Mechanism of Dynamitin-mediated Disruption of Dynactin*

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Dynamitin is a commonly used inhibitor of cytoplasmic dynein-based motility in living cells. Dynamitin does not inhibit dynein directly but instead acts by causing disassembly of dynactin, a multiprotein complex required for dynein-based movement. In dynactin, dynamitin is closely associated with the subunits p150Glued and p24, which together form the shoulder and projecting arm structures of the dynactin molecule. In this study, we explore the way in which exogenous dynamitin affects dynactin disruption. We find that pure, recombinant dynamitin is an elongated protein with a strong propensity for self-assembly. Titration experiments reveal that free dynamitin binds dynactin before it causes release of subunits. When dynamitin is added to dynactin at an equimolar ratio of exogenous dynamitin subunits to endogenous dynamitin subunits (1:4 mol of exogenous dynamitin per mole of dynactin), exogenous dynamitin exchanges with endogenous dynamitin, and partial release of p150Glued is observed. When added in vast excess (≥25×; 100 mol of exogenous dynamitin per mole of dynactin), recombinant dynamitin causes complete release of both p150Glued subunits, two dynactins and one p24, but not other dynactin subunits. Our data suggest that dynamitin mediates disruption of dynactin by binding to endogenous dynamitin subunits. This binding destabilizes the shoulder structure that links the p150Glued arm to the Arp1 filament and leads to subunit release.

Dynactin is a multiprotein complex that plays important roles in microtubule-based motility. Dynactin was first identified as an activator of dynein-driven vesicle movement in vitro (1, 2) and a host of functional studies have verified that it is required for most, if not all, forms of dynein-based movement in vivo (reviewed in Refs. 3 and 4). Dynactin facilitates dynein-based motility in two ways: 1) it serves as an adapter protein that allows dynein to bind a variety of subcellular cargoes, and 2) it increases dynein processivity (reviewed in Ref. 4). Dynactin also interacts with motors of the kinesin superfamily (5, 6) suggesting that its functions are more broad-ranging than previously imagined. In keeping with this idea, we find that dynactin also increases the processivity of kinesin 2 (7).

In addition to facilitating subcellular motility, dynactin contributes to microtubule anchoring at centrosomes (8, 9). Its microtubule binding properties may be the basis for additional anchoring and/or plus-end targeting functions.

Dynactin consists of eleven distinct subunits, some of which are present in multiple copies, that are organized into a structure with a net mass of 1.1 MDa. Rotary shadow electron microscopy, single particle analysis, and biochemical characterization of intact dynactin and dynactin subcomplexes have provided a clear picture of dynactin structure and subunit organization (10–13; reviewed in Ref. 4). Dynactin contains two distinct structural domains: a 37×8 nm cargo-binding structure and a 24 nm-long projecting arm that binds microtubules. These two structures are linked via a triangular shoulder (10, 11, 13). The cargo-binding domain is a short polymer of eight subunits of the actin-related protein, Arp1, plus associated polypeptides. The projecting arm consists of the N-terminal third of the p150Glued dimer. The shoulder assembly is thought to comprise the C-terminal two-thirds of the p150Glued dimer, four monomers of dynamitin (p50), and two monomers of p24. When dynactin disassembly is induced by treatment with potassium iodide (KI) the projecting arm and shoulder assembly separate into two stable complexes: 1) the p150Glued dimer, two dynamitins, and a single p24 (referred to as “shoulder-side-arm” in a previous study (11)), and 2) a smaller assembly of two dynactins and a single p24 (referred to as shoulder previously (11)). Under these conditions, the Arp1 filament depolymerizes completely, and the protein complexes that are present at each end of the filament are released intact (11). p150Glued has been reported to interact with Arp1 directly (14), but it is likely that the other shoulder components, dynamitin and p24, contribute to and stabilize this interaction.

Dynactin has emerged as an especially important element of dynactin structure (reviewed in Ref. 4). The hypothesis that dynamitin might attach p150Glued to the Arp1 filament was first proposed on the basis of dynactin ultrastructure (10). This was later verified biochemically. Overexpression of dynamitin in cultured cells has a dramatic effect on dynactin structure, causing release of p150Glued from the Arp1 filament (15). A similar effect is seen when purified recombinant dynamitin is added to partially purified dynactin (16) or Xenopus egg cytosol (17). In experiments using purified dynamitin (11), excess dynamitin causes release of both p150Glued and p24 from the Arp1 filament, demonstrating that no additional cytosolic factors are required. The Arp1 filament retains four dynamitin subunits, the same number found in intact dynactin. However, it has not been possible to determine whether exogenous dynamitin exchanges for endogenous dynamitin on the Arp1 filament, because the large excess of dynamitin used in all previous work

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completely obscured the behavior of endogenous dynamitin. How exogenous dynamitin interacts with dynactin has important implications regarding dynactin subunit-subunit interactions as well as the mechanism of disruption. Furthermore, it is not clear how p150Glued and p24 are released: as individual polypeptides, in complexes with endogenous dynamitin, or in complexes with exogenous dynamitin.

