin and its complexes were eluted with elution buffer containing 4 mM urea. Fractions were collected and analyzed for presence of thymosin \( \beta_4 \) by quantitative ELISA. The ELISA employed a monoclonal IgG prepared against a peptide corresponding to the C terminus of thymosin \( \beta_4 \) (residues 30–43) which was specific for thymosin \( \beta_4 \) by Western blot.

**Covalently Cross-linked Profilin-Actin Complex**—Covalently linked profilin-actin complex was made as described by Gutsche-Pereholzen et al. (31) with some modifications. After incubation of actin and rat profilin with 2 mM 1-ethyl-3-(3-dimethylamino propyl)-carboximidide and 2 mM sulfo-N-hydroxy succinimidyl carbonate, 50 mM MgCl\(_2\), 50 mM KCl, and 7.3% PEG for 4 h at 4 °C. The sample was pelleted at 150,000 g for 1 h. The resulting pellet contained ~80% cross-linked complex and after depolymerization in buffer G, the concentration was determined using \( \epsilon_{350} = 34.9 \) mM\(^{-1}\) cm\(^{-1}\).

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were performed using absorption optics with data collected at 534 nm (the absorption maximum for rhodamine-labeled proteins or peptides) in a Beckman Optima L-90 in a SW41 angular sector at 4 °C and 140,400 rpm for 48–72 h. Samples for experiments designed to detect a ternary complex contained a mixture of 22 \( \mu \)M profilin, 9.5 \( \mu \)M actin, and 7.5 \( \mu \)M labeled thymosin \( \beta_4 \) or 25 \( \mu \)M thymosin \( \beta_4 \), 8.5 \( \mu \)M actin, and 8.5 \( \mu \)M labeled profilin in buffer G with 20 mM KCl. Samples for detection of thymosin \( \beta_4 \) binding to covalently cross-linked profilin-actin complex contained 3.4 \( \mu \)M labeled thymosin \( \beta_4 \) or with or without 13.5 \( \mu \)M pure protein (as described above), also in buffer G with 20 mM KCl. Buffer density and partial specific volumes were determined as previously reported (26). The data analysis employed implicit constraints as previously described (26, 32). Only the labeled protein or peptide contributes to the observed gradient at 534 nm so that the absorbance at any radius is proportional to the sum of the molar concentrations of all species present. In a model of competitive binding, these species include only labeled protein and labeled protein bound to actin. In a model that includes the formation of ternary complex, the sum also includes the molar concentration of ternary complex. Equilibrium conditions are assumed to be satisfied at all radii (32). The analysis is constrained by the initial concentration of each component, and the data are fit to parameters including the concentration of each component at an arbitrary radius and the equilibrium association constants for each allowed interaction. Error estimates were based on an analysis of the sum of squares of deviations as a function of \( K_d \) (33).

**Fluorescence Anisotropy**—Fluorescence anisotropy measurements and analysis were conducted as previously described (26, 34). All anisotropy experiments utilized labeled thymosin \( \beta_4 \) at a concentration of 0.1 or 0.2 \( \mu \)M. Titrations were conducted with Mg\(^{2+} \)-actin in buffer G converted to Mg\(^{2+} \) by addition of EGTA and MgCl\(_2\) to final concentrations of 0.125 and 0.05 mM, respectively, and after 10 min, KCl was added at a final concentration of 40 mM. Excitation and emission wavelength were 546 and 568 nm, respectively.

**Nucleotide Exchange**—Free ATP was removed by dialysis against buffer containing an amount of ATP equal to that of the actin concentration. Actin (1.7 \( \mu \)M), profilin, thymosin \( \beta_4 \), or thymosin \( \beta_4 \) peptide were incubated in a 300-\( \mu \)l glass cuvette with buffer G without ATP. For experiments using the thymosin \( \beta_4 \) peptide, 10 \( \mu \)M Tris (instead of 5 mM Tris), was used to ensure that the solution was well buffered. Actin was converted to Mg\(^{2+}\)-actin by the addition of 125 \( \mu \)M EGTA and 50 \( \mu \)M MgCl\(_2\). After 10 min, ATP was added to a final concentration of 10 \( \mu \)M to start reaction. After mixing, samples were placed in a spectrofluorimeter and the time course of fluorescence changes was recorded. Exchange rates were obtained by fitting the time course to single exponential or two exponential algorithms (see “Appendix”). Microlab Origin 5.0 Professional Edition (Northampton MA) was used for fitting the experimental data.

