Calmodulin Confers Calcium Sensitivity on Ciliary Dynein ATPase

J. J. BLUM, ALVERNON HAYES, GORDON A. JAMIESON, Jr., and THOMAS C. VANAMAN
Departments of Physiology and of Microbiology and Immunology, Duke University, Durham, North Carolina 27710

ABSTRACT
Extraction of demembranated cilia of Tetrahymena by Tris-EDTA (denoted by the suffix E) yields 14S-E and 30S-E dyneins with ATPase activities that are slightly increased by Ca++. This effect is moderately potentiated when bovine brain calmodulin is added to the assay mixture. Extraction with 0.5 M KCl (denoted by the suffix K) yields a 14S-K dynein with a low basal ATPase activity in the presence of Ca++. Subsequent addition of calmodulin causes marked activation (up to 10-fold) of ATPase activity. Although 14S-K and 14S-E dyneins have Ca++-dependent ATPase activities that differ markedly in the degree of activation, the concentration of calmodulin required for half-maximal saturation is similar for both, ~0.1 μM. Both 30S-K and 30S-E dyneins, however, require ~0.7 μM bovine brain calmodulin to reach half-maximal activation of their Ca++-dependent ATPase activities. Tetrahymena calmodulin is as effective as bovine brain calmodulin in activating 30S dynein, but may be slightly less effective than the brain calmodulin in activating 14S dynein. Rabbit skeletal muscle troponin C also activates the Ca++-dependent ATPase activity of 30S dynein and, to a lesser extent, that of 14S dynein, but in both cases is less effective than calmodulin.

The interaction of calmodulin with dynein that results in ATPase activation is largely complete in <1 min, and is prevented by the presence of low concentrations of ATP. Adenylyl imidodiphosphate can partially prevent activation of dynein ATPase by calmodulin plus Ca++, but at much higher concentrations than required for prevention by ATP. β,γ-methyl-adenosine triphosphate appears not to prevent this activation.

The presence of Ca++-dependent calmodulin-binding sites on 14S and 30S dyneins was demonstrated by the Ca++-dependent retention of the dyneins on a calmodulin-Sepharose 4B column. Gel electrophoresis of 14S dynein that had been purified by the affinity-chromatography procedure showed the presence of two major and one minor high molecular weight components. Similar analysis of 30S dynein purified by this procedure also revealed one major and one minor high molecular weight components that were different from the major components of 14S dynein.

Ca++-dependent binding sites for calmodulin were shown to be present on axonemes that had been extracted twice with Tris-EDTA or with 0.5 M KCl by the use of 35S-labeled Tetrahymena calmodulin. It is concluded that the 14S and 30S dyneins of Tetrahymena contain Ca++-dependent binding sites for calmodulin and that calmodulin mediates the Ca++-regulation of the dynein ATPases of Tetrahymena cilia.

A key role for Ca++ in the control of ciliary and flagellar motility has been well documented (see reference 13 for a recent review). Most of the effects of Ca++ have been studied in intact systems, where Ca++ influx and efflux through the membrane was demonstrated to control the direction and/or frequency of beating. Several effects of Ca++ on demembranated axonemal preparations have also been observed. These include reversal of direction of wave propagation in flagella of Crithidia (26), reversal of direction of beat in cilia of Paramecium (37), and an increase in the pellet height response of...
demembranated cilia of Tetrahymena (8). However, the molecular mechanisms responsible for the effect of Ca" on the axonemal components are unknown. The recent finding that Tetrahymena contain calmodulin (28, 31) and that part of this calmodulin is in ciliary axonemes and is associated with the 14S dynein fraction (28), suggested that calmodulin might confer Ca"-sensitivity on the 14S dynein—possibly on its ATPase activity. Preliminary studies showed that the ATPase activity of crude dynein obtained by Tris-EDTA extraction was indeed enhanced by addition of Ca" if additional calmodulin was present, but the effects were small. Because Doughty (17) has used a KCl extraction procedure to observe the effects of low concentrations of Ca" on Mg"-dependent dynein ATPase activity, it seemed possible that a KCl extraction procedure might be more effective in stripping calmodulin from the dyneins and, hence, in yielding dyneins that were sensitive to Ca" only in the presence of added calmodulin. Greater sensitivity to calmodulin was indeed observed after KCl extraction and it is that sensitivity to Ca" in the presence of added calmodulin which initiated the studies presented in this report.

MATERIALS AND METHODS

Materials

2-Chloro-10-(3-aminopropyl)phenothiazine (CAPP), a generous gift from Dr. Carl Kaiser of the Smith, Kline and French Laboratories, Philadelphia, Pa., was coupled to Sepharose 4B and the resulting CAPP-Sepharose 4B conjugate prepared for use as previously described (27) and used as detailed below under the headings Calmodulin Purification and Affinity Chromatography. Calmodulin-Sepharose 4B was prepared, characterized, and used as previously described (48). Sephade gel filtration media were prepared for use as described by Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. SDS, acrylamide, and bisacrylamide were used as supplied by BDH Chemicals, Ltd., Poole, England, N,N,N',N'-tetramethylenediamine (TEMED) and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PMSF, phenylmethyl-

Analytical Procedures

Procedures for measurement of protein concentration were as described (10), except that the concentrations of bovine brain and Tetrahymena calmodulin and of TnC stock solutions were determined by amino acid analysis after acid hydrolysis (45). ATPase activity was assayed at pH 7.5 for 20 min at 25°C in a total volume of 1.0 ml and a final concentration of 1.0 mM ATP unless otherwise specified, as described earlier (9). All assays were done in duplicate, and reproducibility was within ±5%. At the dye concentrations used, phosphate release was linear over the course of the assay. Gel electrophoretic procedures were performed as detailed in the text and figure legends.

Calmodulin Purification

2S-Labeled Tetrahymena Calmodulin: Two cultures of 100 ml each of Tetrahymena were grown to early stationary phase (~528,000 cells/ml). The cells were collected by centrifugation for 3 min at 900 g at room temperature and resuspended at a density of ~140,000 cells/ml in 60 ml of Wagner's salt solution (45) at 25°C. A culture of E. coli Na-22 (14) containing ~5 Mci of incorporated [35S]sulfate (the generous gift of Dr. F. H. Schachar, Duke University, Durham, N. C.) was then added to the Triton-EDTA containing the Tetrahymena, and the mixed cultures were incubated at 25°C with shaking. No background radioactivity was observed after 20 min. Shaking was continued for an additional 3 h, at which time the cells were pelleted and frozen until processed as described below.