In the present study, we sought to gain a clearer understanding of the mechanism of dynamitin-induced disruption. As a first step, we examined the self-association properties of recombinant, human dynamitin using gel-filtration chromatography and analytical ultracentrifugation. Our data suggest that recombinant dynamitin is present predominantly as monomers and tetramers, both of which are highly elongated. Titration experiments suggest that dynamitin disruption involves binding of exogenous dynamitin monomers to the dynactin molecule. Release of p150Glued can be detected at a submolar ratio of exogenous to endogenous dynamitin (0.5×, 2 mol of exogenous dynamitin per mole dynamitin), but complete disruption requires a molar excess of ~25×. At these high dynamitin concentrations, two exogenous dynamitin subunits are exchanged for two endogenous subunits on the Arp mini-filament. Both p150Glued subunits and a single p24 subunit are released in a complex with endogenous dynamitin. The other two dynamitins and one p24 subunit remain tightly associated with the Arp1 mini-filament in a 19S-20S complex.

EXPERIMENTAL PROCEDURES

Protein Purification—Bovine brain dynactin was purified to 95% homogeneity as described (18). Chick embryo brain dynactin was purified as described (10). p150Glued-coiled-coil 1 (CC1)3 in pVEX (8) was expressed in BL21(DE3) cells and purified as described (19). The double C to A human dynamitin mutant (C245A/C261A) was generated using QuikChange mutagenesis. Human dynamitins (wild type, mutant, untagged, or tagged with the His-/XPress tag; Invitrogen) were expressed in bacteria and purified by Mono Q ion-exchange chromatography and analytical ultracentrifugation. Our data suggest that recombinant dynamitin is present predominantly as monomers and tetramers, both of which are highly elongated. Titration experiments suggest that dynamitin disruption involves binding of exogenous dynamitin monomers to the dynactin molecule. Release of p150Glued can be detected at a submolar ratio of exogenous to endogenous dynamitin (0.5×, 2 mol of exogenous dynamitin per mole dynamitin), but complete disruption requires a molar excess of ~25×. At these high dynamitin concentrations, two exogenous dynamitin subunits are exchanged for two endogenous subunits on the Arp mini-filament. Both p150Glued subunits and a single p24 subunit are released in a complex with endogenous dynamitin. The other two dynamitins and one p24 subunit remain tightly associated with the Arp1 mini-filament in a 19S-20S complex.

Analytical Ultracentrifugation—The partial specific volume (v) was calculated from the amino acid composition using the method of Cohn and Edsall (20). The solvent density (ρ) was calculated from buffer composition as described (21). These calculations along with corrections for temperature were calculated using the computer program SEDNTERP.5

Sedimentation velocity experiments were performed using a Beckman XL-1 analytical ultracentrifuge. Protein concentration was adjusted to an absorbance of 0.6 at 275 nm, which corresponds to 1.4 mg/ml (~30 μM). 0.43-ml samples were loaded into cells with 12-mm double sector charcoal-filled Epon centerpieces and quartz windows. Velocity runs were carried out at 5 °C at 56,000 rpm in an An-60 Ti rotor. Data scans were recorded continuously throughout the experiment using the absorption optical system at 275 nm. All data were initially analyzed using the method of van Holde and Weischedel (23). Extrapolation plots were made and used to generate diffusion corrected integral distribution G(s) plots. Following preliminary analysis, data were further fitted to the Lamm equation using the finite-element method as implemented in the program Ultrascan (24). Initial estimates for the fitting parameters were obtained from the van Holde-Weischedel analysis.

Equilibrium sedimentation experiments were carried out at 4 °C and later at 20 °C using cells fitted with 12-mm double sector charcoal-filled Epon centerpieces and sapphire windows. Cell sectors were loaded with 120 μl of either solvent or protein solution. Samples at two or three different concentrations over the range of 0.4 to 2 mg/ml (~9–45 μM) were run in duplicate in an eight-place An-50 Ti rotor. The lower temperature runs were allowed to reach equilibrium at rotor speeds between 9,500 and 13,000 rpm. The runs at 20 °C were made at 18–19,000 rpm, at which speeds the concentration distributions at equilibrium were essentially depleted at the menisci. Equilibrium was assumed to have been established when no detectable changes in the concentration distributions could be detected over 2- to 3-h intervals. Data from the 4 °C runs were collected by both the absorption (at 280 nm) and interference optical systems; for the runs at 20 °C only the interference system was used. The data from both sets of experiments were analyzed using the ORIGIN-based self-assembly computer program (XL/A/XL-L Data Analysis Software, version 4.0, Beckman Instruments, Inc.). Values for v and ρ were calculated as described above. Dynamitin samples analyzed at 4 °C were in 25 mM Tris-Cl, pH 8.0, 100 mM KC1, 2 mM β-mercaptoethanol, 1 mM EDTA. Those analyzed at 20 °C were in either the solvent just described or in 100 mM KPO4, pH 6.5, 2 mM β-mercaptoethanol, 1 mM EDTA.

