Misakinolide A Is a Marine Macrolide That Caps but Does Not Sever Filamentous Actin*

(Received for publication, November 11, 1996, and in revised form, January 7, 1997)

David R. Terry, Ilan Spector‡, Tatsuo Higa§, and Michael R. Bubb¶

From the Department of Medicine, Health Science Center, University of Florida College of Medicine, Gainesville, Florida 32610; ‡Department of Physiology and Biophysics, Health Science Center, State University of New York, Stony Brook, New York 11794, and §Department of Marine Sciences, University of the Ryukyus, Nishihara, Okinawa 903-01, Japan

We have investigated the biochemical properties of the marine natural product, misakinolide A, a 40-membered dimeric lactone macrolide that differs from swinholide A only in the size of the macrolide ring. Analytical ultracentrifugation and steady-state fluorescence experiments show that misakinolide A binds simultaneously to two actin subunits with virtually the same affinity as swinholide A, suggesting that the modification in the ring size does not change the actin-binding site. Sedimentation equilibrium experiments suggest that binding is independent at each binding site, with a $K_d$ of approximately 50 nM. Remarkably, misakinolide A does not sever actin filaments like swinholide A; rather, it caps the barbed end of F-actin. When capped by misakinolide A, the elongation rate constant at the barbed end is reduced to zero; pointed end growth was affected only to the extent that the compound sequesters unpolymerized actin. Misakinolide A has essentially no effect on the off-rate of actin subunits leaving the barbed end. Energy-minimized models of misakinolide A and swinholide A are consistent with conservation of identical binding sites in both molecules, but a difference in orientation of one binding site relative to the other may explain why swinholide A has severing activity whereas misakinolide A only has capping activity.

In recent years an increasing number of cytotoxic marine natural products have been found to target the actin cytoskeleton and disrupt its organization (1–7). Many of these toxins bind directly to actin (4–7). They represent useful tools in investigations of actin organization, dynamics, and function (8–10) and have potential therapeutic uses, pharmacologically, in the treatment of cancer and other human diseases (11, 12). Identification of compounds with specific actin binding properties that preferentially affect certain aspects of actin filament dynamics will be necessary for further advances in both of these directions.

We previously identified the molecular basis for the cytotoxic effects of swinholide A, a 44-membered dimeric macrolide (6). Swinholide A binds to two actin monomers but also binds to and severs actin filaments. The data suggested that swinholide A did not cap either end of the actin filament.

Misakinolide A, isolated from an Okinawan Theonella sp. sponge, is another dimeric macrolide with potent cytotoxic properties that differs from swinholide A in having a 40-membered dilaactone moiety (13–15). Previously published information suggests that it binds to actin with a stoichiometry of two actin molecules to one misakinolide A molecule (7). In this paper we determined the nature of the interaction between misakinolide A and G-actin at equilibrium and the effects of misakinolide A on F-actin.

**EXPERIMENTAL PROCEDURES**

Steady-state Binding Assays—Pyrenyl-actin1 was prepared (16) from gel-filtered (95-cm column of Sephacryl HR-300, Pharmacia Biotech Inc.) rabbit skeletal muscle actin (17). Steady-state sequestration of actin subunits by misakinolide A was determined in three polymerization buffers using Mg2+-actin in 0.2 mM dithiothreitol, 0.2 mM ATP, 0.01% sodium azide, 0.125 mM EGTA, 0.1 mM CaCl2, and 5 mM Tris, pH 7.9, with either 2.0 mM MgCl2 (Mg2+-F buffer) or 1 mM MgCl2 plus 50 mM KCl (Mg2+-KCl-F buffer) or using Ca2+-actin in 0.2 mM dithiothreitol, 0.2 mM ATP, 0.01% sodium azide, 0.1 mM CaCl2, 100 mM KCl, and 5 mM Tris, pH 7.9 (Ca2+-F buffer). The Mg2+-actin was given 10 min after the addition of EGTA and 50 μM MgCl2 (for exchange of divalent cation), before the addition of KCl and/or MgCl2 to achieve the final concentration. Pyrenyl-actin fluorescence intensity was measured at 21 °C in a Photon Technology International (South Brunswick, NJ) photon-counting spectrofluorometer with excitation at 366 nm and emission at 386 nm.