**RESULTS**

**Steady-state Fluorescence Assay with Capped Actin Filament Barbed Ends Does Not Distinguish between Competitive and Independent Binding of Thymosin \( \beta_4 \) and Profilin to Actin**—Data for the steady-state F-actin concentration for profilin alone or thymosin \( \beta_4 \) alone were best fit with equilibrium dissociation constants of \( K_{DP} = 0.59 \pm 0.04 \) \( \mu \)M for profilin binding to actin and \( K_{DA} = 0.79 \pm 0.02 \) \( \mu \)M for thymosin \( \beta_4 \) binding to actin.
binding to actin (Fig. 1). When profilin and thymosin $\beta_4$ were mixed together, the data did not distinguish between models of competitive and independent binding. Previous investigators may have assumed that independent binding would result in competitive and independent binding. Previous investigators mixed together, the data did not distinguish between models of profilin (downsloping hatch marks; first bar), and a mixture of actin and thymosin $\beta_4$ without profilin (downsloping hatch marks; second bar). Two experiments using a mixture of all three proteins are shown with cross-hatching (third bar) and shading (fourth bar). The profilin peak was in fractions 40 to 43 (not shown). B, using rhodamine label as a chromatophore, 22 $\mu$m profilin, 9.5 $\mu$m actin, and 7.5 $\mu$m labeled thymosin $\beta_4$ (circles) or 25 $\mu$m thymosin $\beta_4$, 8.5 $\mu$m actin, and 8.5 $\mu$m labeled thymosin (squares) were mixed and allowed to reach sedimentation and chemical equilibrium. The gradients, measured by optical density as a function of radius, are directly proportional to the concentration of labeled protein. The data for labeled thymosin $\beta_4$ (solid lines) and labeled profilin (dashed lines) were fit globally to equilibrium dissociation constants for profilin-actin and thymosin $\beta_4$-actin assuming no ternary complex (the poorly fitting lines) and allowing for ternary complex (the better fitting lines). Difference plots for the theoretical curves that assume ternary complex are shown in the middle panel for labeled thymosin $\beta_4$ and in the top panel for labeled profilin. C, fluorescence anisotropy assay showing titration of 0.1 $\mu$m solutions of labeled thymosin $\beta_4$ with actin in of 0 (squares), 2 (circles), 6 (upward triangles), 12 (downward triangles), and 18 (diamonds) $\mu$m profilin. Inset, fluorescence anisotropy assay showing incomplete displacement of thymosin $\beta_4$ from actin by profilin. Labeled thymosin $\beta_4$ was titrated with unlabeled profilin (open symbols) in the presence of 3 (squares) or 1 $\mu$m (triangles) of actin. Labeled thymosin $\beta_4$ was titrated with unlabeled thymosin $\beta_4$ in the presence of 3 $\mu$m actin (closed symbols; circles, squares, and triangles show three separate sets of data). Solid lines represent the best fit to all of the data in C for profilin and labeled thymosin $\beta_4$ assuming the possibility of formation of ternary complex, and the dashed lines represent a competitive binding model. D, data from C (closed symbols), and C, inset (open symbols), are re-plotted showing the dependence on the sum of profilin and actin concentrations, along with the results of global fitting assuming the presence of ternary complex.

For Existence of Ternary Complex—Thymosin $\beta_4$ co-elutes with profilin from a polyproline column only when actin, profilin, and thymosin $\beta_4$ are all present (Fig. 2A). A control with profilin and thymosin $\beta_4$ shows that the interaction of thymosin $\beta_4$ and profilin is specifically mediated by actin, and another control with actin and thymosin $\beta_4$ show that thymosin $\beta_4$ does not remain on the column because of a nonspecific interaction of polyproline with actin. Actin has been previously shown to bind well to profilin that is noncovalently bound to polyproline, and in fact, this is a method used by others to purify non-muscle actin (30, 35). The explanation most consistent with these

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results is that specific interactions between thymosin $\beta_4$ and actin, actin and profilin, and profilin and polyproline can all occur simultaneously, thus providing qualitative evidence for the existence of a ternary complex.

Sedimentation experiments were performed that took advantage of the labeled derivatives of profilin and thymosin $\beta_4$ (Fig. 2B). The resulting gradients reflect the increase in molecular weight that occurs when the labeled protein binds to actin. If profilin and thymosin $\beta_4$ bound to actin competitively and there was a sufficient amount of unlabeled protein present, then the labeled protein would have behaved as though entirely monomeric, and the gradient would have been nearly flat (see example in Fig. 4). Because actin is not completely saturated with unlabeled protein, the theoretical gradients in Fig. 2B for competitive binding are not flat, but they are significantly more shallow than predicted for noncompetitive binding, and more shallow than the actual data. The best global fit to the data were obtained with $K_{d_{AP}} = 0.6 \pm 0.1 \mu M$ and $K_{d_{PT}} = 0.7 \pm 0.2 \mu M$, with equilibrium dissociation constant for thymosin $\beta_4$ binding to profilin-actin complex, $K_{d_{PT}} = 8.1 \pm 2.4 \mu M$. Individual gradients for either labeled profilin or labeled thymosin $\beta_4$ could not alone distinguish between competitive and noncompetitive binding because, for example, a combination of higher $K_{d_{AP}}$ and lower $K_{d_{PT}}$ allowed for a good fit to the data for labeled thymosin $\beta_4$. In combination, however, the data excluded such a possibility because this change in equilibrium constants worsened the fit to the data for labeled profilin. The analysis assumed that the equilibrium dissociation constants were not affected by the attached label (26, 27, 29). The free actin concentration at the base of the centrifuge cell did not exceed 0.6 $\mu M$ for any of the theoretical gradients, a concentration that is well below the critical concentration of actin in this buffer (36).