Dynactin Disruption—For all experiments, an equimolar ratio of exogenous dynamitin to endogenous dynamitin was considered to be 4 mol of exogenous dynamitin per mole dyn-

3 The abbreviations used are: CC1, coiled-coil 1; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody.

4 K. C. Maier and T. A. Schroer, manuscript in preparation.

5 D. Hayes, T. Laue, and J. Philo (2001) Sedimentation interpretation (SENDTERP).
actin, because each mole of dynactin contains 4 mol of dynamitin. 15 μg of bovine dynactin dynamitin was mixed with different amounts of recombinant human dynamitin (0.25–50×, XPress-tagged or untagged, mutant or wild type) or buffer alone, diluted to 0.1 or 0.5 ml in sedimentation buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1 mM EDTA) and then incubated on ice for 30 min. The concentrations of dynactin ranged between 0.06 and 12 μM, and those for dynamitin were between 0.05 and 24 μM. All buffers included 1000-fold dilutions of a protease inhibitor mixture containing 1 mg/ml pepstatin, 1 mg/ml leupeptin, 40 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml N\textsubscript{\textbeta}-p-tosyl-L-arginine methyl ester, 10 mg/ml \textalpha-benzoyl-L-arginine methyl ester, 10 mg/ml \textalpha-p-phenylalaneine chloromethyl ketone, and 10 mg/ml N\textalpha-p-tosyl-L-lysine chloromethyl ketone). Following incubation, the samples were diluted further to 1.5 ml in sedimentation buffer and sedimented into a 5–20% continuous sucrose gradient in a Beckman SW41 rotor at 150,000 × g for 15 h at 4 °C. Fractions were collected, then chloroform/methanol-precipitated and analyzed by SDS-PAGE. Dynactin subunits were detected by Coomassie Blue staining and/or immunoblotting. Control experiments used a 25× molar excess of purified p150\textsuperscript{Glued} amino acids 217–548 ("CC1") in place of dynamitin.

15 μg of purified chicken dynactin was incubated with a 45-fold molar excess of recombinant wild-type human dynamitin monomers to chick dynactin molecules and processed as above. Control dynactin samples supplemented with buffer alone were incubated in parallel. The behavior of chick dynactin subunits and human dynamitin were analyzed by immunoblotting with species-specific antibodies.

PAGE, Immunoblotting, and Densitometry—SDS-PAGE was performed according to Laemmli (25). Proteins were separated on 11% polyacrylamide gels using the Mini-Protean II system (Bio-Rad) and were detected by Coomassie Blue staining. For immunoblots, proteins were transferred to polyvinylidene difluoride (Millipore, Bedford, MA) as described previously (26) using a Mini Trans-Blot apparatus (Bio-Rad). Proteins were detected using dynactin-specific primary antibodies in conjunction with the Western-LightTM protein detection kit (Tropix, Bedford, MA). Quantitation of the dynactin subunit p150\textsuperscript{Glued} was performed on Coomassie blue-stained gels or immunoblots using National Institutes of Health Image 1 software, after scanning on a flat-bed scanner (Microtek).

Antibodies—The XPress tag Ab was purchased from Invitrogen. Others were as follows: for p150\textsuperscript{Glued}, mAb 150B (27, 28) and mAb raised to the N terminus (Transduction Labs); for Arp1, mAb 45A (10); for actin, mAb C4 (29); for p24, R5700 (30); for Cap\textz\textalpha/\textbeta subunits, mAb 5B12 and mAb 3F2 (anti-\textalpha and anti-\textbeta, respectively (31)); and for dynamitin, mAb 50A (32) and rabbit pAb JH2882 against purified, recombinant, human dynamitin. The JH2882 antibody was blot affinity-purified, as previously described (33), with the minor exceptions that the antibodies were absorbed to dynamitin on nitrocellulose strips in phosphate-buffered saline containing 0.1% Tween 20 and 1% bovine serum albumin and the strips were washed with phosphate-buffered saline containing 0.1% Tween 20. An affinity-purified pAb to the Arp11 C terminus was a gift from Dr. M. Way (Cancer Research United Kingdom, London). For p27 (mAb 27A) mice were immunized with purified pointed-end complex from dynactin (11) and mixed with adjuvant (monophosphoryl lipid A plus trehalose dimycolate emulsion, RIBI Immunocore Research Inc., Hamilton, MT). Sera from the immunized mice were screened by enzyme-linked immunosorbent assay and Western blotting on purified dynactin, and hybridomas were prepared by standard methods.