Purification of [35S]Tetrahymena calmodulin was generally as previously described (27). Cells were homogenized in two volumes of 20 mM TES-NaOH/1 mM 2-mercaptoethanol/1 mM EDTA (pH 7.0) containing 1 mM PMSF and 2.5 mg/liter leupeptin (4). The homogenate was centrifuged at 10,000 g for 30 min and the resulting supernate processed batchwise onto 10 g of DEAE-cellulose (DE-52, Whatman Inc., Clifton, N. J.) that had been equilibrated with homogenization buffer. The resin was washed twice with 100 ml of homogenization buffer containing 0.1 M NaCl and the resin-bound material was then eluted with 100 ml of homogenization buffer containing 0.5 M NaCl. After addition of CaCl2 (to 5 mM) to the high-salt eluant, it was added to PP-Sepharose 4B column (27) of 25-ml bed volume. The affinity column was then washed with 100 ml of homogenization buffer containing 0.5 M NaCl and 5 mM CaCl2. Material bound in a Ca"-dependent manner to this affinity column was eluted with homogenization buffer containing 0.5 M NaCl and 10 mM EGTA. Fractions containing EGTA-eluted radioactive material were pooled, Ca" in excess of the EGTA present in the elution buffer added, and the pooled material dialyzed against 1 mM NH4HCO3/1 mM 2-mercaptoethanol and finally freeze-dried. The freeze-dried material was dissolved in 0.5 ml of 10 mM NH4HCO3 and subjected to gel filtration on a column (1.5 x 130 cm) containing Sephadex G-100, using 10 mM NH4HCO3 as the eluant. Fractions (2 ml) were analyzed for radioactivity and by electrophoresis on alkaline urea/10% polyacrylamide gels (23). Calmodulin being eluted was estimated by Coomassie Blue staining and densitometry of the gel after drying. Fractions containing homogeneous [35S]calmodulin were pooled and freeze-dried, and a portion was used to study the binding of calmodulin to twice-extracted axonemes, as described below. The specific activity of the [35S]Tetrahymena calmodulin (~1.3 x 107 cpm/pg) was obtained by subjecting a sample containing a known number of counts to alkaline urea PAGE (polyacrylamide gel electrophoresis) and estimating the protein concentration of the calmodulin band by the level of Coomassie Blue staining (45).

Unlabeled Calmodulins: Tetrahymena calmodulin was isolated exactly as described above for the isolation of radioactive material, except on a larger scale. Bovine brain calmodulin was also purified with this procedure, except that ion-exchange chromatography on DEAE-Sephadex A-50, as described previously (27), was inserted between the CAPP-Sepharose 4B affinity chromatography and Sephadex G-100 gel filtration steps. The yields of Tetrahymena and of bovine brain calmodulin obtained by these procedures were significantly greater than those previously described (28, 45, 46). These methods represent those currently in use in our laboratories for the purification of calmodulin.

Cilia Preparation and Extraction

Cilia were prepared from cultures of Tetrahymena pyriformis strain HSM and demembranated with 0.05% Triton X-100 as described previously (7). The procedures for preparation of crude dynein by extraction of the axonemes with Tris-EDTA (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.2) and for preparation of 30S and 45S dyneins from the crude dynein by sucrose density gradient sedimentation have also been described (6, 7). KCl extraction, procedures were performed as follows: The demembranated axonemes were resuspended in IMT/6 buffer (8.33 mM Tris-HCl/8.33 mM imidazole/12.5 mM MgCl2/0.067 mM EGTA, pH 7.5) containing 0.15 M, 0.3 M, or 0.5 M KCl and gently stirred at 4°C for the times specified in the text. The suspension was then centrifuged (12,000 g for 10 min for the 0.15 or 0.30 M KCl extractions, 27,000 g for 20 min for the 0.5 M KCl extraction) yielding a supernatant fraction, SI, and a pellet fraction, PI, where i is the number of such extraction steps. The supernatant of the 0.5 M KCl extraction step were resolved by sucrose density gradient centrifugation exactly as for crude dynein obtained by the Tris-EDTA extraction procedure. To distinguish between fractions obtained by Tris-EDTA extraction and fractions obtained by KCl extraction, we shall use the suffixes E and K, respectively. Thus, PI-E is the pellet fraction and SI-E the supernatant fraction obtained by one extraction step with Tris-EDTA, whereas PI-K and SI-K are obtained after one extraction with a specified concentration of KCl. Similarly, sucrose-gradient-
purified dyneins obtained from SI-E and SI-K will be referred to as 14S-E or 30S-E and 14S-K or 30S-K dyneins, respectively. Dyneins obtained from a second extraction of PI-E with Tris-EDTA will be designated 14S-E2 and 30S-E2, and those obtained from a second extraction of PI-K with 0.5 M KCl as 14S-K2 and 30S-K2.

In an attempt to optimize conditions for extraction of dyneins with high sensitivity to calmodulin, we have varied the times of extraction of axonemes and of PI-K with 0.5 M KCl. However, no simple relation was found between duration of extraction and degree of calmodulin sensitivity of the dyneins obtained. To avoid uncertainty, the duration of each extraction step will be specified as necessary.

**Affinity Chromatography**

Partially purified 14S and 30S dyneins, prepared by sucrose density gradient sedimentation, were chromatographed separately over three columns connected in series. Each column was equilibrated with IMM/6 buffer containing 1.5 mM Ca"²⁺ (column buffer). The columns contained the following resins: column 1, Sepharose 4B (2.5-ml bed volume); column 2, CAPP-Sepharose 4B (2.5-ml bed volume); column 3, calmodulin-Sepharose 4B (10-ml bed volume, 0.6 mg calmodulin/ml). These columns were used to sequentially bind nonspecific aggregates, calmodulin, and proteins with calmodulin binding sites, e.g., dynein ATPases (see Results). The 14S or 30S dynein was applied after a 1:1 dilution with column buffer and addition of Ca"²⁺ to yield a solution 1.5 mM with respect to this cation. After application of the sample, the columns were washed with 30 ml of column buffer. The columns were then separated and any material bound in a Ca"²⁺-dependent manner was eluted from each column with elution buffer (column buffer with 1 mM EGTA replacing the Ca"²⁺). The fractions obtained from each column were analyzed as described in Results.