We previously reported that the fluorescence anisotropy of labeled thymosin $\beta_4$ increased from $-0.08$ when free to 0.18 when saturated with actin (26). Fig. 2C shows the titration of labeled thymosin $\beta_4$ with actin at various, fixed concentrations of profilin. In the presence of profilin, it would take more actin to saturate thymosin $\beta_4$ with actin if the binding is competitive than if it is noncompetitive, because, if binding is competitive, then the more profilin, the less free actin there is available to bind to thymosin $\beta_4$. Fig. 2C shows that the data better reflect noncompetitive binding. If binding is competitive, then when a mixture of actin and fluorescent thymosin $\beta_4$ is titrated with profilin, the anisotropy will return to baseline (0.08) with saturating amounts of profilin. Instead, a residual amount of complex is observed (Fig. 2C, inset) at high concentrations of profilin, consistent with formation of a ternary complex. The results of global fitting for all of data for both actin and profilin titrations yields $K_{d_{PT}} = 0.16 \pm 0.01 \mu M$ for labeled thymosin $\beta_4$, $K_{d_{AP}} = 0.18 \pm 0.04 \mu M$ for profilin, and $K_{d_{PT}} = 2.2 \pm 0.2 \mu M$ for binding of labeled thymosin $\beta_4$ to a complex of profilin and actin (Fig. 2D). Anisotropy experiments employing ADP-Mg$^2+$-actin prepared using hexokinase as previously described (37) implied that thymosin $\beta_4$ binds to ADP-Mg$^2+$-G-actin with $K_{d_{AP}} = 8 \pm 2 \mu M$ and to profilin-actin with $K_{d_{PT}} = 80 \mu M$ or higher in 40 mM KCl (data not shown). The ratio of $K_{d_{PT}}$ to $K_{d_{AP}}$ of 10 or greater for ADP-Mg$^2+$-G-actin implied that these results do not conclusively determine whether ADP-actin forms a ternary complex less effectively than does ATP-actin.

Because the reported values for $K_{d_{AP}}$ are often significantly higher than those reported here (38), we performed an additional independent assay for measuring this equilibrium dissociation constant. Measurement of intrinsic fluorescence has been used by Perelroizen et al. (30) to measure $K_{d_{AP}}$ for bovine spleen profilin purified using polyproline. For our recombinant human profilin, the data could be fit with $K_{d_{AP}} = 0.21 \pm 0.03 \mu M$ (Fig. 3), a value very similar to that reported by Perelroizen et al. (30) (0.12 $\mu M$). Equilibrium dissociation constants related to ternary complex formation are summarized in Table I.

Thymosin $\beta_4$ Binds to a Covalently Cross-linked Complex of Profilin and Actin—We reinvestigated the reports that covalent cross-linking has failed to produce a ternary complex of thymosin $\beta_4$, profilin, and actin using solution phase assays. Binding of thymosin $\beta_4$ to cross-linked profilin-actin complex was detectable by both analytical ultracentrifugation and fluorescence anisotropy (Fig. 4). Equilibrium dissociation constants for thymosin $\beta_4$ to cross-linked complex were estimated as $K_{d_{PT}} = 6.1 \mu M$ by sedimentation equilibrium and $K_{d_{PT}} = 2.3 \pm 0.1 \mu M$ by fluorescence anisotropy. The significant systematic deviation observed for the sedimentation equilibrium results precluded error analysis and was attributed to a small amount (<5% of total) of higher cross-linked oligomers of profilin-actin complex. A second preparation of covalently cross-linked complex that was more extensively cross-linked resulted in a similar estimate for $K_{d_{PT}}$. A separate analysis showed that there was insufficient non-cross-linked actin contaminating the sample to explain these results (data not shown). The measured equilibrium dissociation constants were remarkably similar to the values obtained for non-cross-linked profilin-actin (Fig. 2), suggesting that major structural alterations in the profilin-actin complex are not necessary to accommodate binding by thymosin $\beta_4$.

