Labeling of *Chlamydomonas* 18 S Dynein Polypeptides by 8-Azidoadenosine 5'-Triphosphate, a Photoaffinity Analog of ATP*

(K. Kevin Pfister‡§, Boyd E. Haley¶, and George B. Witman∥)

From the ‡Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, §Department of Biology, Princeton University, Princeton, New Jersey 08544, and the ¶Division of Biochemistry, University of Wyoming, Laramie, Wyoming 82071

The 18 S dynein from the outer arm of *Chlamydomonas* flagella is composed of an α subunit containing an α heavy chain ($M_r \approx 340,000$) and an $M_r = 16,000$ light chain, and a β subunit containing a β heavy chain ($M_r \approx 78,000$ and 69,000), and seven light chains ($M_r = 8,000\text{--}20,000$). Both subunits contain ATPase activity. We have used 8-azidoadenosine 5'-triphosphate (8-N$_3$ATP), a photoaffinity analog of ATP, to investigate the ATP-binding sites of intact 18 S dynein. 8-N$_3$ATP is a competitive inhibitor of 18 S dynein’s ATPase activity and is itself hydrolyzed by 18 S dynein; moreover, 18 S dynein’s hydrolysis of ATP and 8-N$_3$ATP is inhibited by vanadate to the same extent. 8-N$_3$ATP therefore appears to interact with at least one of 18 S dynein’s ATP hydrolytic sites in the same way as does ATP. When [α- or γ-$^32$P]8-N$_3$ATP is incubated with 18 S dynein in the presence of UV irradiation, label is incorporated primarily into the α, β, and $M_r = 78,000$ chains; a much smaller amount is incorporated into the $M_r = 69,000$ chain. The light chains are not labeled. The incorporation is UV-dependent, ATP-sensitive, and blocked by preincubation of the enzyme with vanadate or ADP. These results suggest that the α heavy chain contains the site of ATP binding and hydrolysis in the α subunit. In the β subunit, the β heavy chain and one or both intermediate chains may contain ATP-binding sites.

Dynein arms project from the outer doublet microtubules of cilia and flagella and convert the chemical energy of ATP into the mechanical energy of interdoublet sliding, which is the basis for ciliary and flagellar motion (Gibbons, 1965, 1981; Gibbons and Gibbons, 1979; Goodenough and Heuser, 1982; Johnson et al., 1984; Satir et al., 1981; Takahashi and Tomonura, 1978; Tsukita et al., 1983; Warner and Mitchell, 1978; Witman and Minervini, 1982). Studies using biochemical, genetic, and immunological techniques have shown that the outer dynein arm of *Chlamydomonas* flagella is compositionally complex (Fay and Witman, 1977; Huang et al., 1979; King and Witman, 1984; Pfister et al., 1982; Pfister and Witman, 1984; Piperno and Luck, 1979) and consists of at least three distinct subunits, each of which contains ATPase activity (Pfister and Witman, 1984). In our purification procedures, two of these subunits are generally isolated together as a single enzyme complex known as 18 S dynein (Pfister *et al.*, 1982; Pfister and Witman, 1984; Watanabe and Flavin, 1976). This complex contains two different heavy chains ($M_r \approx 340,000$), two intermediate chains ($M_r = 69,000$ and 78,000) and eight light chains ($M_r = 8,000\text{--}20,000$) (Pfister *et al.*, 1982). Subfractionation of the complex yields an α subunit containing the α heavy chain ($M_r \approx 340,000$) and one light chain ($M_r = 16,000$), and a β subunit containing the other (β) heavy chain and the remaining intermediate and light chains of 18 S dynein (Pfister and Witman, 1984). The third subunit, 12 S dynein, usually dissociates from the α and β subunits when the outer arm is extracted from the axoneme (Fay and Witman, 1977; Pfister *et al.*, 1982; Pfister and Witman, 1984; Piperno and Luck, 1979); this subunit consists of the γ heavy chain ($M_r = 330,000$) and two light chains ($M_r = 22,000$ and 18,000).