**Binding of [³⁵S]Calmodulin to Pellet 2**

After a second extraction with Tris-EDTA, 0.5 ml of PI-E was added to 0.3 ml IMM/6 buffer (50 mM Tris-HCl, 50 mM imidazole, 7 mM MgCl₂, 4 mM EGTA), pH 7.5, 0.1 ml of either 10 mM EGTA or 12.5 mM Ca"²⁺, and 0.6 ml of [³⁵S]calmodulin (~23,000 cpm) dissolved in H₂O. The mixtures were incubated for 4 min at 25°C and then centrifuged for 20 min at 24,000 g at 0°C. 1 ml of the clear supernatant was carefully removed and placed in a counting vial. The remaining supernate was decanted and the pellet dissolved in 0.5 ml of 0.5 M NaOH and quantitatively transferred to a counting vial by rinsing of the centrifuge tubes with ACS scintillant (Amersham-Searle Corp., Arlington Heights, Ill.). Radioactivity in the vials was determined in a Tri-Carb spectrometer (Packard Instrument Co., Downers Grove, Ill.) equipped with an automatic external standard and corrected to the same quench as that of 0.5 ml of the [³⁵S]calmodulin solution in ACS.

**RESULTS**

A preliminary study showed that very little ATPase activity was extracted when demembranated axonemes were treated with 0.15 M KCl in IMM/6 buffer for 2 h, and that the pellet PI-K remaining after centrifugation still demonstrated a decrease in turbidity (measured at 350 nm) upon addition of ATP. Addition of Ca"²⁺ to the supernate (SI-K) caused a small but reproducible (n > 10) increase in ATPase activity, whereas addition of Ca"²⁺ plus 10 μg of bovine brain calmodulin caused up to a 25% increase in ATPase activity. Similar results were obtained with S2-K and P2-K, obtained after a further 2-h extraction of PI-E with 0.3 M KCl in IMM/6 buffer, and with S3-K and P3-K, obtained after extraction of P2-K with 0.5 M KCl in IMM/6 buffer. In these experiments, the ATPase assay was initiated by adding 0.1 ml of Si to 0.9 ml of reaction mixture containing ATP, Ca"²⁺, bovine brain calmodulin, and buffer. It was then discovered that if the calmodulin was preincubated with the supernatant fraction, a larger degree of activity enhancement could be obtained, as shown in Table I. Activity enhancement was almost complete in 30 s, and increasing the amount of calmodulin added to the assay from 10 to 20 μg resulted in only a small, but significant, additional increase in ATPase activity. All subsequent experiments containing calmodulin were performed with 10 μg in the assay mixture unless otherwise specified. Calmodulin alone (10 μg) had no ATPase activity.

In the absence of a large supply of purified *Tetrahymena* calmodulin, we have used homogeneous bovine brain calmodulin for most of these experiments. Because of the similarity in amino acid composition and other physicochemical properties between *Tetrahymena* calmodulin and bovine brain calmodulin (28) and the similarity in their Ca"²⁺-dependent activities (28)—i.e., both proteins (a) activate partially purified "activator-depleted" bovine brain cyclic nucleotide phosphodiesterase (30), (b) form complexes with rabbit skeletal muscle troponin inhibitory subunit (TnI), and (c) bind to phenothiazines—it seemed unlikely that there would be any appreciable differences between these two calmodulins in their ability to enhance dynein ATPase activity. To test this assumption, sufficient *Tetrahymena* calmodulin was prepared to perform the experiments shown in Fig. 1. It can be seen that *Tetrahymena* calmodulin is as effective as bovine brain calmodulin as an activator of the Ca"²⁺-dependent ATPase activity of 30S-E dynein. Similar results were obtained with 30S-E2. It appears that bovine brain calmodulin may be a slightly more effective activator of 14S-E dynein ATPase than the *Tetrahymena* calmodulin. Another experiment with 14S-E2 dynein confirmed this observation. Further clarification of the differences between the ATPase stimulating ability of vertebrate and *Tetrahymena* calmodulins, if any, must await the preparation of more *Tetrahymena* calmodulin. In any case, bovine brain calmodulin is at least as effective as *Tetrahymena* calmodulin in enhancing the Ca"²⁺-dependent ATPase activities of both 14S-E and 30S-E dyneins, so that studies using the brain protein are likely to yield essentially the same results as would be obtained with *Tetrahymena* calmodulin. In what follows, the term "calmodulin" refers to the bovine-brain-derived protein unless otherwise specified.

**Effects of Calmodulin on Dyneins Prepared by Tris-EDTA- and KCI-Extraction Procedures**

Table II presents results obtained from studies in which demembranated axonemes were washed in IMM/6 buffer and divided into two equal portions that were treated separately as
follows: One portion was extracted with Tris-EDTA, yielding S1-E and P1-E, containing, respectively, 51 and 39% of the original total ATPase activity. The other portion was extracted for 2 h with 0.3 M KCl in IMT/6 buffer and then centrifuged, yielding S1-K and P1-K, containing 25 and 57% of the original ATPase activity. P1-K was then extracted for ~20 h with 0.5 M KCl in IMT/6, yielding S2-K and P2-K, each containing 47% of the activity in P1-K. 3 ml each of S2-K and S1-E were layered onto sucrose density gradients (made up in IMT/6 buffer), and the peak fractions were assayed for ATPase activity in the presence and absence of Ca"++. Calmodulin, when present at the concentrations shown on the abscissa, was preincubated with the dynein for 4.0 min at 25°C before the 0.1 ml of 10 mM ATP was added to initiate the ATPase activity assay. Circles, 30S dynein; squares, 14S dynein; open symbols, bovine brain calmodulin; filled symbols, Tetrahymena calmodulin.

The supernatant (S1-K) and pellet (P1-K) fractions obtained from a 2-h extraction of axonemes with 0.3 M KCl exhibited ATPase activities that were only mildly sensitive to Ca"++ in the presence of calmodulin (Table II). When P1-K was extracted with 0.5 M KCl in IMT/6 buffer, the ATPase activity of the resulting supernatant fraction, S2-K, was more sensitive to calmodulin stimulation than any of the fractions obtained from the Tris-EDTA fractionation procedure. Although both the 30S-K2 and 14S-K2 dynein ATPases obtained from sucrose density fractionation of S2-K were more sensitive to calmodulin stimulation than the corresponding fractions obtained from the Tris-EDTA fractionation, it is clear that the major effect of the 0.5 M KCl extraction procedure was to yield a 14S-K2 dynein ATPase that showed a marked enhancement of activity in the presence of Ca"++ plus calmodulin. In one KCl-extraction experiment, in which the basal ATPase activity of the 14S-K fraction was very low, addition of calmodulin gave a 10-fold enhancement.

The above experiments were performed with no added Ca"++ (i.e., 0.13 mM EGTA) or a sufficient excess of Ca"++ to yield >0.1 mM free Ca"++ in the assay mixtures. Several experiments were performed to estimate the free Ca"++ concentration required to produce the full calmodulin-dependent Ca"++ sensitivity of the ATPase activity. For a preparation of 14S-K dynein (from an 0.5 M KCl extraction of axonemes), full activation (2.6-fold) was obtained at <1.1 x 10^-5 M free Ca"++.