RESULTS

Purified Recombinant Dynamitin Self-associates—To learn more about the interaction between exogenous dynamitin and dynactin and how this causes release of p150\textsuperscript{Glued} and p24 from the Arp1 filament, we performed a hydrodynamic characterization of recombinant human dynamitin. Superose12 HR 10/30 gel-filtration chromatography yielded a major population of molecules with a Stokes radius of 6.3 nm (Fig. 1A). Comparison of this elution profile with that of native, heterotrimeric shoulder complex (i.e. two dynamitins and one p24 (11)) revealed that dynamitin eluted from the column slightly earlier (Stokes radius ~6.3 versus ~6.1 nm; see Table 1). The larger dimensions of recombinant dynamtin suggest self-association into a larger structure.

We then used sedimentation equilibrium analytical ultracentrifugation analysis to obtain a rigorous determination of the molecular weight of recombinant human dynamitin. To obtain interpretable data a homogeneous sample is required. However, we had noted that even our most highly purified samples contained a high molecular weight contaminant (Fig. 1B, faint upper band in lanes 15–17). The presence of high concentrations of reducing agents decreased but did not completely remove the contaminant, and it reappeared in the samples after storage. The contaminant reacted with antibodies to dynamitin (data not shown), suggesting that it corresponded to an irreversibly cross-linked oligomer. To obtain dynamitin that would not form this contaminant, we mutantized its two cysteine residues (amino acids 245 and 261) to alanines. This yielded a protein that behaved identically to wild-type dynamitin on Mono Q and Superose12 columns but no longer yielded the high molecular weight species. The cysteine-to-alanine double mutant (C245A/C261A) was fully active for dynactin disruption (Fig. 2) and was used for most experiments.

Sedimentation equilibrium experiments using purified dynamitin (in 100 mM KPO\textsubscript{4}, pH 6.5, 2 mM \textbeta-mercaptoethanol maintained at 20 °C) indicated that the predominant species was tetramers, with clear evidence of monomer. We also observed a larger oligomer that could be modeled as an octamer (see fit in Fig. 1C). No evidence of dimers was seen in either the equilibrium runs or in sedimentation velocity runs. The concentration sensitivity of the association constants calculated for the formation of tetramer and octamer indicated that the assembly of the oligomers is not reversible (i.e. oligomers are not in chemical equilibrium with monomers). Assuming that self-association is completely irreversible, the relative amounts of the three species present can be estimated from the “pseudo” association constants K\textsubscript{1} and K\textsubscript{2}. The percentages (on a mass scale) of monomer, tetramer, and octamer present in the sample shown in Fig. 1C are 17%, 74, and 9%, respectively. The amount of each species varied somewhat from sample to sam-
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![Graph](image)

**FIGURE 1. Biochemical characterization of dynamitin.** Human dynamitin (wild type) was expressed in *Escherichia coli* and purified by ion-exchange chromatography, as described under “Experimental Procedures.” A, the protein was purified further on a Superose 12 size-exclusion column in the presence of 100 mM KCl. The $V_0$, $V_e$, and elution positions of protein standards are indicated. Protein standards and Stokes radii: apoferritin, 6.0 nm; alcohol dehydrogenase, 4.5 nm; carbonic anhydrase, 2.5 nm. B, Coomassie Blue-stained SDS-PAGE illustrating the composition of column fractions spanning the major absorbance peak. C245A/C261A dynamitin shows the same elution pattern, except that it lacks the high molecular weight contaminant that is faintly visible in fractions 15–17. L, column load. Molecular weight markers are on the left. C, sedimentation equilibrium of C245A/C261A dynamitin at 20 °C was analyzed using the ORIGIN-based self-association program. Two milligrams/ml dynamitin (45 μM, in 0.1 M KPO$_4$, pH 6.5, 2 mM β-mercaptoethanol, and 1 mM EDTA) was brought to equilibrium at 19,000 rpm. The bottom graph shows protein concentration (fringe displacement; 3.33 fringes = 1 mg/ml) versus radius (cm). Data collected by the interference optical system could be fitted to a monomer-tetramer-octamer assembly model. In this case, the concentration of the three species at any point in the distribution is given by $K_s = \frac{\text{[tetramer]} \cdot \text{[monomer]}^3}{\text{[octamer]} \cdot \text{[monomer]}^3} = 39$ and $K_s = \frac{\text{[octamer]} \cdot \text{[monomer]}^3}{\text{[tetramer]} \cdot \text{[monomer]}^3} = 135$ (measured in reciprocal fringes raised to the appropriate power). The direct relationship between tetramer and octamer can easily be shown to equal $\frac{\text{[octamer]}^2}{\text{[tetramer]}^2} = K_s = 0.09$. The molecular weight of the C245A/C261A dynamitin monomer was assumed to be 44,941 based on amino acid composition. The fit generated a variance of 8.9E−5. The top graph shows the residuals of the data points plotted against radius.

We also evaluated the behavior of dynamitin using sedimentation velocity. These runs yielded a $s_{20,w}^0$ of 5.8 S and an $f/f_0$ of 1.873. The latter figure indicates that the dynamitin tetramer is non-globular and can only be modeled by a shape with high axial ratio. If fitted to a rod and assuming a reasonable degree of hydration based on amino acid composition ($s = 0.44$), the dimensions of the tetramer would be 43 nm (length) by 3.1 nm (diameter), although the exact shape of the molecule cannot be rigorously determined from this analysis.