Sedimentation equilibrium and velocity experiments were performed using absorption optics in a Beckman XLA analytical ultracentrifuge with procedural techniques and analyses as described previously (6, 18). The actin concentration was 15 μM in buffer G (Ca2+-F buffer without KCl) in all experiments. The Marquardt-Levenberg nonlinear curve-fitting algorithm was used to estimate the $K_d$ of the interaction between misakinolide A and actin (19). The curve-fitting algorithm for the sedimentation equilibrium data (14,500 rpm at 4 °C) assumed that actin interacted independently and identically with either of two binding sites on misakinolide A, that the molecular weight of misakinolide A was insignificant, and that misakinolide A had negligible optical absorbance at the measured wavelength of 280 nm. Data obtained in the absence of misakinolide A were used to obtain a best fit for the partial specific volume of actin, $\bar{\rho}$, equal to 0.737 cm3/g. Sedimentation velocity experiments at 52,000 rpm and 20 °C compared 15 μM actin plus either 7.5 μM MgCl2 or swinholide A in buffer G.

Effects of Misakinolide A on F-actin—To compare the effects of misakinolide A and swinholide A on actin depolymerization, Mg2+-actin (5% pyrenyl-actin) was polymerized in Mg2+-F buffer and diluted, and fluorescence intensity was measured as described previously (6). For the effect of misakinolide A on seeded polymerization, cross-linked F-actin seeds were prepared by cross-linking F-actin with N,N′-pyrenylmaleimide as described previously (20), except that the cross-linked actin was gel-filtered after extensive dialysis against buffer G and the fractions containing small actin oligomers were pooled. Mg2+-actin (100% pyrenyl-labeled) was mixed with misakinolide A in Mg2+-KCl-F buffer, and polymerization was initiated by the addition of cross-linked seeds. Using these assay conditions, the initial rate of polymerization was proportional to the concentration of added seeds.

To measure the effect of misakinolide A on the elongation rate at the pointed end of F-actin, we made seeds from F-actin capped with a

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† This research was conducted while M. R. B. was a Pfizer Scholar. To indicate this fact.

¶ The abbreviation used is: pyrenyl-actin, N′pyrenyl-carboxamidomethyl actin.
recombinant purified fragment of gelsolin corresponding to amino acids 1–406 (provided by Dr. Frederick Southwick, University of Florida), which caps the barbed end of actin filaments with affinity similar to intact gelsolin (21) but is calcium-insensitive. The gelsolin fragment was mixed with Mg\(^{2+}\)-actin subunits in a ratio of 1:20, and the material was polymerized with Mg\(^{2+}\)-KCl-F buffer.

**RESULTS**

**Steady-state Sequestration of Actin Subunits by Misakinolide A**—The critical concentration determined by steady-state fluorescence of 5% pyrenyl-actin polymerized in Mg\(^{2+}\)-KCl-F buffer in the presence of 0 (○), 0.1 (△), 0.25 (△), and 0.4 (○) μM misakinolide A. The solid lines represent the expected result if the fluorescence intensity is proportional to the amount of filamentous actin, with the proportionality constant determined from the 0 μM misakinolide A data, if each of two binding sites on misakinolide A binds independently to monomeric actin with \(K_d = 50\) nM, and if the misakinolide-actin dimer complex binds to one end of the actin filament with \(K_d = 50\) nM where the average actin filament length is 90 subunits. The steeper gradient seen with misakinolide A reflects a change in the apparent molecular weight of actin caused by dimerization. The solid line fitting these data shows the expected result for monomeric actin in the case of no added misakinolide A and dimeric actin for 7.5 μM misakinolide A. The rightward shift of the data, extrapolated to an F-actin concentration of zero (i.e., fluorescence intensity of zero), is consistent with two binding sites on misakinolide A interacting with actin with a \(K_d\) of 50 nM. The increasing slope of the data for increasing actin concentrations could be accounted for by assuming that the misakinolide actin-dimer complex binds one end of the actin filament with \(K_d = 50\) nM and that the average filament length is 90 subunits; thus at higher F-actin concentrations less misakinolide A is available to bind to monomeric actin, and the apparent shift in critical concentration is correspondingly less. Measured \(K_d\) values were indistinguishable when the experiment was repeated with Ca\(^{2+}\)-actin in Ca\(^{2+}\)-F buffer and were 30% less with Mg\(^{2+}\)-actin in Mg\(^{2+}\)-F buffer (data not shown). Although the steady-state fluorescence data fit the expected results for independent binding, they do not rule out a cooperative effect.