Full activation of the pellet fraction, P1-E, obtained after Tris-EDTA extraction of demembranated axonemes, was evident...
at \(<10^{-5}\) M free \(\text{Ca}^{++}\). (Computations, performed with a program provided by Professor C. Tanford of Duke University, included the four \(\text{Ca}^{++}\) ions bound to the calmodulin.) Although further studies will be necessary to establish the concentration of \(\text{Ca}^{++}\) required for half-maximal activation of dynein ATPases in the presence of calmodulin, it is clear that the effect occurs in the same range \((10^{-9}-10^{-5}\) M\) as required for the \(\text{Ca}^{++}\) effect on the pellet height response of *Tetrahymena* axonemes (8). It is important to note that the range of minimum \(\text{Ca}^{++}\) concentration required for maximum ATPase stimulation correlates well with calmodulin’s affinity for \(\text{Ca}^{++}\) (four binding sites, \(K_d = 1 \times 10^{-8}\) M [40]). It should also be mentioned that there was practically no change in ATPase activity with free \(\text{Ca}^{++}\) in the range 0.1-2 mM in the presence or absence of calmodulin.

**Effect of CPZ on the Ability of \(\text{Ca}^{++}\) to Cause Enhancement of Dynein ATPase Activity in Presence of Calmodulin**

In the presence of \(\text{Ca}^{++}\), calmodulin has a high affinity for CPZ (32, 49), a fact that serves as the basis for the use of an analogue of CPZ (i.e., CAPP) as a ligand for the affinity-column purification of calmodulin (27) (see Materials and Methods). CPZ has been shown to inhibit a number of \(\text{Ca}^{++}\)-calmodulin-activated enzymatic activities (44, 49), and in our earlier report (28), it was noted that CPZ partially inhibited the ATPase activities of axonemes and of \(14\)S and \(30\)S dyneins. It was therefore of interest to ascertain whether low concentrations of CPZ might negate the activity enhancing effects of calmodulin in the presence of calcium. In the experiment shown in Table III, axonemes were extracted for 40 min with 0.3 M KCl, followed by 1.2 h with 0.5 M KCl, yielding an \(\text{S}_{2-K}\) that was then subjected to sucrose density gradient sedimentation. Addition of calmodulin in the absence of added \(\text{Ca}^{++}\) (0.13 mM EGTA; \(\approx 10^{-8}\) M free \(\text{Ca}^{++}\)) caused a slight increase in ATPase activity of both 14S-K2 and 30S-K2 dynein ATPases, as did addition of \(\text{Ca}^{++}\) alone. In the presence of both \(\text{Ca}^{++}\) and calmodulin, the 30S-K2 dynein ATPase activity was stimulated \(~\text{1.7-fold, and that of 14S-K2 dynein ATPase}\) as did addition of \(\text{Ca}^{++}\) alone. The range of free \(\text{Ca}^{++}\) concentrations which 30S-K and 14S-K dyneins, prepared by extraction with 0.5 M KCl, showed a high affinity for \(\text{Ca}^{++}\) (calmodulin) that was then subjected to sucrose density gradient sedimentation of the supernate, \(51-K\). It was noted above (see text pertaining to Table I) that the protein concentrations during the ATPase assays were 155 and 213 nmol/min/mg, respectively, and the protein concentrations during the ATPase assay were then initiated by the addition of 0.1 ml of 10 mM ATP to both sets of assay mixtures (thus bringing the final volume to 1 ml). Exp. I was performed on a different preparation of cilia that was extracted for 3.8 h with 0.5 M KCl in IMT/6 buffer. After centrifugation, the pellet, \(P_{1-K}\), was resuspended in IMT/6 and assayed as described for exp. I, as was the 14S-K dynein obtained by sucrose density sedimentation of the supernate, \(S_{2-K}\). For exp. I, 100% ATPase activity of the 30S-K2 and 14S-K2 dyneins was 226 and 213 nmol/min/mg, respectively, and the protein concentrations during the assays were 17.5 and 14.0 \(\mu\)g/ml, respectively. For exp. II, 100% ATPase activity was 80.5 nmol/min/mg for 14S-K and 79.9 nmol/min/mg for the 14S-K dynein, and the protein concentrations during the ATPase assays were 155 and 128 \(\mu\)g/ml, respectively.

**Inhibition of ATPase Activation by Adenine Nucleotides**

It was noted above (see text pertaining to Table I) that the effect of calmodulin in causing enhancement of the ATPase activity of crude dynein required a brief preincubation of the crude dynein fraction with calmodulin in the absence of ATP. In those experiments, not only was a crude dynein used but the ATP concentration was 1 mM, the standard concentration used in our ATPase assay procedure. It was therefore of interest to ascertain whether the prevention of the \(\text{Ca}^{++}\)- and calmodulin-dependent enhancement of ATPase activity occurred with succrose-density-purified dyneins, whether it was a function of ATP concentration, and whether AMP-PNP and AMP-PCP, nonhydrolyzable analogues of ATP that have proven useful in probing the properties of dynein ATPases (10, 38), could also prevent the enhancing effect of calmodulin.

Exp. I of Table IV shows the results of an experiment in which 30S-K and 14S-K dyneins, prepared by extraction with 0.5 M KCl, were added to the assay mixtures (thus bringing the final volume to 1 ml). Exp. II was performed on the same cilia preparation described in the legend to Table III. Samples under start were preincubated for 240 s in the presence of \(0.13\) mM free \(\text{Ca}^{++}\), calmodulin, and the indicated nucleotide. Control assays (under end) received the nucleotide after 260 s of incubation. The ATPase assay was then initiated by adding 0.1 ml of 10 mM ATP to both sets of assay mixtures (thus bringing the final volume to 1 ml). Exp. I was performed on a different preparation of cilia that was extracted for 3.8 h with 0.5 M KCl in IMT/6 buffer. After centrifugation, the pellet, \(P_{1-K}\), was resuspended in IMT/6 and assayed as described for exp. I, as was the 14S-K dynein obtained by sucrose density sedimentation of the supernate, \(S_{1-K}\).