**Assessment of the Fate of Dynactin Subunits after Dynamitin-induced Disruption**—Dynamitin treatment of dynactin releases p150Glued and p24 as slowly sedimenting (4 to 9 S) species but it leaves Arp1 and p62 in complex that still sediments rapidly (19 to 20 S) (8, 11, 15, 16, 22). To explore the possibility that dynamitin treatment might cause loss of additional dynactin subunits, we used dynamitin to disrupt dynactin and analyzed the sedimentation properties of all dynactin subunits for which antibodies are available (Fig. 2). For all disruption experiments, the relative concentrations of exogenous to endogenous dynamitin are expressed as the molar ratio of exogenous dynamitin subunits to endogenous dynactin subunits, keeping in mind that each mole of dynactin contains 4 mol of dynamitin. As expected, excess dynamitin (25× molar excess: 100 mol of dynamitin per mole dynactin) caused almost all the p150Glued to be released in a form that sedimented at ~8 to 9 S. Under these same conditions about half the p24 remained associated with the Arp1 minifilament (Fig. 2; see also Ref. 22). This finding was highly reproducible. In this and all other experiments, free p24 showed a broader sedimentation peak than p150Glued (see Figs. 2 and 5B) suggesting that the subunits of the dynactin shoulder and sidearm may not be not released in a single, stable complex. The approximate S value of the “light” pool of p24 (Table 1) is similar to that of the dynactin shoulder complex released by KI treatment (dynamitin/p24 in a 2:1 ratio) (11). By contrast, Arp1, actin, capZ, and components of the pointed end complex (Arp11 and p62) all cosedimented as a tight peak in the presence or absence of excess dynamitin (Fig. 2), indicating that the integrity of the Arp1 minifilament was fully maintained. Surprisingly, the dynactin subunit, p27, was seen to spread across the gradient from ~17 S to ~7 S in either the presence or
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TABLE 1
Hydrodynamic parameters of dynactin subunit complexes

All available data regarding the hydrodynamic parameters of the dynactin subunit complexes discussed in this study are provided here. Most of this information came from ultracentrifugation approaches (velocity sedimentation into sucrose gradients, or analytical sedimentation velocity, or sedimentation equilibrium analysis) rather than gel filtration because of the problems associated with protein losses on gel-filtration columns. The values shown correspond to a dynamitin tetramer and a 2:1 heterotrimer of dynamitin/p24. $M_r$ value: approximate sedimentation coefficients estimated from sucrose gradient velocity sedimentation. The sedimentation coefficient of recombinant dynamitin was determined to be 5.8 S by sedimentation velocity analytical ultracentrifugation.

| Protein complex | $M_r$ (Da) | Stokes radius (nm) | S value | $M_r$ (Da) | $M_r$ (Da) |
|-----------------|-------------|--------------------|-------|------------|------------|
| Intact dynactin | 1.1 (MDa)   | ND                 | 19–20 | NA         | ND         |
| Dynamitin (recombinant) | 44,941 (monomer) | 6.3 | 4–5 | 133,000 | 179,764 (tetramer) |
| p150Glued (released by dynamitin treatment) | NA | ND | 8–9 | NA | ND |
| p150Glued/dynamitin/p24 (shoulder/sidearm released by KI treatment) | 386,373 | 10.7 | 9 | 396,550 | ND |
| Dynamitin/p24 (heavy pool released by dynamitin treatment) | NA | ND | 7–9 | NA | ND |
| Dynamitin/p24 (light pool released by dynamitin treatment) | NA | ND | 4–6 | NA | ND |
| Dynamitin/p24 (shoulder released by KI treatment) | 110,850 | 6.1 | 4.4 | 110,800 | ND |
| Dynamitin/p24 complex (recombinant) | 115,686 | 6 | 4–5 | 111,000 | 115,686 |

- Native molecular weights of protein complexes that best fit the equilibrium sedimentation data.
- Not available (NA); the Stokes radius could not be determined because of protein instability or loss on gel-filtration columns.
- Refers to the sucrose gradient fractions containing the p150Glued that was released by dynamitin disruption. This sedimentation behavior was highly consistent and was also seen when dynamitin present in cell lysates was disrupted by overexpressed dynamitin. These same fractions also contained the more rapidly sedimenting pool of endogenous dynamitin and p24.
- Refers to sucrose gradient fractions containing the slower sedimenting light pool of endogenous dynamitin and p24.