Misakinolide A caused actin to shift its apparent molecular weight from that of monomer to dimer when present in a molar ratio of 1:2, misakinolide A to actin (Fig. 1B). The theoretical curve for dimeric actin (Fig. 1B) is indistinguishable from a theoretical curve that assumes reversible equilibrium between 7.5 μM misakinolide A and 15 μM actin with \(K_d\) of 50 nM at each binding site, i.e., these conditions have sufficiently high concentrations of both components to ensure that essentially all of the actin is dimeric. Sedimentation velocity analysis of the actin dimer induced by misakinolide A under the same conditions as
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sedimentation equilibrium in Fig. 1B yielded a sedimentation coefficient, $s_{20,w} = 5.1$ S, which was indistinguishable from that induced by swinholide A in a simultaneous experiment under identical conditions (data not shown).

If the interaction between actin and misakinolide A was highly cooperative rather than independent, then an excess of misakinolide A would not cause significant dissociation of the ternary complex. Instead, the data (Fig. 1C) show that when misakinolide A is in molar excess with 25 $\mu$m misakinolide A and 15 $\mu$m actin, nearly 50% of the dimeric actin has been shifted to monomer (virtually all of the monomer would be expected to be in a 1:1 complex with misakinolide A). Therefore, these data are consistent with a model of independent binding of actin at each of the two binding sites on misakinolide A. A site-specific dissociation constant of $K_d = 55$ nM at each of two proposed actin-binding sites on misakinolide A was used to calculate the theoretical curve for equilibrium binding in Fig. 1C.

Effects of Misakinolide A on the Rate of Depolymerization of F-actin and on the Rate of Filament Growth in a Seeded Polymerization Assay—Misakinolide A had little effect on the depolymerization of Mg$^{2+}$-F-actin (Fig. 2A). Swinholide A served as a positive control, causing rapid depolymerization under identical circumstances. The slight increase in depolymerization rate in the presence of a large excess of misakinolide A can be qualitatively explained by sequestration of actin subunits, with subsequent decreases in the on-rate at the filament ends. While it is true that even the lower concentration of misakinolide A shown in Fig. 2A could sequester essentially all unpolymerized actin at steady state, this experiment takes place in a dynamic environment where the on-rate of misakinolide A could be rate-limiting, causing the depolymerization rate to be concentration-dependent.

In the absence of misakinolide A, actin polymerizes when seeded with cross-linked actin filament nuclei (18 nM in subunit concentration) in Mg$^{2+}$-KCl-F buffer at a rate proportional to the actin concentration (Fig. 2B). The x-intercept of the solid line drawn for the data in the absence of misakinolide A approximates the critical concentration (0.15 $\mu$m) for these conditions. Using the binding constant for misakinolide A to actin calculated in Figs. 1 and 3, the amount of actin that 70 nM misakinolide A should sequester can be calculated and a prediction can be made of how much the elongation rate will decrease when this much misakinolide A is added, assuming that misakinolide A only sequesters actin but does not cap filaments. As shown in Fig. 2B (dashed line), 70 nM misakinolide A should sequester a little more than 0.1 $\mu$m actin, and therefore the expected effect on $dF/dt$ at any concentration of actin should be equivalent to the effect of decreasing the actin concentration by 0.1 $\mu$m. Instead, the actual inhibition is much greater (Fig. 2B, squares) and implies that misakinolide A is preventing elongation by capping the barbed end of the actin filament. Similar experiments using seeds that have been previously capped at the barbed end show that misakinolide A inhibits polymerization from the pointed end in a manner that is quantitatively consistent with sequestration of actin subunits (Fig. 2B, inset), proving that the pointed end does not interact with misakinolide A or misakinolide-A-actin complex. The critical concentration was 1.1 $\mu$m for filaments capped at the barbed end. The data for pointed end growth cannot distinguish between stoichiometric binding and that of relatively high affinity, e.g. $K_d = 50$ nM.

In these experiments, misakinolide A did not lower the critical concentration of actin filaments to that of the pointed end at steady state (Fig. 1A) as might be expected for a barbed end-capping molecule. However, the large inhibition of polymerization seen in a seeded polymerization assay strongly suggests that the barbed end is capped by this toxin (Fig. 2B). These results can be reconciled by assuming that the misakinolide-actin dimer complex caps the barbed ends but not free misakinolide A. Thus in steady-state conditions with low free actin there is a low concentration of misakinolide-actin dimer complex so that not enough filament ends get capped to cause a switch to the pointed end critical concentration. In a seeded polymerization assay employing high concentrations of unpolymerized actin, actin saturates misakinolide A, creating much of the misakinolide-actin dimer complex, which then effectively inhibits elongation. The data in Fig. 1A were fit assuming that only the misakinolide-actin dimer complex has capping activity.
InsightII and Sybyl were used to generate three-dimensional structures of swinholide A and misakinolide A (Fig. 3, C and D). Identical structures were obtained independent of program or fitting strategy. In both molecules, the “first guess” structure had the branching side chains extending from the opposite sides of a plane formed by the ring, and the programs converged without obvious local minima into structures with the side chains on the same side of the ring. In the energy-minimized structures for both swinholide A and misakinolide A, the ring folds in half, forming a C-shaped structure when viewed from the side. The side chains attach to the folded “crease” in the ring, and both side chains extend on the convex side of the ring. The differences between misakinolide A and swinholide A are modest. The side chains and the rings of both molecules have nearly superimposable substructures. However, the ring is twisted relative to the side chains in misakinolide A so that when viewed with the position of the side chains held constant, the ring is rotated approximately 30° relative to the ring for swinholide A (Fig. 3D). In the image in Fig. 3D, this axis of rotation is horizontal and in the plane of the paper. The published crystallographic structure of swinholide A (22) was compared with the computer-predicted structure, and no atom varied by more than an angstrom, suggesting that both the modeling is accurate and the crystallographic structure probably is representative of the structure in solution.

