Multiple Sites of Phosphorylation within the α Heavy Chain of *Chlamydomonas* Outer Arm Dynein*

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We have examined the phosphorylation of the α dynein heavy chain (DHC) from the outer arm of the *Chlamydomonas* flagellum. Quantitative analysis indicates that this DHC is phosphorylated at a minimum of six sites. Using previously identified proteolytic and photocleavage sites (King, S. M., and Witman, G. B. (1988) *J. Biol. Chem.* 263, 9244-9255), we have mapped two regions that are phosphorylated in *vivo*. One is located in a 20-kDa section immediately N-terminal to the site of V1 photocleavage. Thus, this region is close to the ATP hydrolytic site and also to the predicted junction between the head and stem domains of the particle. The second encompasses the 90-kDa C-terminal region of the molecule. In this latter section, at least one site is found in an ~2-kDa region close to domains that are predicted to adopt a coiled-coil structure in those DHCs that have been sequenced. The α DHC also is specifically labeled by endogenous kinases in demembranated, washed axonemes, suggesting that at least one α DHC kinase is located close to, or is a component of, the outer arm *in situ*.

Dyneins are molecular motors involved in a variety of motile events. One of the best characterized of these enzymes is the outer dynein arm from *Chlamydomonas* flagella. This dynein consists of three heavy chains (the α, β, and γ DHCs; ~500 kDa) associated with two intermediate chains (IC70 and IC78; 63 and 76 kDa, respectively) and at least 10 light chains (8–22 kDa) (Pfister et al., 1982; Piperno and Luck, 1979; see Witman et al., 1994, for review). Structurally, each DHC consists of a large globular head domain and a flexible stem domain; the stems connect the heads to a common base (Goodenough and Heuser, 1984; Johnson and Wall, 1983; Witman et al., 1983). Several lines of evidence indicate that the C-terminal ~350-kDa portions of the DHCs form the head domains of the dynein particle (King and Witman, 1990; Mocz and Gibbons, 1990; Witman et al., 1983); this region of each chain contains the site(s) of ATP hydrolysis and the presumed ATP-sensitive microtubule binding site (Gibbons et al., 1991; King and Witman, 1987; King et al., 1988; Lee-Efford et al., 1985; Ogawa, 1991; Sakakibara et al., 1993; Wilkerson et al., 1994). The N-terminal regions of the heavy chains comprise the stems of the structure and associate at the base with the ICs and several of the LCs, which are organized as an IC/LC complex (King and Witman, 1990; Sakakibara et al., 1993; Witman et al., 1991). In addition, each DHC is tightly associated with a single LC that does not appear to be part of the IC/LC complex (Pfister and Witman, 1984; Pfister et al., 1982). Recent evidence supports the localization of these components to the stem regions of the dynein (Sakakibara et al., 1993).

Analysis of motility mutants of *Chlamydomonas* has identified 12 distinct loci (oda1-10, pf13A, and pf22) that are essential for the assembly and function of the outer arm (Kamiya, 1988). All of these mutants fail to assemble an outer arm, and several have been shown to be defective in structural genes for dynein polypeptides (Luck and Piperno, 1989; Mitchell and Kang, 1991; Sakakibara et al., 1993). The oda mutants swim slowly (~60 μm/s versus ~190 μm/s for wild-type cells); both pf13A and pf22 have paralyzed flagella. In wild-type cells, the cis and trans flagella exhibit different intrinsic beat frequencies (62 ± 11 and 74 ± 9 Hz, respectively), whereas the flagella of the oda mutants fail to show this imbalance (Sakakibara and Kamiya, 1989). When outer arm dynein from wild-type cells is rebound to axonemes of the mutant oda1 *in vitro*, both flagella beat at the same higher frequency. This observation led to the suggestion that the outer arm dyneins from the cis and trans flagella are different, perhaps as the result of a posttranslational modification such as phosphorylation (Sakakibara and Kamiya, 1989). At present, the α DHC is the only component of the *Chlamydomonas* outer dynein arm known to be subject to such modification (King and Witman, 1989; Piperno and Luck, 1981).