At present little is known about the separate functions of these ATPases or how they interact in an intact outer arm. To better understand the roles of the individual chains of the dyneins, we recently used a radioactively labeled photoaffinity analog of ATP, 8-N$_3$ATP,$^*$ to identify the polypeptides associated with the site of ATP binding and hydrolysis in isolated 12 S dynein (Pfister *et al.*, 1984). Exposure of 8-N$_3$-ATP to UV irradiation converts the azido group into a highly reactive nitrene which can form a covalent bond with amino acids at the nucleotide-binding site of the protein being studied (Czarnecki *et al.*, 1979; Guillory and Jeng, 1983). The polypeptides can then be separated from one another by SDS-polyacrylamide gel electrophoresis and the chains containing the azido nucleotide identified by autoradiography. We found that the analog was incorporated in a UV-dependent manner into the heavy chain of 12 S dynein, but not its light chains. This incorporation was progressively eliminated by the addition of increasing amounts of ATP to the reaction mixture, but not by the addition of adenosine or other nucleotides that do not affect ATP hydrolysis. The incorporation was also blocked by preincubation of the enzyme with vanadate and ADP, which inhibits 12 S dynein’s ATPase activity. Therefore, the heavy chain appears to contain the site of ATP binding and hydrolysis in 12 S dynein (Pfister *et al.*, 1984).

We now report that 8-N$_3$ATP similarly labels the two heavy chains and the 78,000 intermediate chain of 18 S dynein. The $M_r = 69,000$ chain is also labeled, but to a much lesser degree. The eight light chains are not labeled. These results suggest that in the α subunit, the heavy chain contains the site of

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† To whom correspondence should be addressed.

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1 The abbreviations used are: 8-N$_3$ATP, 8-azidoadenosine 5'-triphosphate; SDS, sodium dodecyl sulfate; 8-N$_3$AMP, 8-azidoadenosine 3',5'-monophosphate; 8-N$_3$AMP, 8-azidoadenosine 5'-monophosphate.
ATP binding and hydrolysis; in the β subunit, the heavy chain and one or both intermediate chains may be involved in ATP binding.

EXPERIMENTAL PROCEDURES

Culture of *Chlamydomonas reinhardtii*, cell harvesting, axoneme isolation, hydroxylapatite purification of 18 S dynein, SDS-polyacrylamide gel electrophoresis, and enzyme assays were all as described previously (Pfister et al., 1984) except that the hydroxylapatite column was eluted at 4–6 drops/min. Protein concentration was measured according to the method of Bradford (1979).

For photoaffinity labeling, hydroxylapatite-purified 18 S dynein was dialyzed against TEM buffer (30 mM Tris, 0.5 mM EDTA, and 5 mM MgSO₄, pH 7.5) for 18 h with two changes of buffer to remove diethiothreitol and the phosphate used to elute the enzyme from the hydroxylapatite column. Twenty-microliter aliquots of the dialyzed enzyme were placed in 0.5-ml microfuge tubes to which 10 μl of 8-N3ATP in the same buffer with or without added nucleotide were added. Unless otherwise noted, the protein concentration was 0.2–0.45 mg/ml, and the final concentration of 8-N3ATP was 0.3–20 μM at a specific activity of 4–50 Ci/mmol. The tubes were then vortexed to mix the solution and either irradiated with UV light by resting a UVS-11 or UVG-11 lamp (Ultra-Violet Products, Inc., San Gabriel, CA) on the top of the tube or allowed to incubate without UV exposure. After the indicated times, each sample was mixed with an equal volume of twice the normal concentration of sample buffer and then analyzed by electrophoresis. Silver-stained gels (Merril et al., 1981) were dried and autoradiographed or analyzed to quantify the radioactivity incorporated into specific polypeptides, as previously described (Pfister et al., 1984). To determine the effect of vanadate on 8-N3ATP labeling of the dynein polypeptides, 5 μl of TEM buffer containing sodium metavanadate plus ATP or ADP were added to the aliquot of dynein and incubated for 30 min. Five μl of 8-N3ATP at twice the standard concentration were then added and irradiation begun. The sample was then analyzed by electrophoresis as described above.

RESULTS

We have previously shown that *Chlamydomonas* 18 S dynein can be subfractionated into two distinct subunits, both of which contain ATPase activity (Pfister and Witman, 1984). However, because our present subfractionation and purification methods yield only analytic amounts of the two subunits and because the ATPase activities of the purified subunits are quite labile, the experiments reported here were carried out on the intact 18 S dynein.