**Table IV**

| Additions | % Basal ATPase activity |
|-----------|-------------------------|
| **Calmodulin** | **Chlorpromazine** | **30S-K2 dynein** | **14S-K2 dynein** |
| + | + | 127 | 118 |
| + | + | 114 | 104 |
| + | + | 165 | 148 |
| + | + | 120 | 103 |
| + | + | 134 | 129 |

| Exp. | Nucleotide | Start* | End* | Start | End |
|------|-----------|--------|------|------|-----|
| I | - | - | (100) | (100) |
| + | - | 127 | 118 |
| + | ATP, \(10^{-5}\) M | 148 | 132 |
| + | AMP-PNP, \(10^{-4}\) M | 158 | 149 |
| + | AMP-PCP, \(10^{-4}\) M | 117 | 132 |
| II | - | - | (100) | (100) |
| + | ATP, \(10^{-4}\) M | 121 | 152 |
| + | ATP, \(2 \times 10^{-5}\) M | 148 | 151 |
| + | ATP, \(5 \times 10^{-8}\) M | 158 | 134 |
| + | AMP-PNP, \(2 \times 10^{-4}\) M | 117 | 132 |

Exp. I was performed on the same cilia preparation described in the legend to Table III. Samples under start were preincubated for 240 s in the presence of \(0.13\) mM free \(\text{Ca}^{++}\), calmodulin, and the indicated nucleotide. Control assays (under end) received the nucleotide after 260 s of incubation. The ATPase assay was then initiated by adding 0.1 ml of 10 mM ATP to both sets of assay mixtures (thus bringing the final volume to 1 ml). Exp. II was performed on a different preparation of cilia that was extracted for 3.8 h with 0.5 M KCl in IMT/6 buffer. After centrifugation, the pellet, \(P_{1-K}\), was resuspended in IMT/6 and assayed as described for exp. I, as was the 14S-K dynein obtained by sucrose density sedimentation of the supernate, \(S_{1-K}\). For exp. I, 100% ATPase activity of the 30S-K2 and 14S-K2 dyneins was 226 and 213 nmol/min/mg, respectively, and the protein concentrations during the assays were 17.5 and 14.0 \(\mu\)g/ml, respectively. For exp. II, 100% ATPase activity was 80.5 nmol/min/mg for 14S-K and 79.9 nmol/min/mg for the 14S-K dynein, and the protein concentrations during the ATPase assays were 155 and 128 \(\mu\)g/ml, respectively.

- **Start**, nucleotide added at start of preincubation interval; **End**, nucleotide added at end of preincubation interval.
0.5 M KCl, were preincubated for 4 min in the presence of 0.1 mM ATP, AMP-PNP, or AMP-PCP. As controls, the same concentrations of these nucleotides were added at the end of the 4-min preincubation period, just before addition of 1 mM ATP for the 20-min ATPase assay. With no additions to the reaction mixtures containing 14S-K dynein and calmodulin (plus Ca**+), a 3.2-fold enhancement of ATPase activity occurred. Similar results were obtained when 0.1 mM ATP or AMP-PNP was added at the end of the preincubation period. However, when the same concentration of ATP or AMP-PNP was present at the start of the preincubation, only a 1.3-fold (for ATP) or a 2.5-fold (for AMP-PNP) activation was obtained. Hence, 0.1 mM ATP almost completely prevented the stimulation effect of calmodulin on 14S-K dynein ATPase. Though 0.1 mM AMP-PNP was less effective, it nevertheless prevented some of the Ca**+-calmodulin-induced activation of ATPase activity. AMP-PCP is a stronger inhibitor of dynein ATPase than is AMP-PNP (10). This is evident in Exp. I of Table IV. The inhibition of 14S-K dynein activity by 0.1 mM AMP-PCP was about the same whether the AMP-PCP was added at the beginning or end of the preincubation period with calmodulin, suggesting that AMP-PCP did not prevent Ca**+-calmodulin ATPase-activity enhancement but acted only as an inhibitor of ATPase activity. As shown in Table IV, similar results were obtained for 30S-K dynein, but because the enhancement effect observed was much smaller than for 14S-K dynein, conclusions similar to those presented above must be regarded as tentative.

Exp. II of Table IV shows that considerable inhibition of the stimulatory effect of Ca**+-calmodulin on 14S-K dynein ATPase can be obtained by the presence of an ATP concentration as low as 5.6 ÌM during preincubation. Equivalent inhibition of 14S-K ATPase activity by Ca**+-calmodulin was obtained with 1.1 x 10^-4 M ATP and 2.2 x 10^-9 M AMP-PNP. These results also demonstrate that ATP can partially prevent the Ca**+-calmodulin enhancement of ATPase activity in the pellet, P1-K, obtained from a 3.8-h extraction of axonemes with KCl. However, it appears that a much higher concentration of ATP is required for prevention of the Ca**+-calmodulin stimulatory effects on the pellet ATPase. Again this statement must be regarded as tentative because of the small degree of activity stimulation observed. Based on these results, all subsequent assays employed a 4-min preincubation of the calmodulin with dynein before initiation of the assay, providing ample time for the calmodulin effect to occur.

**Sensitivity of 14S and 30S Dynesins to Ca**+-calmodulin-induced Enhancement of ATPase Activity**

In the experiments so far described, bovine brain calmodulin was used at a concentration of 10 Ìg/assay, with one exception (Table I), in which 20 Ìg yielded only a slight increase in enhancement over that obtained with 10 Ìg. In that experiment, however, the dyneins were not preincubated with the calmodulin before addition of ATP. Figs. 1 and 2 show the results of detailed analyses of the effects of calmodulin concentration on dynein ATPase activity. The effect of added calmodulin on the Ca**+-dependent ATPase activity of 14S-E dynein differs from that of 30S-E dynein (Fig. 1). The ATPase activity of 30S-E dynein rises almost linearly with increasing calmodulin concentration up to at least 2.2 ÌM calmodulin. On the other hand, 14S-E dynein ATPase activity rises sharply at very low concentrations of calmodulin and then only slowly as the calmodulin concentration increases above 0.2 ÌM. Identical results were obtained with 14S-E and 30S-E dyneins obtained from a different preparation of axonemes. The response of 14S-K2 dynein, obtained from extraction of axonemes by 0.5 M KCl for 6 h followed by a second extraction with 0.5 M KCl for 21 h is shown in Fig. 2. The 14S-K2 dynein ATPase activity was stimulated more than eightfold by calmodulin (in the presence of Ca**+), half-maximal enhancement occurring, as with 14S-E dynein, at ~0.1 ÌM calmodulin. The Ca**+-dependent ATPase activity of 30S-K2 dynein was similar to that of 30S-E, i.e., a slowly increasing activity with increasing calmodulin concentration that appeared to saturate at ~2.2 ÌM (Fig. 2). Thus 14S dynein, whether prepared by Tris-EDTA or KCl extraction, has a Ca**+-dependent ATPase activity that is sensitive to a much lower concentration of calmodulin than is the activity of 30S dynein.