Purified bovine dynamitin was mixed with different amounts of recombinant human dynamitin (wild type or C245A/C261A) and incubated for 30 min on ice. The mixtures were then sedimented into 5–20% sucrose gradients to separate intact dynactin and Arp1 minifilaments, which sediment at 19S–20S, from free p150Glued and free dynamitin, which sediment at lower S values. As a control, purified dynamitin was sedimented by itself. Individual gradient fractions were analyzed on immunoblots (p150Glued and Arp1) or on Coomassie Blue-stained gels (dynamitin). In the absence of exogenous dynamitin, the hallmark dynactin subunits p150Glued and Arp1 sedimented at ~20 S (Fig. 3B). Increasing amounts of recombinant dynamitin yielded increasing amounts of p150Glued in the 8 to 9 S fractions. Complete release of p150Glued was first observed when exogenous dynamitin was present at approximately a 25-fold stoichiometric excess relative to endogenous dynamitin subunits (e.g. 6 μM dynamitin to 0.6 μM dynactin; Fig. 3A).

To verify that this effect was specific for dynamitin we tested the ability of another elongated protein, CC1, to cause release of p150Glued. CC1 is a coiled-coil fragment of p150Glued that has a similar monomer mass as dynamitin (8, 19). CC1 caused no alteration in the sedimentation behavior of dynactin subunits, even when present at a 45X molar excess (data not shown). This agrees with our observation that the structural integrity of dynactin is maintained in cells overexpressing CC1 (8) and verifies the selective effect of dynamitin on dynactin structure.

Exchange of Exogenous Dynamitin for Endogenous Dynamitin—Both intact dynactin (10, 18) and the Arp1 minifilament that is left following dynamitin disruption (11) contain four subunits of dynactin. Because recombinant dynamitin shows such a strong propensity to form tetramers, dynamitin tetramers may be the species that mediate dynamitin disruption. If this is the case, disruption might occur in one of two ways. A tetramer might exchange, en masse, for the endogenous dynamitin tetramer that is complexed with p24 and p150Glued, resulting in release of p150Glued, p24, and all endogenous dyna-

absence of excess dynamitin (Fig. 2, bottom), p27 showed a broader sedimentation profile than other dynactin subunits under a wide range of buffer conditions and also when some unfractonated cytosols were analyzed by velocity sedimentation (data not shown), suggesting that this subunit is not as tightly associated with the Arp1 minifilament as other dynactin components.

Concentration Dependence of Dynamitin Disruption—Dynamitin disrupts dynactin when it is vastly overexpressed in cultured cells, but the minimum amount required for complete release of p150Glued in vitro has never been explored. To learn more about the disruption process, we examined the effects of increasing amounts of dynamitin on dynactin integrity (Fig. 3).
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**FIGURE 3.** Concentration dependence of p150\(^{\text{Glued}}\) release. Purified bovine dynamitin was mixed with increasing amounts of recombinant human dynamitin (both wild-type and C245A/261A dynamitin were used in this series of experiments), incubated on ice for 30 min, and then the mixtures were sedimented into 5–20% sucrose gradients as described under “Experimental Procedures.” Dynamitin disruption was evaluated by immunoblotting for Arp1 tetramers competitively extract p150\(^{\text{Glued}}\) and p24 from dynactin. To gain a clearer picture of the way in which exogenous and endogenous dynamitins partition between the free pool and the 19 S Arp1 minifilament-associated pool during disruption, we used two approaches. In the first, excess human dynamitin was used to disrupt chicken dynactin, and the behaviors of exogenous human and endogenous chicken dynamitin were compared using species-specific antibodies (Fig. 4). Human dynamitin readily disrupted chicken dynactin, as expected based on the fact that it can disrupt *Xenopus* dynamitin (17). Excess dynamitin once again caused all p150\(^{\text{Glued}}\) and half of p24 to be released from the Arp1 filament (data not shown). Although the majority of the exogenous dynamitin sedimented slowly at ~6 S, immunoblot analysis revealed a small amount of human dynamitin in the rapidly sedimenting ~19 S fractions (Fig. 4, lanes 2 and 3). This suggests that some exogenous dynamitin might be associated with the Arp1 minifilament. Parallel immunoblot analysis of the behavior of endogenous chicken dynamitin (Fig. 4C) revealed that approximately half (*i.e.* two subunits) remained associated with the Arp1 minifilament, and the other half (*i.e.* two subunits) was released into a pool that sedimented at ~6 S. This finding suggests that disruption involves exchange of two exogenous dynamitin subunits for two endogenous subunits.