To ascertain that 8-N3ATP was a suitable analog for probing the ATP hydrolytic sites of 18 S dynein, we investigated whether it interacted with 18 S dynein in the same manner as ATP.

Fig. 1 shows a Lineweaver-Burk plot of 18 S dynein ATPase activity in the presence and absence of 8-N3ATP. In the absence of 8-N3ATP, the plot is linear, suggesting that, in the intact 18 S particle, either the ATPases of the α and β subunits have very similar kinetic parameters or one of the two subunits is so much more active than the other that the latter contributes negligibly to the plot. We do not have information on the kinetic properties or relative activities of the two ATPases in the intact particle; however, the isolated β subunit has a specific activity 10 times that of the isolated α subunit (Pfister and Witman, 1984), so the second possibility is quite likely. There is no evidence in the plot for a noncatalytic (regulatory) ATP-binding site.

The plots of ATPase activity in the presence of 8-N3ATP are also linear, and the lines intersect on the ordinate, indicating that the analog is a competitive inhibitor of the enzyme. 8-N3ATP must therefore bind to at least one of the active sites of 18 S dynein. Under our assay conditions, the apparent Kᵦ of ATP = 1.05 μM; 8-N3ATP binds to 18 S dynein with an apparent Kᵦ = 10.3 μM.

That 8-N3ATP binds to at least one of the sites of ATP hydrolysis of 18 S dynein was confirmed by the observation that the analog is a substrate of the enzyme (Fig. 2). Hydrolysis of 8-N3ATP by 18 S dynein was linear with time, although slower than that of ATP. In the presence of 1 μM substrate, 18 S dynein hydrolyzed 8-N3ATP at a rate of 4.1 nmol/min/mg versus 58.7 nmol/min/mg for ATP.

Additional evidence that 8-N3ATP and ATP interact with 18 S dynein in a similar way came from a comparison of the effect of vanadate on the hydrolysis of the two nucleotides. Vanadate inhibits dynein’s hydrolysis of ATP by acting as a phosphate analog; in combination with ADP it binds to the
was observed in the heavy chain region and the high molecular weight polypeptides were subsequently analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, a considerable amount of label was observed in the heavy chain region and the \( M_r = 78,000 \) chain (Fig. 4, A and B). A much smaller amount of label was incorporated into the \( M_r = 69,000 \) polypeptide. The results were qualitatively the same regardless of whether \([\alpha-^{32}P]-\) or \([\gamma-^{32}P]8\text{-N3ATP} \) was used. In the absence of UV irradiation, no dynein polypeptides were labeled. The fact that the same UV-dependent labeling pattern was observed with both \([\alpha-^{32}P]\) and \([\gamma-^{32}P]8\text{-N3ATP} \) indicates that incorporation of label into the polypeptides was not due to a phosphorylation or other enzymatic reaction.

The 5–15% acrylamide gradient gel system used to resolve the intermediate and light chains of 18 S dynein does not resolve the heavy chains. However, a 3–5% acrylamide, 0–8 M urea gradient SDS gel system provides good resolution of the high molecular weight polypeptides (\( M_r > 200,000 \)) (Pfister et al., 1982). An autoradiograph of such a gel (Fig. 5A) showed that both the \( \alpha \) and \( \beta \) heavy chains of 18 S dynein were labeled in a UV-dependent manner by \([\gamma-^{32}P]8\text{-N3ATP} \).

In the course of these experiments, we observed that the relative amount of label incorporated into the \( \alpha \) and \( \beta \) heavy chains varied (compare Figs. 5, 6, and 7), suggesting that the two polypeptides had different and possibly variable labeling kinetics. Indeed, a difference in the relative amount of label incorporated into the two chains occurred even when the same preparation of dynein was irradiated for different lengths of time (Fig. 5B). When 18 S dynein was exposed to UV light for a short period of time, the \( \beta \) chain was heavily labeled while the \( \alpha \) chain was only lightly labeled; with a longer exposure to UV irradiation, the two chains appeared to incorporate more nearly equal amounts of label. Possible reasons for these results are discussed in the following section.

Quantitation of the amount of \( ^{32}P \) incorporated into the 18

**Fig. 3.** Vanadate is equally effective in inhibiting the hydrolysis of 8-N3ATP and ATP by 18 S dynein. The graph shows the effect of increasing concentrations of vanadate on the relative rates of hydrolysis of ATP and 8-N3ATP by 18 S dynein. Assay conditions as in Fig. 2 with the addition of the indicated concentrations of vanadate to the reaction mixture.