The results presented above show that 14S-K and, to a lesser extent, 30S-K dyneins have Ca**+-dependent ATPases that are more sensitive to stimulation by calmodulin than those obtained by EDTA extraction. It was therefore of interest to examine the effect of KCl treatment on 30S and 14S dyneins that had been prepared from Tris-EDTA-extracted axonemes. As can be seen in Table V, incubation with 0.5 M KCl caused
an increase in the basal ATPase activity for both dyneins. However, a concomitant increase in activatability of ATPase activities was not observed. It appears, therefore, that conditions during extraction from the axonemes (and possibly during subsequent sedimentation through the sucrose density gradient), rather than the effect of KCl on the extracted dyneins, determine the subsequent sensitivity of Ca**+-dependent ATPase activity to calmodulin.

**Effect of TnC on Dynein ATPase Activities**

Bovine brain calmodulin has considerable structural homology with the Ca**+-regulatory protein of actomyosin ATPase, TnC (22, 42, 46, 47), interacts Ca**+-dependently with the inhibitory subunit of the actomyosin regulatory complex, TnI, and can substitute for TnC in conferring Ca**+-sensitivity on actomyosin ATPase (1). It was therefore of interest to ascertain whether TnC could replace calmodulin in conferring Ca**+-dependent activation on dynein ATPase activities. As shown in Fig. 2, TnC activated both 14S-K2 and 30S-K2 ATPases to a limited extent. The activation was less than that achieved with similar concentrations of calmodulin, and TnC stimulated the activity of 14S-K2 dynein much less than it did 30S-K2 dynein. This difference in sensitivity to TnC between 14S and 30S dyneins was also observed with dyneins prepared by Tris-EDTA extraction of axonemes (Fig. 3). Fig. 2 further shows that although addition of 1.5 μM calmodulin to the assay system increased the Ca**+-dependent ATPase activities of 14S-K2 and 30S-K2 dyneins eightfold and threefold, respectively, the same concentration of TnC yielded less than a twofold increase of the ATPase activity of either dynein. At the highest concentration of TnC studied (5.4 μM), the same increase in ATPase activity (~3.2-fold) was obtained for 30S-K2 dynein as was obtained with calmodulin, but the activation of 14S-K2 by this amount of TnC was much less than that caused by calmodulin (Fig. 2). Thus, the ATPase activity of 14S dynein is much less responsive to Ca**+-dependent stimulation by TnC than is the ATPase activity of 30S dynein ATPase, regardless of whether the dyneins are prepared by KCl or Tris-EDTA extraction of axonemes. In preliminary experiments, we have found 0.4 μM TnC to have no effect on the ATPase activities of either P1-K or P1-E.

**Affinity Chromatography of 14S and 30S Dyneins**

The ability of calmodulin to confer Ca**+-sensitivity on 14S and 30S dynein ATPases implies the presence of a calmodulin-binding site(s) on these enzymes. Direct evidence for the presence of such a binding site(s) was sought by subjecting the dyneins to Ca**+-dependent affinity chromatography on a calmodulin-Sepharose 4B column. In an effort to remove any aggregates that might be present, and to remove any endogenous calmodulin present in the samples, the material to be analyzed (pooled 30S-E or 14S-E fractions from Tris-EDTA-extracted axonemes resolved by sucrose density gradient sedimentation) was passed sequentially through a Sepharose 4B column and a CAPP-Sepharose 4B column connected serially to and mounted vertically above the calmodulin-Sepharose 4B column (see Materials and Methods). The columns were washed with a Ca**+-containing buffer, and then eluted separately with a buffer containing EGTA to release any proteins that bound in a Ca**+-dependent manner. The results of one such experiment are presented in Fig. 4; essentially identical results were obtained with 30S-E and 14S-E dyneins from a different preparation of Tris-EDTA-extracted axonemes. Analysis of the material that passed unretracted through the chromatographic analysis described above revealed little ATPase activity or protein to be present (see Figs. 4 and 5). For both the 14S and 30S dynein affinity-chromatography separations, EGTA elution of the Sepharose 4B column also yielded an eluant virtually devoid of protein and ATPase activity, suggesting that few if any large aggregates had been nonspecifically adsorbed to the Sepharose 4B resin. Similarly, the EGTA eluant from the CAPP-Sepharose 4B column contained very little protein and had no ATPase activity (Fig. 4).
FIGURE 4 Serial Sepharose 4B/CAPP-Sepharose 4B/Calmodulin-Sepharose 4B affinity chromatography of 14S and 30S dyneins prepared from Tris-EDTA-extracted axonemes. 9 ml of pooled 14S dynein (1.0 mg protein), lower panel, and 11.8 ml of pooled 30S dynein (1.9 mg protein), upper panel, were diluted 1:1 with IMT/6 + 1.5 mM Ca++, pH 7.5, and applied onto a column of Sepharose 4B that was connected to a CAPP-Sepharose 4B column and finally to a column of calmodulin-Sepharose 4B, as described in Materials and Methods. After sample application was complete, the columns were washed with ~30 ml IMT/6 (pH 7.5) buffer containing 1.5 mM Ca++. The columns were then disconnected and eluted separately with buffer containing 1.0 mM EGTA instead of the calcium. The A₂₅₀ (A) of each fraction was determined and aliquots were taken from selected fractions for assay of ATPase activity (1.25 mM total Ca++) in the absence of added calmodulin (O) or after addition of 10 μg of bovine brain calmodulin (●). For further details, see text.

FIGURE 5 Analysis of fractions from affinity chromatography of 14S and 30S dyneins by gel electrophoresis. Fractions from the experiments shown in Fig. 4 were subjected to slab gel electrophoresis on SDS-7.5% polyacrylamide gels. Lanes: A and H, 4 μg each of Mr standards (phosphorylase b, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; and carbonic anhydrase, 30,000); B, 0.5 ml of fraction 30 of the 14S dynein affinity-column separation; C and D, 2.5 and 5.0 μg, respectively, of the 14S dynein eluted from the calmodulin-Sepharose 4B column; E, 0.5 ml of fraction 50 of the 30S dynein affinity-chromatography separation; F and G, 2.5 and 5.0 μg, respectively, of the 30S dynein eluted from the calmodulin-Sepharose 4B column. The gel was electrophoresed until the dye band was ~1 cm from the bottom of the gel.

of the CAPP-Sepharose 4B EGTA-eluant fractions by alkaline urea-PAGE did not reveal the presence of calmodulin, but the presence of small amounts of calmodulin below the limits of sensitivity of the analysis cannot be excluded. Recent experiments using [³⁵S]Tetrahymena calmodulin support this conclusion. Over 99% of the [³⁵S]calmodulin added to the material applied to an identical set of serial affinity columns was retained by the CAPP-Sepharose 4B column in the presence of Ca++. When the calmodulin-Sepharose 4B column was eluted with the EGTA-containing buffer, a single sharp peak of protein and of basal ATPase activity was eluted (Fig. 4) demonstrating the presence of a Ca++-dependent binding site(s) for calmodulin on both 14S and 30S dyneins. In one experiment, the pooled fractions from the calmodulin-Sepharose 4B column of 30S dynein were resedimented in a sucrose density gradient to ascertain whether the eluted ATPase was still a “30S” dynein. Most of the ATPase activity sedimented as 30S dynein, with only a small amount of material—thought to be aggregates—sedimenting at a greater S value.