Recently, an additional mutation (oda11) affecting the outer dynein arm has been described (Sakakibara et al., 1991). Cells defective at this locus swim at a speed intermediate between wild-type cells and oda cells lacking the complete outer arm and do not show the cis/trans frequency imbalance. Biochemical analysis of flagella from oda11 indicates that these cells do form an outer arm but that the structure lacks the α DHC and its tightly associated 16-kDa light chain. Restriction fragment length polymorphism analysis suggests that this mutation occurs within the structural gene for the α DHC. Thus, although not essential for the stable assembly of the outer arm, the α DHC appears to function in force transduction and to be involved in determining the beat frequency of the cis versus trans flagellum.

In this report, we have examined the phosphorylation of the α DHC *in vivo*. We demonstrate that this molecule is phosphorylated at a minimum of six sites and that rapid acylation/deacylation at several of these sites occurs within the flagellum. Moreover, we have located the regions containing these sites on the previously described map of this DHC (King and Witman, 1988) and, thus, can predict the location of these sites within the dynein particle.

**Footnotes:**

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1 The abbreviations used are: DHC, dynein heavy chain; IC, intermediate chain; LC, light chain.
EXPERIMENTAL PROCEDURES

In Vivo Labeling with [32P]Phosphate—Chlamydomonas reinhardti strains 1132D and pf18 were grown in minimal medium containing one-tenth the normal amount of phosphate (MP/10 medium) for several weeks to reduce the internal phosphate stores (Piperno and Luck, 1981). For continuous labeling experiments, cells were grown for 4 days in 4 liters of MP/10 medium containing -20 mCi of carrier-free [32P]orthophosphoric acid (DuPont NEN). For pulse labeling, cells were harvested, washed twice with phosphate-free medium, and then incubated with gentle stirring in the presence of the radionuclide. Routinely, 60–70% of the label was taken up by the cells within 10 min. Cells were harvested and deflagellated with dibucaine as described previously (Witman, 1986).

Purification and Cleavage of Dynein—Flagella were demembranated with Nonidet P-40 and the resulting axonemes extracted with 0.6 M KCl. All buffers contained 10 mM NaF to inhibit protein phosphatase activity. The αβ dimer and γ subunits of the outer dynein arm were subsequently purified by sucrose gradient centrifugation as described previously (King et al., 1986). Dynein heavy chains were fragmented by digestion with trypsin and elastase as described by King and Witman (1988) and by vanadate-mediated photolysis (Lee-Eiford et al., 1986; King and Witman, 1987).

Phosphoamino Acid Analysis—Purified 32P-labeled dynein was subjected to acid hydrolysis in 6.5 M HCl at 110°C for 2.5 h. Samples were lyophilized, spotted onto a cellulose thin-layer plate (E. Merck, Darmstadt, Germany), and chromatographed in pyridine/acetic acid/water (10:100:1890). The locations at which the phosphoamino acid standards (Ser(P), Thr(P), and Tyr(P)) migrated were determined by staining the plate with ninhydrin.

Gel Electrophoresis and Autoradiography—Samples were separated in either 5–15% acrylamide, 0–2.4 M urea or 5–15% acrylamide, 2–8% SDS-midgap gradient gels as described previously (King et al., 1986). Gels were stained with Coomassie Brilliant Blue or by the silver stain method of Merril et al. (1981). Dried gels were autodradiographed for 1–5 days using the software package Quantity One (pdi, Huntington Station, NY) running on a Sparc workstation (Sun Microsystems, Inc., Mountain View, CA).