**Fig. 4.** Photoaffinity labeling of 18 S dynein polypeptides by 8-N3ATP. A, purified 18 S dynein (0.45 mg/ml) was incubated with 0.32 \( \mu \)M \([\gamma-^{32}P]8\text{-N3ATP} \) (specific activity = 50 Ci/mmol) in the presence and absence of UV irradiation for 1 min and its polypeptides separated on a 5–15% polyacrylamide gradient-SDS gel and autoradiographed (AR). A representative silver-stained lane (SS) shows the unresolved heavy chains (\( M_r = 340,000 \)) (H), the two intermediate chains (\( M_r = 78,000 \) and 69,000), and the light chains (\( M_r = 8,000–20,000 \)). The minor bands in the midregion of the gel are artifacts created by the electrophoretic conditions (Hashimoto et al., 1983; Tasheva and Dessev, 1983). The autoradiograph shows that in the presence of UV irradiation, one or both heavy chains and the \( M_r = 78,000 \) intermediate chain were labeled. Less easily seen at this exposure is faint labeling of the \( M_r = 69,000 \) chain. The light chains were not labeled. None of the dynein polypeptides were labeled in the absence of UV irradiation. Autoradiograph was exposed for 14 days. B, autoradiograph of a similar gel of 18 S dynein (0.25 mg/ml) that was incubated with 10 \( \mu \)M \([\alpha-^{32}P]8\text{-N3ATP} \) (specific activity = 4.6 Ci/mmol) in the presence and absence of UV irradiation for 1.0 min. Label was incorporated into one or both heavy chains and the two intermediate chains. No label was incorporated into the light chains. None of the polypeptides were labeled in the absence of UV irradiation. Autoradiograph was exposed for 2 days.
Fig. 5. Photoaffinity labeling of the heavy chains of 18 S dynein. A, purified 18 S dynein (0.3 mg/ml) was incubated with 18 \( \mu M \) \([\gamma-\text{32P}]\)8-N3ATP (specific activity 18 Ci/mmol) in the presence and absence of UV irradiation for 0.8 min, and its polypeptides were separated on a 0-8 urea, 3-5% polyacrylamide gradient-SDS gel which was silver-stained and autoradiographed for 2 days. Only the high molecular weight region of the gel is shown. A representative silver-stained lane (SS) shows the \( \alpha \) and \( \beta \) heavy chains (\( M_r = 340,000 \)) and polypeptide 11, a proteolytic fragment of the former (Fizeter and Witanman, 1984). The autoradiograph (AR) shows that in the presence of UV irradiation, both heavy chains were labeled. Neither of the polypeptides were labeled in the absence of UV irradiation. B, autoradiograph of a similar gel of 18 S dynein which was incubated with \([\gamma-\text{32P}]\)8-N3ATP and irradiated with UV light. In the lane labeled .25, a 20-\( \mu l \) aliquot of purified 18 S dynein (0.25 mg/ml) was mixed with 10 \( \mu l \) of 30 \( \mu M \) 8-N3ATP (specific activity = 4.6 Ci/mmol) and irradiated with UV light for 0.25 min. The bulk of the label was incorporated into the \( \beta \) heavy chain. In the lane labeled 3, the same concentrations of 18 S dynein and 8-N3ATP were irradiated with UV light for 1 min and then a 10-\( \mu l \) aliquot of the sample was removed from the tube and mixed with an equal volume of electrophoresis sample buffer. 10 \( \mu l \) of 30 \( \mu M \) 8-N3ATP was then added to the tube, the mixture irradiated with UV light for a second min, and another 10-\( \mu l \) aliquot removed. The process was then repeated a third time, to give a total UV exposure of 3.0 min and a final 18 S dynein concentration of 0.07 mg/ml. An autoradiograph of a gel of this third sample (3) shows that under these conditions a more nearly equal amount of label was incorporated into the \( \alpha \) and \( \beta \) heavy chains.