Fractions containing the calmodulin-Sepharose 4B affinity column—purified dyneins were also assayed for ATPase activity in the presence of 10 μg of bovine brain calmodulin and Ca++. It can be seen (Fig. 4) that, unlike the 14S or 30S dyneins that had been loaded onto the column, which had calmodulin stimulation indices (+ calmodulin/− calmodulin) of 1.2 and 1.9, respectively, the ATPase activities of the eluted dyneins in this experiment were only marginally sensitive to the addition of 10 μg of calmodulin.
However, in another affinity-chromatography experiment with 14S-E and 30S-E dyneins, sensitivity to 10 µg calmodulin was still apparent, and it was confirmed that by adding more calmodulin (>10 µg per reaction mixture) to the 30S dynein eluted from the calmodulin-Sepharose 4B column, a larger degree of (Ca"+-dependent) enhancement of the ATPase activity was achievable. In one experiment with 30S-K dynein eluted from the calmodulin-Sepharose 4B, an ATPase activity ratio of 1.6 (with 10 µg calmodulin per assay) was observed. Thus, although there may be a variable reduction in sensitivity to added calmodulin—perhaps because of the variable loss of other regulatory components—both the 14S and 30S dynein ATPases that are eluted from calmodulin-Sepharose 4B columns are still stimulated by the addition of calmodulin. The presence of other regulatory components is also suggested by the variable sensitivity of dyneins prepared by several methods to calmodulin stimulation of ATPase activity (e.g., 14S-K1 vs. 14S-K2 or EDTA-vs. KCl-extracted material). The reasons for this variable, partial loss of sensitivity to calmodulin stimulation of ATPase activity are the subject of current investigations.

Analysis of Affinity-column-purified Dyneins by PAGE

Portions of the fractions from the affinity-chromatography separation shown in Fig. 4 were subjected to electrophoresis in 7.5% SDS polyacrylamide gels. Fig. 5 shows that, in agreement with the very low A280 levels, there were no discernible protein bands in the unretarded fractions from either column. It also shows that a majority of the Ca"+-dependently bound 14S and 30S dyneins consisted of high molecular weight components. At these high loads, at least 10 intermediate and low molecular weight components were observed in addition to the high molecular weight components of both the 14S and 30S dyneins. Because the high molecular weight components predominated, there is some question as to whether these intermediate and low molecular weight bands are stoichiometric components of the dyneins, minor contaminants, or limited degradation products. To obtain further information about the high molecular weight bands, which scarcely penetrate the 7.5% gel in the time required for the dye front to reach 1 cm from the anodal end of the gel, suitable aliquots were analyzed on the same gels electrophoresed for twice this time (Fig. 6). It can be seen that two major (~260,000 and 253,000 Mr) and one minor (~270,000 Mr) components compose the bulk of the isolated 14S dynein. The 30S dynein was composed of one major component (~270,000 Mr), possibly corresponding to the minor band of 14S dynein, and a lesser amount of a 246,000-dalton component that is not found in the 14S dynein. It is also of interest that 14S-E2 and 30S-E2 dyneins appear to consist of components identical to their E1 counterparts (Fig. 6). Because these Mr values were obtained by extrapolation of the graph of log molecular weight vs. migration distance for the standards, the largest of which was myosin (200,000), β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 68,000; and ovalbumin, 43,000; B, 4 µg of the affinity-purified 14S dynein (fraction 68, Fig. 4); C and D, 1.5 and 6 µg, respectively, of affinity-purified 30S dynein (fraction 85, Fig. 4); E and F, 1.5 and 3 µg, respectively, of a preparation of 14S-E2 dynein; G and H, 1 and 2 µg, respectively, of 30S-E2 dynein obtained from the same preparation as that used for isolation of the 14S-E2 dynein used in lanes E and F. For details of the preparation of 14S-E2 and 30S-E2 dyneins, see Materials and Methods.

Calmodulin Binding to Tris-EDTA-extracted Axonemes

We have earlier shown (3) that even after a second extraction with Tris-EDTA, a residual ATPase activity remains associated with the twice-extracted axonemal pellet (P2-E) and that this residual ATPase activity differs in several ways from that of extracted dyneins (3, 5, 9). Having established the presence of binding sites for calmodulin in 14S and 30S dyneins, we found it of interest to ascertain whether any calmodulin binding sites remained in twice-extracted axonemes. An experiment was therefore performed in which P2-E (2 mg of protein from the same preparation of cilia as used in Fig. 2) was added to tubes containing 0.75 mM EGTA, or 0.75 mM free Ca"++, and [35S]calmodulin (pre pared as described in Materials and Methods). Quadruplicate samples with EGTA or Ca"++ were centrifuged and the counts per minute remaining in the supernate and in the pellet were measured (see Materials and Methods). The amount of added [35S]calmodulin (sum of counts per minute in pellet plus supernate) was 22,750 ± 940 cpm (SD; n...
Calmodulin Induction of Dynein ATPase Ca++-sensitivity and the Nature of the Calmodulin-binding Sites

Examination of the differential abilities of calmodulin and the closely related calcium-binding protein TnC to activate the dynein ATPase activities has proved useful in demonstrating the specificity of calmodulin in this system. The Ca++-dependent ATPase activity of 14S dynein is stimulated by a lower concentration of calmodulin than is required for stimulation of 30S dynein, regardless of whether the dyneins are prepared by Tris-EDTA or by KCl extraction of the axonemes. TnC also stimulates the Ca++-dependent ATPase activities of the dyneins, but to a limited extent. It stimulates 30S-E dynein more effectively than 14S-E (Fig. 3). The activities of 14S-K2 and 30S-K2 dyneins are stimulated approximately equally (Fig. 2), but the concentration of TnC required for half-maximal saturation of 30S-K2 dynein ATPase is much less than that for 14S-K2 dynein ATPase. Thus, whereas calmodulin confers calcium sensitivity to both 14S and 30S dynein ATPase, TnC confers significant sensitivity only to 30S dynein ATPase. This suggests that the calmodulin-binding sites on the two dyneins may differ.