Nucleotide Exchange Rates of the Ternary Complex Are Slow and Comparable to Actin Alone—One of the functional consequences of the formation of ternary complex will be its effect on the rate of actin nucleotide exchange. Data for nucleotide exchange on actin in the presence of profilin and saturating amounts of thymosin $\beta_4$ show that the rate approaches 0 (Fig. 5). These data do not distinguish between competitive and noncompetitive models of binding, but assuming that thymosin $\beta_4$ and profilin form a ternary complex on actin, then the nucleotide dissociation rate from the complex can be determined using Equation 4 of the “Appendix.” This equation describes the relationship between observed exchange rates (determined by fitting the time course of nucleotide exchange to a single exponential) and the concentrations of thymosin $\beta_4$ and profilin and is applicable when conditions of rapid equilibrium are satisfied as defined in Equation 3 of the “Appendix.” Then, given that the rate constant for nucleotide exchange on actin alone is 0.0039 s$^{-1}$, as determined from an independent experiment (data not shown), and that $K_{d_{AP}}$ is 0.1 $\mu M$, this equation
The result was not sensitive to the rate of nucleotide dissociation. The equilibrium dissociation constant for binding of labeled thymosin to actin is $K_d = 0.01\text{ Kd}$ shows the expected result if cross-linked complex binds to actin with its theoretical mass, shown relative to that expected ($K_d = 6.1 \mu M$) for its theoretical mass, shown relative to that expected ($K_d = 6.1 \mu M$).

These results imply that, in agreement with results of Safer et al. (12) and De La Cruz et al. (13), the C terminus of thymosin $\beta_4$ likely contributes to the binding of the intact protein by direct interactions with actin. It is unlikely that the 5 residues N-terminal to our peptide are responsible for binding of intact thymosin $\beta_4$ in the presence of the peptide because others have shown that these residues are not important in this regard (9). The distal N terminus is important, however, to achieve a steric effect that inhibits actin polymerization (9).

Despite Low Affinity, the N-terminal Thymosin $\beta_4$ Peptide Dissociates Slowly from Actin—The data for nucleotide exchange by the N-terminal thymosin $\beta_4$ peptide in the absence of profilin can be fit with a single exponent. Using Equation 4 of the “Appendix,” these data then yield an estimate for $K_{N}$ of 20 $\mu M$ (shown by line of Fig. 6A, inset). However, as noted in the “Appendix,” this situation is more precisely described by two rate constants of similar order, and when the original nucleotide exchange data are fit by Equation 9 of the “Appendix,” then $K_{N} = 33.2 \pm 0.9 \mu M$, the rate of dissociation of nucleotide from free actin is $k_{-} = 0.00781 \pm 0.00004 \text{ s}^{-1}$, the dissociation rate constant for nucleotide from peptide-actin complex is $k_{-} = 0.00158 \pm 0.00003 \text{ s}^{-1}$, and the dissociation rate for peptide from actin is $k_{P} = 0.0029 \pm 0.0001 \text{ s}^{-1}$. This is in contrast to a dissociation rate constant of $-5 \text{ s}^{-1}$ reported previously for...
intact thymosin $\beta_4$ (13). Thus, the dissociation rate of the low-affinity peptide is slower by a factor of $-10^3$ than intact thymosin $\beta_4$. Note that values for $k_−$ varied from 0.0039 (see text describing Fig. 5) to 0.0078 s$^{-1}$ for slightly different buffer conditions.

Nucleotide exchange in the presence of both thymosin $\beta_4$ peptide and profilin reveals a departure from first-order kinetics (Fig. 6B, inset). A qualitative explanation for this observation is provided by assuming that the peptide is inhibiting exchange on the population of actin subunits to which it is bound because of both slow dissociation of nucleotide from the peptide-actin complex and slow dissociation of peptide from actin. Thus, the free and profilin-bound actin exchanges nucleotide more slowly, and the rate-limiting step for exchange of nucleotide on the peptide-bound actin is the sum of these slow dissociation steps. This interpretation is confirmed by the ability to fit the wide range of data shown in Fig. 6B using this assumption and Equations 6–8 of the “Appendix.” Global fitting of these data, using the same nomenclature as above, results in $K_{\text{INT}} = 9 ± 36 \mu M$, $k_− = 0.077 ± 0.004$ s$^{-1}$, $(k_{N,L} + k_{N}) = 0.0020 ± 0.0005$. The analytical solution confirms the qualitative impression that peptide dissociation from actin is slow, and is consistent with $k_{N,L}$ as calculated from data obtained in the absence of profilin. Slow dissociation of the N-terminal thymosin $\beta_4$ peptide was independently confirmed by fluorescence anisotropy. Data for the time course of displacement of thymosin $\beta_4$ from actin after addition of 200 $\mu M$ N-terminal peptide to solution were best fit by $k_{N,L} = 0.00047$ s$^{-1}$ with $K_{\text{INT}} = 0.30 \mu M$, $K_{\text{INT}} = 35 \mu M$, and $K_{\text{INT}} = 2.1 \mu M$ (Fig. 6C). Dissociation rate constants are summarized in Table II.