Fig. 6. Effect of ATP on the incorporation of 8-N3ATP into 18 S dynein. A, autoradiograph of a 0-8 urea, 3-5% acrylamide gradient-SDS gel. 18 S dynein (0.2 mg/ml) was irradiated with UV light for 1.0 min in the presence of 3 \( \mu M \) \([\gamma-\text{32P}]\)8-N3ATP (specific activity = 4.8 Ci/mmole) and the indicated concentrations of ATP. The autoradiograph was exposed for 4.5 days. B, autoradiograph of a 5-15% acrylamide gradient-SDS gel of 18 S dynein (0.2 mg/ml) which was irradiated with UV light for 0.25 min in the presence of 20 \( \mu M \) \([\gamma-\text{32P}]\)8-N3ATP (specific activity = 6.5 Ci/mmol) and the indicated concentrations of ATP. As was observed for the heavy chains, there was a progressive decrease in the amount of label incorporated into the intermediate chains upon the addition of increasing concentrations of ATP to the incubation mixture. Autoradiograph was exposed for 1 day.

S dynein polypeptides when the particle was incubated with \([\alpha-\text{32P}]\)8-N3ATP in the presence of UV irradiation for 2 min showed that the two heavy chains and the \( M_r = 78,000 \) polypeptide incorporated approximately equal amounts of label, while the \( M_r = 69,000 \) chain incorporated about 1/3 as much as the others (Table I). The light chains incorporated 50-70-fold less analog than the three heaviest chains.

Several additional experiments were carried out to investigate whether all the polypeptides labeled by 8-N3ATP were related to sites of ATP hydrolysis in 18 S dynein. Addition of increasing amounts of ATP to the reaction mixture resulted in a progressive decrease in the amount of label incorporated into the heavy (Fig. 6A) and intermediate chains (Fig. 6B). ATP therefore competes with 8-N3ATP for the sites that are labeled. In contrast, cAMP, AMP, N3AMP, N3AMP, and adenosine, which do not interfere with the hydrolysis of 8-N3ATP by 18 S dynein (data not shown), did not reduce the amount of label incorporated into any of the polypeptides (Fig. 7A). In similar experiments, cAMP, AMP, adenosine and NaN3 were tested and found to have no effect on the amount of label incorporated into the intermediate chains (data for cAMP shown in Fig. 7B).

When 18 S dynein was preincubated with vanadate and a low concentration of ADP or ATP prior to addition of \([\alpha-\text{32P}]\)- or \([\gamma-\text{32P}]\)8-N3ATP and irradiation, almost no label was incorporated into the heavy chains (Fig. 8) or the intermediate chains (data not shown). Vanadate together with ADP or ATP therefore blocks the analog from the labeled sites.

No label was incorporated into 18 S dynein that had first been denatured by boiling for 3 min (data not shown). A specific three-dimensional conformation of the protein is therefore necessary for 8-N3ATP labeling.

Discussion

The ATP photoaffinity analog 8-N3ATP interacts with at least one of 18 S dynein's ATP hydrolytic sites in the same manner as ATP, as evidenced by the observations that (i) the analog was a competitive inhibitor of 18 S dynein's ATPase activity; (ii) the analog itself was hydrolyzed by 18 S dynein;
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and (iii) the hydrolysis of ATP and 8-NT3ATP by 18 S dynein was inhibited by vanadate to the same extent. 8-NT3ATP therefore should be a suitable analog for photoaffinity labeling of one or both of 18 S dynein's sites of ATP hydrolysis.

Incubation of 18 S dynein with either [α-32P]- or [γ-32P]8-NT3ATP in the presence of UV irradiation resulted in the incorporation of approximately equal amounts of label into the α, β, and M₁ = 78,000 chains of 18 S dynein. A smaller amount of label was incorporated into the M₂ = 69,000 chain; almost none was incorporated into the light chains. The incorporation of label into these polypeptides was reduced in a concentration-dependent manner by ATP, but not by nucleotides which do not interfere with the hydrolysis of ATP, and was blocked by preincubation with vanadate plus ATP or ADP, which inhibits 18 S dynein's ATPase activity.

Both the α and β subunits of 18 S dynein possess ATPase activity (Pfister and Witman, 1984). The α subunit consists of two polypeptides, the α heavy chain and the M₁ = 16,000 light chain (Pfister and Witman, 1984). Because the α heavy chain was the only one of these polypeptides which was labeled by 8-NT3ATP, it must contain that subunit's site of ATP hydrolysis. It therefore seems likely that both the α and β heavy chains contain ATP hydrolytic sites.