RESULTS

Phosphorylated Dynein Proteins and the Stoichiometry of Labeling—A sucrose gradient profile of axonemal dynein purified from cells grown in the presence of [32P]phosphate is shown in Fig. 1. As reported previously (Piperno and Luck, 1981), both 18 S and 12/10.5 S dynein peaks contain phosphorylated proteins. Within the 12/10.5 S peak, label is associated with inner arm dynein heavy chains and with unknown axonemal components of both higher and lower Mr. (Fig. 2a). However, no label is found in the outer α DHC from the outer arm. In the 18 S peak, which contains the αβ dimer from the outer arm, only the α DHC is phosphorylated (Fig. 2b). Similar patterns of labeling of the dynein polypeptides are found in samples prepared from pulse-labeled cells (not shown).

Under the above conditions, approximately 4.8 nmol of phosphate are incorporated per mg of αβ dimer. Using a mass for the dimer of 1.27 MDA and assuming that phosphate pools have become equilibrated during the 4-day experiment, the stoichiometry of labeling is calculated to be 6.1 mol of phosphate/mole of dynein. Thus, there are at least six sites of phosphorylation within the α DHC. Phosphoamino acid analysis of dynein from pulse-labeled cells indicates that this molecule is phosphorylated mainly on serine residues (not shown).

Location of Phosphorylation Sites within the α DHC—Previously, we examined the pathways by which the α DHC was proteolyzed (King and Witman, 1988). In combination with vanadate-mediated photolysis at the V1 and V2 sites and epitope mapping with monoclonal antibodies specific for the α DHC, the proteolytic cleavage sites were located on a linear map of the molecule and thereby the origin of the various fragments determined (see Fig. 7).

Photolysis of the α DHC at the V1 site generates two fragments of Mr, 190,000 and 290,000 (King and Witman, 1987). When prepared from continuously labeled cells, ~53% of the 32P label was found in the smaller N-terminal fragment (Fig. 3). Interestingly, dynein from pulse-labeled cells showed a somewhat different pattern of incorporation with ~44% of the label in the N-terminal fragment (not shown). This indicates that phosphorylation sites are located to both sides of the V1 site. Furthermore, as axonemal polypeptides are turned over very slowly (Remillard and Witman, 1982), the data suggest that phosphorylation/dephosphorylation at sites located in both sections of the α DHC occurs rapidly in vivo.

To further determine the location of phosphorylated residues in the α DHC, the purified αβ dimer was digested with trypsin and elastase. This experiment was possible only after using dynein from pulse-labeled cells; insufficient amounts of material were obtainable from continuously labeled cells to make this further analysis practical. Proteolytic fragments were identified by comparison with the previously established digestion pattern (King and Witman, 1988), and the amount of 32P incorporated into the fragments was quantitated. Tryptic digestion of the α DHC first occurred at a site (T1) located 90 kDa from the C terminus (Fig. 4b). Both fragments (α390 and α315) were labeled (Fig. 4a). Subsequent digestion of α390 occurred at several discrete locations. The major pathway involved cleavage at T3 (Fig. 5c) to yield an N-terminal fragment (α272) and α315, which represents the central portion of the molecule. The α272 fragment was not labeled; all of the label located within α390 was found in the central α315 fragment (Fig. 5, a and b).

Digestion of α317 then proceeded via cleavage at T2, T7, and T10 to yield the relatively stable fragment α45 (Fig. 5b). This fragment spans the V1 photolysis site (Fig. 5c) and contained most (if not all) of the label found in α317. As the N-terminal V1 fragment was labeled (see above), phosphorylation

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1 This estimate utilizes a value of ~510 kDa for the mass of each Chlamydomonas axonemal DHC (Mitchell and Brown, 1994; Wilkerson et al., 1994) and is entirely consistent with the scanning transmission electron microscopic analysis, which gave a mass of 1.22 ± 0.117 MDA (Witman et al., 1983).

2 Molecular cloning now permits the map to be oriented with regard to the N and C termini.
Phosphorylation of Dynein Polypeptides. Sucrose gradient purified dyneins continuously labeled with [γ-32P]Posphate were electrophoresed in a 3–5% acrylamide gradient gel. The silver-stained gels (SS) and corresponding autoradiographs (AR) are shown. a, fractions across the 12 S region of the sucrose gradient. Note that the γ DHC (γ), which is found mainly in the first two lanes, is not labeled but that several inner arm DHCs (IA) in lanes 3 and 4 and several other axonemal components are labeled. b, the peak fraction from the 18 S region. Only the α DHC and its breakdown product (111) are phosphorylated. Lower molecular weight components of the outer arm are not labeled (not shown).