**Assuming That Cytoplasmic Excluded Volume Effects Are as Large as Theoretically Predicted, the Existence of Ternary Complex Would Have a Large Effect on the Amount of Sequestered Actin**—While precise data are unavailable, molecular crowding in the cytoplasm would be expected to increase actin self-association so as to decrease the critical concentration by more than an order of magnitude (39). Attempts to measure the effects of molecular crowding of actin in vitro have been limited to single observations or data points, and previous reports have described results varying from no effect (40) to more than a 10-fold decrease in Mg$^{2+}$-containing buffers (41, 42). Using 6% PEG 8000 in a steady-state assay of critical concentration, we find that the critical concentration decreases by more than a factor of 10 in 2.0 mM MgCl$_2$ and 40 mM KCl (Fig. 7, inset). Steady state experiments in the presence of up to 15% PEG and various actin-sequestering proteins confirmed that PEG has no influence on the fluorescence of either G- or F-actin. Also, profilin and thymosin $\beta_4$ are sufficiently small that binding of either to actin is unlikely to be affected by molecular crowding (39), and this was confirmed experimentally by fluorescence anisotropy and steady-state assay (data not shown). Given a 10-fold decrease in c without corresponding changes in $K_{\beta_4}$ or $K_{\text{INT}}$, $(K_{\beta_4}K_{\text{INT}})^{1/2}$ is then significantly larger than c, and significant differences in actin sequestration are predicted at steady state, depending on competitive or noncompetitive binding. With competitive binding, very little actin could be sequestered under these conditions, even with large amounts of both thymosin $\beta_4$ and profilin (Fig. 7). In contrast, noncompetitive or independent binding predicts that a much larger pool of actin could be sequestered. Experiments to test these predictions are ongoing but have been complicated by nonlinear effects of both profilin and thymosin $\beta_4$ (6, 29), which are particularly prominent in excluded volume conditions.

**DISCUSSION**

Previous methods used to detect the presence of a ternary complex between profilin, thymosin $\beta_4$, and actin were unsuc-
cells, is present as ternary complex. The functional significance of this finding remains to be elucidated. We have estimated the nucleotide exchange rate of the ternary complex as similar to that of actin alone, but we have no information regarding its effect on nucleation, capping, and filament elongation.

These results provide a possible resolution to the paradox as to how significant amounts of actin can be sequestered assuming that molecular crowding lowers the critical concentration of actin in vivo. That is, if the intracellular critical concentration of actin is as low as predicted by experimental data reflecting the molecularly crowded environment of the cytoplasm, then profilin or thymosin \( \beta_4 \) acting alone or together would be able to sequester only small amounts of actin. However, if binding is noncompetitive, then because of the disparity between \( c \) and the equilibrium dissociation constants for profilin and thymosin \( \beta_4 \), these proteins will act synergistically to form ternary complex. This is illustrated in Fig. 7 in an example relevant to human polymorphonuclear cells in which the concentrations of profilin and thymosin \( \beta_4 \) are \( \sim 40 \) and \( 150 \) \( \mu \)M, respectively (2).

This observation provides other potential mechanisms for regulating actin sequestration. Whereas competitive binding results in a shallow linear increase in sequestered actin with increasing profilin, noncompetitive binding results in a steep dependence on profilin concentration that cells could readily regulate, for example, by regulation of production or turnover of phosphophoinositides, another ligand of profilin.

We observe that the equilibrium dissociation constant for profilin-actin reported here is lower than often reported in the literature. However, the range of reported results for mammalian profilin is quite large. Gutsche-Perelroizen (30) obtained \( K_{\text{D}} = 0.12 \) \( \mu \)M by intrinsic fluorescence measurements on polypropylene-purified bovine profilin, and \( K_{\text{D}} = 0.13 \) to 0.15 \( \mu \)M from metal nucleotide exchange experiments (45). Selden et al. (46) obtained \( K_{\text{D}} = 0.60 \) \( \mu \)M from data for nucleotide exchange.