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We refined this analysis by examining the incorporation and release of exogenous and endogenous dynamitin subunits at
much lower stoichiometric ratios (0.25×, 0.5×, and 1× molar excess). For this work, we used an XPress-tagged form of human dynamitin that migrates slightly more slowly than endogenous dynamitin in SDS-PAGE (Fig. 5). XPress-tagged dynamitin behaved indistinguishably from untagged dynamitin in dynactin disruption and sedimentation equilibrium experiments (data not shown). At the substoichiometric concentration of 0.25×, (i.e. one dynamitin subunit per dynactin molecule) exogenous dynamitin bound to and cosedimented with dynamitin, but it did not induce release of p150Glued (Fig. 5A). This suggests that the pathway to dynactin disruption involves incorporation of free dynamitin monomers. Exogenous dynamitin most likely binds to endogenous dynamitin, p150Glued, and/or p24. At slightly higher concentrations (0.5 and 1×) we could observe release of both endogenous dynamitin and p150Glued. We typically observed that roughly equal amounts of exogenous dynamitin became bound to Arp1 filament as endogenous dynamitin that was released (see boxes in 0.5× and 1× samples). We could not extend this analysis to determine how much dynamitin was released in completely disrupted samples, because the large amounts of exogenous dynamitin required obscured the behavior of endogenous dynamitin. However, these results are consistent with the results of the experiment in Fig. 4, where we saw that complete disruption involved exchange of exogenous for endogenous dynamitin.

**Subunit Release by Exogenous Dynamitin**—KI treatment depolymerizes the Arp1 minifilament and releases p150Glued in a complex along with dynamitin and p24 (the shoulder/sidearm complex described previously (11)). The p150Glued that is released by dynamitin treatment overlaps partially on sucrose gradients with the endogenous dynamitin that is also released, suggesting that these two proteins might be released as a complex. This was the conclusion of our earlier analysis of dynamitin-induced dynactin disruption in vitro (11). However, our original analysis was limited by the fact that the samples contained a vast excess of recombinant dynamitin. The availability of tagged, exogenous dynamitin allowed us to address this question more carefully, because we could directly follow the behavior of exogenous and endogenous dynamitin as well as p150Glued and p24 during disruption. For this work, we used a 5× molar excess of exogenous dynamitin. This is the highest amount that can be added that still allows endogenous and exogenous dynamitins to be distinguished from one another. Under these conditions, a third of p150Glued is released (Figs. 3A and 5B). The endogenous dynamitin that was released sedimented in a broad peak in the sucrose gradient (Fig. 5B, boxed DM-wt in fractions 8–11), similar to what we observed previously (Figs. 4C and 5A and Table 1). The p24 that was released showed very similar sedimentation behavior to endogenous dynamitin (Fig. 5B, boxed p24 in fractions 8–11), suggesting that these two proteins are released together as a complex. This behavior was highly reproducible. p150Glued, by contrast, was present in a narrower, more rapidly sedimenting peak (Fig. 5B, fractions 8 and 9, and Table 1). This pool of p150Glued may comprise free dimers, or it may be associated with dynamitin and p24 in a complex that is analogous to the dynactin shoulder/sidearm (11). The lighter sedimenting pool of endogenous dynamitin and p24 shows a similar sedimenta-
**Dynamitin Disruption of Dynactin**

Dynamitin profile to the shoulder assembly (2:1, dynamitin/p24) that is released when dynactin is disassembled with KI. Importantly, none of the shoulder/sidearm subunits showed an obvious cosedimenting peak with free endogenous dynamitin (peak in fractions 10 and 11), suggesting that they do not form complexes with exogenous dynamitin.

**Irreversibility of Dynactin Disruption**—The fact that the subunits that are released do not rebinding the remaining Arp1 filament indicates that the process of disruption is irreversible. This may be because the disassembly process yields “dead-end” reaction products. One of these would be the Arp1 filament, which now contains a different shoulder structure made up of four dynamitins, one p24, and no p150Glued. This assembly is clearly different from the shoulder that is present in native dynamitin and might therefore be incapable of rebinding the complexes that have been released. The other reaction product(s) may also be dead-end complexes that cannot reassociate. Our data indicate that one disruption product is the dynamitin/p24 shoulder. To test the hypothesis that this complex is also a dead-end reaction product, we prepared recombinant dynamitin/p24 heterotrimer and tested their ability to disrupt dynactin (Fig. 5C). Release of p150Glued was not observed. This suggests that the dynamitin/p24 heterotrimer is a stable reaction product that does not interact with and destabilize dynactin in the same way as free dynamitin subunits.

**DISCUSSION**

The present study represents a detailed biochemical analysis of recombinant dynamitin and the way it interacts with purified dynactin in vitro. Sedimentation equilibrium studies indicate that dynamitin monomers readily self-associate. Excess free dynamitin causes release of two p150Glued subunits and one p24 subunit from the Arp1 minifilament in a process that involves exchange of two dynamitin exogenous monomers for two endogenous monomers. The Arp1 filament that remains contains Arp1, Arp11, p62, p27, actin, α- and β-capping proteins, the other p24 subunit, and four monomers of dynamitin (two endogenous and two exogenous). Dynamitin-mediated dynactin disruption is concentration-dependent, and complete release of p150Glued requires ~25 mol of exogenous dynamitin per mole of endogenous dynamitin in dynactin (i.e. 100 mol of dynamitin per mole dynactin). At substoichiometric concentrations, free dynamitin monomers bind to dynactin, most likely by associating with endogenous dynamitin and/or p24, but do not cause subunit release. However, only slightly more dynamitin is required for release of p150Glued, p24, and dynamitin. Taken together, our results suggest that binding of free dynamitin monomers to the dynactin shoulder structure perturbs the protein-protein interactions that are required for optimal stability of this complex.