TABLE I

| Polypeptide | 32P incorporated/ protein | mmol/mol |
|-------------|--------------------------|----------|
| α (~340,000) | 14.0                    | 16.3     |
| β (~340,000) |                         |          |
| 78,000      | 15.5                    |          |
| 69,000      | 5.5                     |          |
| 20,000      | 0.4                     |          |
| 19,000      | 0.4                     |          |
| 16,000      | 0.2                     |          |
| 14,000      | 0.4                     |          |
| 11,000      | 0.3                     |          |
| 8,000       | 0.3                     |          |

*Quantitative densitometry of Coomassie Brilliant Blue-stained gels indicates that all polypeptides are present in equimolar amounts, except for one or both of the M₁ = 8,000 light chains (see text). *

b Two-dimensional gel electrophoresis indicates that each of these bands contains two polypeptides (Pfister et al., 1982).

The β subunit consists of the β heavy chain, two intermediate chains (M₁ = 69,000 and 78,000), and seven light chains (M₂ = 8,000-20,000) (Pfister and Witman, 1984). There are several possibilities that might result in the apparently specific labeling of three of these polypeptides by 8-NT3ATP. First, all three polypeptides might be part of a single hydrolytic site and be accessible to the nitrene group of the irradiated analog. Second, the β subunit might contain more than one site of ATP hydrolysis. Third, other ATP catalytic activities might be associated with the β subunit, for example protein kinase, adenylate kinase, or nucleoside diphosphokinase activity (Watanabe and Flavin, 1976). Finally, the subunit might contain a noncatalytic nucleotide-binding site.

To test the possibility that the β subunit may contain more than one ATPase activity, we attempted to subfractionate 18 S dynein further. In one preliminary experiment, the α and β heavy chains were separated from the intermediate and most of the light chains, which sedimented as a complex in a sucrose density gradient (data not shown). ATPase activity was detected only in the fractions containing the heavy chains. The specific activity of these fractions was similar to that of native 18 S dynein and higher than that of the purified α subunit, suggesting that the β heavy chain contains a complete site of ATP hydrolysis. It therefore seems likely that both the α and β heavy chains contain ATP hydrolytic sites.

K. K. Pfister and G. B. Witman, unpublished results.

FIG. 7. Incorporation of 8-NT3ATP into 18 S dynein is not affected by nucleotides that are not substrates for dynein. A, 18 S dynein (0.3 mg/ml) was irradiated with UV light for 1.0 min in the presence of 5 μM [γ-32P]8-NT3ATP (specific activity = 4.5 Ci/mmol), and 50 μM of either adenosine (Ado), cAMP, N₆cAMP, AMP, N₆AMP, ADP, or ATP and electrophoresed as described in Fig. 5. The first lane (C) shows the amount of label incorporated into the heavy chains of 18 S dynein when incubated with 8-NT3ATP alone. The next lanes show that adenosine, cAMP, N₆cAMP, AMP, or N₆AMP, which do not interfere with 18 S dynein's hydrolysis of 8-NT3ATP, did not reduce the amount of label incorporated into the heavy chains. ADP and ATP, which do interact with the hydrolytic site of 18 S dynein, did reduce the amount of label incorporated. Similar results were obtained with [α-32P]8-NT3ATP (data not shown). Autoradiograph was exposed for 25 days. B, autoradiograph of a 5–15% acrylamide gradient SDS gel of purified 18 S dynein which was incubated with UV irradiation for 2.0 min in the presence of 10 μM [γ-32P]8-NT3ATP (specific activity = 17 Ci/mmol) alone (C) or with 100 μM cAMP. Cyclic AMP did not reduce the amount of label incorporated into the intermediate chains of 18 S dynein. Autoradiograph was exposed for 25 days.