There are several possibilities that can explain why the intact thymosin \( \beta_4 \) peptide binds more rapidly to actin than does the N-terminal peptide. The C terminus could augment the rate of conversion from unfolded to a folded, binding competent, intermediate in solution, or could orient the peptide so as to catalyze a zipper effect where the N terminus binds quickly after the C terminus is bound, or could catalyze a conformational change in actin that facilitates rapid binding. The explanation for the slow dissociation rate constant of N-terminal peptide restricts these possibilities. An orientation effect in which the C terminus lines up the N terminus for binding would result in more rapid dissociation of the N-terminal peptide relative to intact protein. Similarly, rapid dissociation would be expected for the N-terminal peptide if the C terminus induced a conformational change in actin that facilitates binding of the N terminus. In contrast, if a conformational change in the N terminus was the rate-limiting event in binding, and if the presence of the C terminus resulted in more rapid interconversion between the various conformers of thy-

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**TABLE II**

| Reaction mechanism | Constant | Nucleotide exchange | Fluorescence anisotropy |
|--------------------|----------|---------------------|-------------------------|
| PA-ATP \( \rightarrow \) PA + ATP | \( h_1 \) | 0.097 ± 0.004 | 0.00047 ± 0.00015 |
| NA-ATP \( \rightarrow \) NA + ATP | \( h_2 \) | 0.00158 ± 0.00003 | |
| PAT-ATP \( \rightarrow \) PAT + ATP | \( h_3 \) | 0.005 ± 0.007 | |
| NA-ATP \( \rightarrow \) N + A-ATP | \( h_{N-} \) | 0.0029 ± 0.0001 | |
| | \( h_{2-} + h_{N-} \) | 0.0020 ± 0.0005 | |

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**FIG. 7.** Theoretical dependence of the amount of sequestered actin at low critical concentration according to three models. Competitive binding model (1, dashed line), model allowing ternary complex formation (2, solid line), and independent binding model (3, dotted line) are shown for critical concentration, \( c = 0.01 \) \( \mu \)M, \( K_{\text{D}} = 0.2 \) \( \mu \)M for thymosin \( \beta_4 \) binding to actin, \( K_{\text{D}} = 0.2 \) \( \mu \)M for profilin-actin binding, \( K_{\text{D}} = 3.0 \) \( \mu \)M (used in ternary complex model only) for binding of thymosin \( \beta_4 \) to profilin-actin complex. The models assume a total thymosin \( \beta_4 \) concentration of 150 \( \mu \)M, the amount found in polymorphonuclear cells (2). Inset, steady-state pyrene fluorescence assay showing decrease of actin critical concentration in presence of PEG. Pyrenyl-Mg\(^{2+}\) actin (4% labeled) diluted to indicated concentrations in the absence (circles) or presence of 6% PEG 8000 (triangles). Lines represent the best linear fits to the data with critical concentration in the absence of PEG, \( c = 0.048 ± 0.009 \) \( \mu \)M, and with PEG, \( c = 0.003 ± 0.008 \) \( \mu \)M.

---

Successful. Methods employing steady-state measurements of F-actin concentrations were not sensitive to differences between competitive and noncompetitive binding. Covalent cross-linking also failed to detect the ternary complex, but several explanations are possible. The site of covalent cross-linking on actin could have been identical for both profilin and thymosin \( \beta_4 \), so that both could not cross-link simultaneously even if both were bound, or more subtly, profilin may alter the interface of the interaction of thymosin \( \beta_4 \) and actin so that thymosin \( \beta_4 \) no longer cross-links to actin. Our data which imply competition between the profilin and the N-terminal thymosin \( \beta_4 \) suggest that the latter explanation is reasonable. Alternatively, one of the many demonstrable allosteric effects of profilin (26) may be to alter actin conformation so as to decrease the efficiency with which thymosin \( \beta_4 \) cross-links to actin.

The concentrations of thymosin \( \beta_4 \) and profilin varies in different cells, with as much as 560 \( \mu \)M thymosin \( \beta_4 \) and 55 \( \mu \)M profilin in platelets (38, 43), and perhaps more typically, embryonic chick brain has 50–60 \( \mu \)M thymosin \( \beta_4 \) and 5–6 \( \mu \)M profilin (44). These concentrations are sufficiently high that given a value of \( K_{\text{D}} \) of 2 to 8 \( \mu \)M, it is likely that significant amounts of the sequestered actin, indeed, most of it in some
mosin $\beta_4$, then the effective dissociation rate would be slow in the absence of C terminus. Detached thymosin $\beta_4$ would be much more likely to re-bind than unfold in the absence of the C terminus. This mechanism is consistent with the previously reported finding that the interaction of actin with thymosin $\beta_4$ is not diffusion limited (13). The C terminus may effect catalysis of conformational switching in solution or when bound to actin, but given the random coil structure of the C terminus in solution, cooperative effects on conformation are perhaps more likely to be transmitted in the bound state.