Despite numerous differences, there are a large number of similarities between the kinetic properties of dynein ATPase and myosin ATPase (see reference 13 for review). The only documented role for calmodulin in actomyosin regulation in vivo is through the activation of myosin light-chain kinase (16, 24, 36). Murofushi (35) reported that three cyclic AMP-independent protein kinase activities could be found in Tetrahymena axonemes. Although these have not yet been systematically examined for possible Ca++-calmodulin sensitivity, it is possible that calmodulin may regulate a kinase that modulates the activity of one or both of the dyneins.

The finding that TnC is an effective activator of 30S dynein ATPase raises the possibility that although calmodulin acts as the endogenous activator of 14S dynein ATPase, an as yet undetected TnC-like protein is the endogenous activator of 30S dynein ATPase. Recent studies by Gitelman and Witman (21) indicate that although calmodulin is present in Chlamydomonas flagella, it is not found associated with the isolated dyneins. In addition, the flagella of Chlamydomonas contain a TnC-like protein. Proc. Nat. Acad. Sci. U. S. A. In press.

2 The ability of extracts of Tetrahymena axonemes to phosphorylate casein (using an assay similar to that described by Murofushi [35]) was not altered by addition of calcium plus calmodulin (unpublished data). We have not, however, attempted purification of the kinase activities by column chromatography or examined the individual kinases for possible calcium-calmodulin regulation.

3 VanEldik, L. J., G. Piperno, and D. M. Watterson. Similarities and dissimilarities between calmodulin and a Chlamydomonas flagellar protein. Proc. Nat. Acad. Sci. U. S. A. In press.
than AMP-PNP, did not appear to prevent activation. The discovery here that low concentrations of ATP (~5 μM) and moderate concentrations of AMP-PNP (~200 μM) partially prevent Ca**+-calmodulin stimulation of dynein ATPase activity, whereas AMP-PCP does not, (a) correlates with our previous results (10), (b) suggests that 14S dynein also has a high-affinity ATP-binding site, and (c) indicates a close connection between the calmodulin-binding site and the high-affinity ATP-binding site.

Although the interaction of calmodulin with dynein that results in Ca**+-dependent activation of dynein ATPase activities was determined to be rapid, it does not appear from the data presented here that activation occurs through a simple, diffusion-limited process. Clearly, detailed kinetic analyses will be required to further clarify this point.

**Affinity Chromatography and Gel Electrophoresis of 14S and 30S Dyneins**

Calcium-dependent affinity chromatography on calmodulin-Sepharose 4B conjugates (45) is one of the major tests used to demonstrate calmodulin binding by putative calmodulin-regulated enzymes. This affinity-chromatography procedure has here been modified for use in demonstrating calmodulin-binding sites on the dyneins in an attempt to rule out possible artifacts and improve its effectiveness. Passage of the sample through Sepharose 4B and CAPP-Sepharose 4B column ensures (a) the removal of any aggregates or of material interacting nonspecifically with Sepharose 4B and (b) removal of most, if not all, endogenous calmodulin, thus enhancing the specificity and effectiveness of the calmodulin-Sepharose 4B affinity step. One likely contaminant to accompany the eluted dyneins is, of course, any other protein(s) that has a calmodulin-binding site. Analysis of 14S dynein obtained using the serial column procedure on SDS polyacrylamide gels revealed the 14S dynein to be composed of two major high molecular weight polypeptides and one minor high molecular weight component, whereas analysis of the 30S dynein revealed one major and one minor high molecular weight component (see Fig. 6). Both the 14S and 30S affinity-column-purified dyneins contained low levels of lower molecular weight components that may represent (a) other components of the dyneins, such as those in dynein I of sea urchin sperm flagella (18); (b) other ciliary calmodulin-binding proteins; or (c) products of limited proteolysis of the dyneins. It should be noted that although tubulin may be present in the 30S dynein fractions recovered from calmodulin-Sepharose 4B chromatography, no such components were observed in similar preparations of 14S dynein. Because 14S dynein has molecular weight of ~600,000 (20) and is composed primarily of two polypeptides of 260,000 and 253,000 daltons (see Fig. 6) or 358,000 and 375,000 daltons (25), the calmodulin-binding site that was demonstrated to be present by the affinity-column procedure (Fig. 4) is very likely to be localized on one or both of these polypeptides.

**Studies with Radiolabeled Calmodulin**

The development of a simple procedure for preparing endogenously radiolabeled Tetrahymena calmodulin of high specificity activity provides a useful tool for studies of calmodulin-binding proteins in Tetrahymena. Because of the high degree of structural and functional similarity between Tetrahymena calmodulin and bovine brain calmodulin (this paper and reference 28), the [35S]Tetrahymena calmodulin should also prove useful for studies on other calmodulin-regulated systems. In the present studies, [35S]calmodulin was used to demonstrate the presence of Ca**+-dependent binding sites in twice-extracted axonemal pellets. Although such pellets contain a low amount of Ca**+-calmodulin-activatable ATPase activity, it cannot be concluded that the binding sites on pellet 2 are on this residual ATPase, as calmodulin binds Ca**+-dependently to an affinity column of tubulin-Sepharose 4B (31). In those experiments, Kumagai et al. (31) used porcine brain tubulin with microtubule-associated proteins (MAPs) to prepare their affinity column. Whether calmodulin interacts directly with tubulin or with the MAPs was not determined in their studies. As it is likely that a twice-extracted axonemal pellet would contain MAPs, the [35S]calmodulin might interact with these proteins in addition to any remaining dyneins or to other, as yet undefined, residual ATPases (3). Further studies will be required to clarify the nature and function of the calmodulin-binding components in the twice-extracted axonemal preparations.

**Final Remarks**

The in vitro consequences of the Ca**+-calmodulin-dependent increase in dynein ATPase activity may be varied. Ca**+-dependent changes in symmetry of beat form (2, 15), spontaneous starting and stopping (19, 41), and reversal of beat direction (26, 27) have been observed in demembranated axonemes of different species. We have not found any convincing evidence that addition of calmodulin to demembranated Tetrahymena axonemes causes any change in the turbidity response (measured at 350 nm in the presence or absence of added Ca**+) even though the addition of Ca**+ alone consistently causes a small increase in this response. Determination of the means by which the effects of Ca**+ are relayed by calmodulin (and possibly other dynein-associated regulatory components) to Tetrahymena ciliary dynein ATPases and of how this translates into the control of directional ciliary movement awaits further study.

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Preliminary reports of part of these studies have appeared (11, 12, 29).

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