Twenty microliters of purified 18 S dynein (0.2 mg/ml) were mixed with 10 μl of [α-32P]8-NT3ATP (specific activity 4 Ci/mmol, final concentration 9.5 μM) and irradiated for 2 min. The polypeptides were then separated by electrophoresis on 5–15% polyacrylamide gradient-SDS gels, the silver-stained high molecular weight region and the intermediate and low molecular weight bands excised from the gel, and the radioactivity in each determined by liquid scintillation counting (Pfister et al., 1984). The relative proportion of label in the α and β heavy chains was determined by quantitative densitometry of an autoradiograph of a 3–5% acrylamide gel on which the same sample had been loaded at 1/50 the protein concentration used in the 5–15% gel.
The labeling of the intermediate chains was also blocked by preincubation with vanadate and ADP (results not shown). Autoradiograph of a 0–8 M urea, 3–5% acrylamide gradient-SDS gel. The first lane (C) shows the amount of label incorporated when 18 S dynein (0.33 mg/ml) plus 5.3 μM [γ-32P]β-N3ATP (specific activity = 53 Ci/mmol) was irradiated with UV light for 1 min. The second and third lanes show that preincubation with 100 μM vanadate and 5.3 μM ATP or ADP, respectively, for 30 min prior to the addition of 8-N3ATP and irradiation with UV light blocked the incorporation of label into the heavy chains. Similar results were obtained if [α-32P]β-N3ATP was used (data not shown). The labeling of the intermediate chains was also blocked by preincubation with vanadate and ADP (results not shown). Autoradiograph was exposed for 6 days.

The reason for the labeling of the intermediate chains in the β subunit is less clear. We do not have any evidence that they possess ATPase activity independent of the heavy chains. We have not investigated the possibility that 18 S dynein contains other enzymatic activities; however, Watanabe and Flavin (1976) found that the adenylate kinase and nucleoside diphosphokinase activities of *Chlamydomonas* flagella did not copurify with the Mg2+-ATPase activity (dynein). As discussed below, our 18 S dynein preparations do not contain a protein kinase that can use the polypeptides of 18 S dynein as substrates. However, in one experiment there was a UV-independent labeling of a very minor intermediate molecular weight contaminant (results not shown), suggesting that in at least one preparation the dynein was associated or contaminated with a protein kinase. We have not tested the interesting possibility that 18 S dynein itself may contain a protein kinase activity specific for polypeptides not usually present in our 18 S dynein preparations.

The Lineweaver-Burk plot of 18 S dynein ATPase activity was linear and therefore provides no evidence for a regulatory nucleotide-binding site.

An alternative possibility for the labeling of the intermediate chains is that, although they do not actually contribute to the β subunit's catalytic groups, they may be located sufficiently close to its hydrolytic site that they are labeled during the specific interaction of the analog with that site. Depending on the exact orientation of the analog in the site, the UV-generated nitrone could react with amino acids sterically adjacent to the site. This possibility is supported by the observation that labeling of the intermediate chains was all-ways kinetically similar to that of the β chain (results not shown; see below) and was blocked by preincubation with vanadate and ATP or ADP. Clearly, further experiments on isolated subfractions of 18 S dynein will be needed to choose between the various possibilities.

The situation with the light chains of the β subunit is much simpler. There is almost no detectable label incorporated into any of these polypeptides. Quantitative analysis of Coomassie Blue-stained gels indicates that all of the 18 S dynein polypeptides are present in equimolar ratios (except for one or both of the *M*<sub>r</sub> = 8000 light chains, which may be present in multiple copies). Therefore, label should have been observed in the light chains if they formed a significant part of the ATP-binding site.

In our experiments, approximately 50 mmol of [α-32P]β-N3ATP were incorporated per mol of 18 S dynein or about 15 mmol each per mol of α, β, or *M*<sub>r</sub> = 78,000 intermediate chain. Although photoaffinity analogs in general give low levels of labeling (Campbell and MacLennan, 1983; Czarnecki et al., 1979; Kerlavage and Taylor, 1980), the relatively small amount of incorporation that we obtained may have been due in part to the conditions that we employed. To minimize nonspecific labeling, all of our experiments were carried out with very low concentrations of 8-N3ATP. At the concentration of 9.5 μM 8-N3ATP used for the quantitated experiments, a maximum of only about one-half of the 18 S hydrolytic sites would have been occupied at the beginning of the incubation, and this would have decreased rapidly with increasing time of incubation. Control experiments using [γ-32P]β-N3ATP showed that under these conditions most of the analog was hydrolyzed in 1–2 min. Therefore, it is not surprising that only a small per cent of the available sites were labeled. As expected, sequential addition of 8-N3ATP and irradiation with UV light significantly increased the amount of label incorporated into the 18 S dynein polypeptides (Fig. 5B), confirming that nucleotide-binding sites were still available for labeling at the end of our normal incubation procedure.