We note that an alternative interpretation of the data is that the N-terminal thymosin $\beta_4$ peptide does not bind and release slowly from actin, but rather exerts its effect on nucleotide exchange slowly after rapidly binding. This would necessarily involve both conformational change and allosteric effects in actin. While these conformational changes and allosteric effects very likely exist (13, 26), there is no precedent nor simple explanation that can elucidate how rapid detachment of the peptide would result in a slower conformational change in actin than does similarly rapid detachment of the intact protein. Still another interpretation of the data is that it is the missing 5 residues from the N terminus of thymosin $\beta_4$ rather than the missing 21 C-terminal residues that causes the thymosin $\beta_4$ peptide 6–23 to bind with different kinetics than intact thymosin $\beta_4$. However, Vancompernolle et al. (9) present evidence that these N-terminal residues have no effect on binding of thymosin $\beta_4$ to actin (although these residues do contribute to a steric effect that contributes to the inhibition of actin polymerization), whereas others have presented considerable evidence that the C terminus does influence actin binding activity (14, 15). Thus the presumption that C-terminal residues, rather than the N-terminal residues, are affecting the kinetics of binding is reasonable.

**APPENDIX**

**Actin Nucleotide Exchange Rates Complicated by Slow Releasing Ligands or by Formation of a Ternary Complex.**—Currently published algorithms for analysis on nucleotide exchange on actin assume that the data can be fit by a single exponential. This will not be the case when the dissociation rate constant of a ligand is slow relative to that of the dissociation rate of nucleotide itself. A general solution is derived here and the theory is extended to allow for multiple ligands (in this example, thymosin $\beta_4$ and profilin) interacting simultaneously with actin. We have observed similar data to those shown in Fig. 6B, inset, with certain marine natural products that bind to actin with high affinity (data not shown), for which the assumption of a single exponential fit is clearly inadequate, and as shown in Fig. 6A, even in some cases when the data can be fit to a single exponential, such an approach introduces a significant error. We therefore anticipate that the approach described here will be of much practical utility.

The reaction mechanism includes reactions for binding and dissociation of ATP or $\varepsilon$-ATP to nucleotide-free actin, actin-profilin complex, actin-thymosin $\beta_4$ complex, and a ternary complex of actin, profilin, and thymosin $\beta_4$ with the following rate and equilibrium constants: $k_{+}, k_{-}, K_{d} = k_{+}/k_{-}$ (for actin alone), $k_{+1}, k_{-1},$ and $K_{d1} = k_{+1}/k_{-1}$ (for actin-profilin complex), $k_{+2}, k_{-2},$ and $K_{d2} = k_{+2}/k_{-2}$ (for actin-thymosin $\beta_4$ complex), $k_{+3}, k_{-3},$ and $K_{d3} = k_{+3}/k_{-3}$ (for the ternary complex of actin, profilin and thymosin $\beta_4$), and for binding and dissociation of profilin and/or thymosin $\beta_4$ to nucleotide-bound actin with the following rate and equilibrium constants: $k_{+4}, k_{-4}, K_{d4} = k_{+4}/k_{-4}$ (for profilin to actin), $k_{+5}, k_{-5}, K_{d5} = k_{+5}/k_{-5}$ (for thymosin $\beta_4$ to actin), $k_{+6}, k_{-6}, K_{d6} = k_{+6}/k_{-6}$ (binding of thymosin $\beta_4$ to actin-profilin complex), $k_{+7}, k_{-7}, K_{d7} = k_{+7}/k_{-7}$ (binding of profilin to actin-thymosin $\beta_4$ complex). We assume that all rate constants involving ATP are identical for $\varepsilon$-ATP. The total concentrations of each species is represented by: $[N]$ (ATP), $[E]$ (ATP-bound), $[T]$ (thymosin $\beta_4$), $[A]$, $[AN]$ (nucleotide-free, ATP-bound, $\varepsilon$-ATP-bound actin, respectively), $[PA]$, $[PAN]$ (nucleotide-free, ATP-bound, and $\varepsilon$-ATP-bound profilin-actin complexes, respectively), $[AT]$, $[ATN]$, $[ATE]$ (nucleotide-free, ATP-bound, and $\varepsilon$-ATP-bound thymosin $\beta_4$–actin complexes, respectively), $[PAT]$, $[PATN]$, $[PATE]$ (nucleotide-free, ATP-bound, and $\varepsilon$-ATP-bound ternary complexes, respectively).