**DISCUSSION**

The close relationship between actin filament severing and capping was suggested by the observation that subtle mutations resulting in undetermined structural changes convert cap-G from a capping protein to an actin filament-severing protein with activity like that of gelsolin (23). The difference between misakinolide A and swinholide A, a single double bond in positions 2–3 and 2′-3′, likely represents a minimal structural difference that can cause similar alterations in activity.

The 2-fold axis of rotational symmetry seen in the two-dimensional structure is well preserved in the three-dimensional structures for both molecules, implying that both of the actin-binding sites in misakinolide A (and swinholide A) are identical. Moreover, the most likely explanation for the result
that misakinolide A binds to two actin subunits with similar affinity to that seen previously with swinholide A is that the actin-binding sites of these two molecules are identical. This is consistent with the predicted structures of these molecules in that the substructure of the ring and the branching side arms are nearly identical for both molecules. What, then, explains the difference in severing and capping activities? If in the heterocomplex of actin and toxin the toxin is a hinge between two actin subunits, then the orientation of the two actin-binding sites in the toxin will determine the orientation of the two actin subunits to each other in the actin dimer. In this way, subtle differences in the structure of misakinolide A and swinholide A could result in relatively large differences in the conformation of the actin dimer they create. We speculate that differences in the orientation of the two actin-binding sites affect the way the toxin binds to adjacent actin subunits either at a filament end as a cap or along the side as a severing molecule. Furthermore, we speculate that the differences in relationship between the ring and the branching sidearms of swinholide A and misakinolide A cause a difference in orientation of the actin binding surface without changing the binding site itself. Crystallographic data would provide the means to confirm these hypotheses. The interaction has sufficient affinity (and inhibits polymerization) so that the formation of crystals of the heterotrimeric complex is a reasonable proposition.

The dimer resulting from cross-linking the swinholide A-actin dimer complex (6) and that produced by cross-linking actin dimers generated by gelsolin (24) results from a covalent Cys-374 to Cys-374 cross-link and has been previously termed “antiparallel” because the geometry is assumed to be a linear alignment of the two subunits. Since actin filaments are polar, an antiparallel geometry would be incompatible with interactions involving two adjacent subunits along the filament or with two subunits at a filament end. The terminology is misleading, however, as zero-length Cys-374 to Cys-374 cross-links could just as well cross-link parallel adjacent subunits, even those with some degree of “stagger,” given the flexibility of the C terminus of actin. Therefore, the previously reported Cys-374 to Cys-374 cross-links (6) do not rule out the possibility that both actin-binding sites of these toxins bind simultaneously to filamentous actin, and since subunits in an actin filament cannot be covalently cross-linked to give the Cys-374 to Cys-374 cross-link, we speculate that misakinolide A caps and swinholide A severs actin filaments by distorting normal filament structure.

Misakinolide A duplicates one property of actin filament-capping proteins in that it prevents filament elongation but does not have the property of stabilizing actin filaments because it has essentially no effect on depolymerization rates. In cells that it readily enters, such as fibroblasts, it should be useful for dissecting cellular pathways specifically dependent on assembly of actin filaments. Furthermore, since capping proteins may function as inhibitors of polymerization or as stabilizers of filamentous actin, misakinolide A may prove useful for clarifying the activity of capping proteins. For example, in cells such as the yeast Saccharomyces cerevisiae, where capping proteins are postulated to function as filament stabilizing agents (25), misakinolide A should have little effect in reversing the consequences of a deficiency of capping protein, but in Dictyostelium where capping protein is necessary for motility (26), misakinolide A may be able to replace deficient capping protein.

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