The relative amount of label incorporated into the α and β heavy chains varied from experiment to experiment; in some experiments the two chains incorporated approximately equal amounts of label (Fig. 5A), whereas in others one or the other chain was labeled more heavily (Figs. 6A and 7A). Preparation-to-preparation variability in the labeling pattern could have occurred for at least two reasons. First, differential inactivation of one of the subunits during isolation of 18 S dynein could lead to differences in the relative ATPase activities of the two subunits. That such differential inactivation could occur is suggested by the observation that the purified α subunit is more labile than the β subunit. Second, the α chain is more sensitive to low concentrations of protease than the β chain (Pfister and Witman, 1984), and differential proteolysis of the α chain during the isolation of 18 S dynein frequently reduces the amount of protein migrating in the α chain band relative to that in the β chain band (see e.g. Figs. 5A SS and Figs. 5 and 6A in Pfister and Witman, 1984). Such differential proteolysis would reduce the relative amount of intact α chain available for labeling.

We also observed that the amount of label incorporated into the α chain increased relative to that in the β chain with increased time of UV irradiation (Fig. 5B). The reason for such a change in the relative rate of incorporation of label into the two polypeptides is not clear, but the rates of incorporation of the two subunits could vary if their activities were affected differently by UV irradiation. A short period of UV irradiation caused a stimulation of 18 S dynein ATPase activity, whereas longer exposures decreased the rate of hy-
drolysis (data not shown). We do not know what affect the UV irradiation had on the activity of the individual subunits, but differential activation of the α subunit or inactivation of the β subunit could have resulted in the observed kinetics of labeling. A particular labeling pattern was not correlated with the concentration of analog used, so the increased rate of incorporation into the α chain probably was not because that chain was more effective than the β chain at binding 8-N$_3$ATP at the lower nucleotide concentrations present as the incubation progressed. Increased labeling of the α chain with increased time of irradiation was observed with 8-N$_3$P in both the α and γ positions of the analog, so the pattern was not due to the α chain selectively binding and incorporating [α-8$^3$P]-8-N$_3$ATP produced during the incubation.

In certain of our experiments, we observed proteolysis of one or both heavy chains of 18 S dynein (see e.g. Fig. 5). In some cases the fragments were labeled; this was particularly true of polypeptide 11, a fragment of the α heavy chain (Figs. 5 and 7) (King et al., 1985; Pfister and Witman, 1984). If this fragment were generated before the photoincubation, as seems likely (Pfister and Witman, 1984), then it probably retains ATPase activity. In any case, the fragment must contain the ATP hydrolytic site. Further analysis of the 8-N$_3$ATP-labeled fragments generated by controlled proteolysis of both the α and β chains in our experiments was not due to a protein kinase. When [γ-8$^3$P]-8-N$_3$ATP was mixed with our highly purified 18 S dynein, no detectable label was incorporated into any of the 18 S dynein polypeptides (data not shown). Purified 18 S dynein with detectable label was incorporated into any of the 18 S dynein polypeptides in the absence of UV irradiation. If the incorporation was due to protein kinase activity, it would have occurred in the absence as well as the presence of UV irradiation. Furthermore, [α-8$^3$P]-8-N$_3$ATP gave an identical labeling pattern, even though its β$^3$P would not be transferred to the heavy chain by a protein kinase. Finally, incubation of the purified 18 S dynein with [γ-8$^3$P]-ATP in the presence or absence of UV irradiation resulted in no detectable labeling of the 18 S dynein polypeptides (data not shown). Purified 18 S dynein therefore does not appear to be phosphorylated by a protein kinase under the incubation conditions used in these experiments.

In conclusion, the results presented here, together with our previous findings on 12 S dynein (Pfister et al., 1984), strongly suggest that the heavy chain of each of the three major subunits of the Chlamydomonas outer dynein arm contains that subunit's ATP hydrolytic site. It may be a general rule that the heavy chains contain the catalytic groups responsible for dynein's ATPase activity. The results also raise the possibility that one or both intermediate chains of the β subunit of 18 S dynein may contain an ATP-binding site.

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