Our ability to dissect the dynactin disassembly pathway to the extent we have here relies upon the availability of highly sensitive antibody reagents that permit detection of very small amounts of p150Glued and dynamitin. These proteins are both released at the same substoichiometric concentrations of exogenous dynamitin. Unfortunately, the p24 Abs currently available do not allow us to detect trace amounts of p24, so we cannot say for certain how closely p24 release correlates with release of p150Glued and dynamitin at substoichiometric dynamitin concentrations. Despite this limitation, our findings are consistent with the disassembly pathway cartooned in Fig. 6. Free dynamitin monomers bind dynactin, which destabilizes its structure and leads to release of two molecules of p150Glued, two molecules of dynamitin, and one molecule of p24. These are the same subunits that comprise the dynactin shoulder/sidearm assembly that is released by KI treatment, suggesting this may also be the species that is released by dynamitin. However, we consistently observe a pool of dynamitin and p24 that is not associated with p150Glued. This light pool of dynamitin and p24 may be the product of dissociation of shoulder/sidearm into free p150 dimers and dynamitin/p24 shoulder complexes. The shoulder/sidearm that is released by KI does not exhibit this behavior, suggesting that the presence of free dynamitin may be exerting a destabilizing influence. Dynamitin may perturb contacts between p24 and p150Glued, leading first to release of shoulder/sidearm from dynactin and then disintegration of this complex into the p150Glued dimer and shoulder.
Our findings emphasize the important role interactions between p150 Glued, p24, and dynamitin play in dynactin integrity. Biochemical studies (herein and in Ref. 11) indicate that dynamitin serves as a structural scaffold for multiple dynactin subunits, including p24, p150 Glued, and Arp1. Although p150 Glued can bind Arp1 (14) and p24 (16) directly in vitro, it is highly likely that dynamitin supports and stabilizes the interactions between these proteins in native dynactin. We show here that the native dynactin shoulder/side-arm structure, which is structurally robust under normal conditions (13), is exquisitely sensitive to the presence of exogenous dynamitin. The polypeptides that comprise this structure (two p150 Glued, two p24, and four dynamitin subunits) all have a strong propensity for α-helix formation, suggesting that the structural basis of the assembly is a supercoil or spectrin repeat motif. Apparently, the incorporation of just one or two extra dynamitin subunits remodels and destabilizes this otherwise sturdy structure.

Our sedimentation equilibrium data fit well to a dynamitin self-assembly mode of monomer:tetramer:octamer. The finding that dynamitin can form tetramers is not surprising. Each dynactin molecule is known to contain four subunits of dynamitin. The dynamitin primary sequence predicts multiple coiled-coil and triple-coil motifs, all of which may support oligomerization. The sedimentation equilibrium data can also be made to fit a monomer:trimer assembly mode,6 but this model is difficult to reconcile with the stoichiometry of dynamitin in dynactin and the pattern of dynamitin exchange we observe. Sedimentation equilibrium analysis yielded no evidence of dimer formation, suggesting that dynamitin-mediated disruption involves either monomers or tetramers. That two exogenous dynamitin monomers ultimately become bound to the Arp1 minifilament suggests that subunit exchange involves incorporation of individual monomers rather than an assembled tetramer. In keeping with this, we find that small amounts of exogenous dynamitin (i.e. 0.25 ×; 1 mol of dynamitin per mole dynactin) bind completely to dynactin. This suggests that at low concentrations (e.g. 0.05 μM versus the ≥9 μM required for sedimentation equilibrium analysis) dynamitin self-assembly is minimal or disfavored due to subunit depletion. Either way, our data indicate that free exogenous monomers readily associate with endogenous dynamitin subunits. Pure dynamitin is able to oligomerize further into octamers, suggesting a very strong propensity for self-association. By contrast, the dynamitin assembly that is present in dynactin is highly selective and accommodates only one or two additional dynamitin monomers. This indicates that the structural or surface features of dynamitin in its usual context (i.e. as a subunit of dynactin) are somewhat different from those of dynamitin in isolation. All available evidence suggests that extensive self-assembly of dynamitin is a strictly in vitro phenomenon.

Although recombinant dynamitin shows no evidence of dimer formation by itself, it is highly stable when it is complexed with one molecule of p24 in a heterotrimer. Apparently, p24 suppresses the intrinsic tendency of dynamitin to self-associate into tetramers and higher order complexes. p24 is predicted to be largely α-helical and may stabilize dynamitin dimers by associating with them in a triple-coil structure.

The ability of dynamitin to self-associate and to bind to other dynactin subunits, particularly p24, makes it an intriguing subject for further study. Ongoing work in our laboratory is focused on identifying and characterizing the protein structural domains and motifs that support interactions between dynamitin and p24, as well as dynamitin and itself.

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