When all three proteins are present, the rates of change of the five fluorescent species are,

\[
\begin{align*}
\frac{d}[AE]dt &= k_{+} [A][E] - k_{-} [AE] - k_{+5} [AE][P] + h_{2} [PAE] - k_{+7} [AE][T] + k_{T} [ATE] \\
\frac{d}[PAE]dt &= k_{+1} [PA][E] - k_{-1} [PAE] + k_{+3} [AE][P] - k_{+7} [PAE] \\
\frac{d}[ATE]dt &= k_{+2} [AT][E] - k_{-2} [ATE] + k_{+7} [AE][T] - k_{+7} [ATE] \\
\frac{d}[PAT]dt &= k_{+3} [AT][E] - k_{-3} [PAT] + k_{+7} [AE][T] - k_{+7} [PAT] + k_{T} [PATN] \\
\frac{d}[PATE]dt &= k_{+5} [AT][E] - k_{-5} [PATE] + k_{+7} [AE][T] - k_{+7} [PATE] + h_{2} [PATE] \\
\frac{d}[E]dt &= -[AE] - [PAE] - [ATE] - [PAT] - [PATE] \\
&= e - [AE] - [PAE] - [ATE] - [PAT] - [PATE] \quad (Eq. 2)
\end{align*}
\]

We assume that nucleotide-free actin and its complexes with profilin and/or thymosin $\beta_4$ are in fast equilibrium with their nucleotide-bound forms. Indeed, according to Ref. 36, $K_{d} = 10^{-10}$ M, and $k_{e} = 2 \times 10^{4}$ M$^{-1}$ s$^{-1}$, and in our experiment $a = 1.7 \mu$M, and $e = 10$ $\mu$M. The rate of reaching local equilibrium for $[A]$, $[AE]$, and $[AN]$ should be $k_{e}([E] + [N]) + k_{r} \approx k_{e}([E] + n - a) + k_{r} = 20$ s$^{-1}$, which is faster than any other rate in solution. Since the concentrations of nucleotide-binding species of actin greatly exceeds the concentration of nucleotide-free species, the nucleotide-free species can be neglected when accounting for total actin.

The solution of the set of differential Equation 1 can be found in form of $A + Bexp(-kt)$, and if,

\[
\begin{align*}
k_{p}, k_{T}, k_{P}, k_{n}, \gg k_{+, k_{-}, k_{1}},
\end{align*}
\]

then only one slow exponent is observed,

\[
(1 + r_{0})/(1 + \beta + \gamma + R \cdot \beta \cdot \gamma) \quad (Eq. 4)
\]

where $r_{0} = alf = n + e - a$, $\beta = [P]/K_{dP}$, $\gamma = [T]/K_{dT}$, and $R = K_{dP}/K_{dT}$. $K_{dP}$ is the equilibrium constant of $\varepsilon$-ATP.

The values of $\beta$ and $\gamma$ can be found from equilibrium conditions, because addition of $\varepsilon$-ATP does not change the equilibrium between the complexes of nucleotide-bound actin. For our experiment with profilin and full-length thymosin $\beta_4$, the concentration of profilin was low enough relative to total actin concentration that it could be neglected in the calculation of the amount of nucleotide-bound actin. In this case, $X = \left[AN\right] + \left[AE\right] = \left((K_{dP} + t - a)^{2} + 4 \cdot a \cdot K_{dP}t^{2} - (K_{dP} + t - a)^{2}/2 \gamma + (t/K_{dT} + X) \beta = p/K_{dP} + X(1 + R))\right]$. In the experiment with thymosin $\beta_4$ peptide, Equation 3 is no longer true, but the analytical solution still can be found if no ternary complex is assumed, and if,
then two exponents of different order may be observed, and fluorescence, \( F \), as a function of time, \( t \), is described as,

\[
F = F_0 + \Delta F(1 - C_1 \exp(-k_1 t) - C_2 \exp(-k_2 t))
\]

(6)

where, \( F_0 \) is the initial fluorescence when all \( e \)-ATP is free, \( \Delta F \) is the total change of fluorescence, \( C_1 = \gamma m(1 + \beta + \gamma) \), \( C_2 = 1 - C_1, m = (1 + \beta + \gamma)/(1 + \beta(1 + r_0) + \gamma), k_1 = (k_{-2} + k_{-T})(1 + r_0)m \), and \( k_2 = (k_+ + k_{-2} \cdot \beta)(m(1 + \beta)) \).

If \( K_{RT} \) is much greater than the total actin concentration, as in Fig. 6, then,

\[
\gamma = t/K_{RT}
\]

(7)

\[
\beta = ((k_{sp}(1 + \gamma) + a - p)^2 + 4 \cdot p \cdot K_{sp}(1 + \gamma))^{1/2} - (k_{sp}(1 + \gamma) + a - p)/(2 \cdot K_{sp})
\]

(8)

In the case when no profin is present in solution, as in the experimental data used to generate Fig. 6A, inset, an exact analytical solution is possible without any assumptions for the relative values of dissociation constants. In this case the fluorescence time course is still described by Equation 6, but the two rate constants are of approximately the same order, so, the result will have appearance of the first-order kinetics,

\[
k_{12} = ((a_1 + a_2) 
\]