Photocleavage of phosphorylated α DHC at the V1 site. The α@ dimer was photocleaved at the V1 site by UV irradiation in the presence of MgATP2- and vanadate and the resulting fragments separated in a 3–5% acrylamide gel. The locations at which the α DHC and the 290- and 190-kDa V1 fragments migrated are indicated in the left panel (silver stain). The corresponding autoradiograph for a sample derived from continuously labeled cells is shown at right.

Elastase digestion of the α DHC occurred sequentially at the sites El and E2 (Fig. 6b), yielding a heavily labeled central fragment of M, 317,000 and terminal fragments of M, 75,000 and 88,000 (Fig. 6a). Consistent with the data from trypsin digestion, the α390 fragment, which represents the N-terminal region, was not labeled. The C terminal fragment α88 was labeled but to a much lesser extent than α390 (cf. Fig. 4). Quantitative analysis indicates that ~53.5% of the [32P]phosphate incorporated into the α DHC is found within α88; only ~25% is within α390. This suggests that at least one and probably more sites of phosphorylation are located in the small region of ~2 kDa between the E1 and T1 sites. The α88 fragment appears to be further digested to yield a labeled fragment of M, 76,000; the precise origin of this peptide within α88 is uncertain.

The location of phosphorylated regions of the α DHC is shown diagrammatically in Fig. 7.

Phosphorylation of the α DHC in Situ and in Vitro—Incubation of washed demembranated axonemes with [γ-32P]ATP resulted in the incorporation of label into a variety of axonemal polypeptides (Fig. 8). Under these conditions, the α DHC and several inner arm DHCs became modified, whereas the β DHC (Fig. 8) and tubulin (not shown) did not. The addition of [γ-32P]ATP to sucrose gradient-purified dynein resulted in the labeling of both α and β DHCs and tubulin (not shown). This suggests that the phosphorylation
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Fig. 5. Tryptic digestion of the fragment α"DHC. Autoradiographs of 5–5% (a) and 5–15% (b) acrylamide gradient gels showing the tryptic digestion of the fragment α"DHC to yield α"317 (a) and the α"317 fragment to yield α"16 (b). For a, 6 µg of dynein were digested with (from left to right) 0, 2.5, 5, 25, 100, 250, 500, 1500, 2500, and 3750 ng of trypsin. For b, only the latter eight digests are shown. The origin of the various fragments and the location of the tryptic cleavage sites are shown in (c).

DISCUSSION

In this study, we have investigated the number, nature, and location of phosphorylation sites within the α DHC in vivo. This polypeptide apparently is the only component of the Chlamydomonas outer arm that is phosphorylated in vivo (King and Witman, 1989; Piperno and Luck, 1981), and phenotypic analysis of the mutant odal1 suggests that this molecule is involved in the determination of both swimming speed and flagellar beat frequency (Sakakibara et al., 1991). Moreover, biochemical studies indicate that the association of axonemal components in situ is specific and that this specificity is imparted by the structural organization of the axoneme. The incorporation of label into the α DHC was not affected by the addition of cAMP, cGMP, Ca²⁺, or calmodulin to the reaction mixture (not shown).⁴

Fig. 6. Elastase digestion of the phosphorylated α DHC. α, autoradiograph of pulse-labeled dynein (6 µg) digested with 0, 12.5, 25, 37.5, 50, 100, 250, 375, 500, 1000, 1500, 2500, and 3750 ng of trypsin. The samples were separated in a 5–15% acrylamide gradient gel. The α DHC is cleaved first at E₂ to give rise to α"E₃ and α"E₄, the latter is only very weakly labeled. α"E₅ is then digested at E₁ to yield α"E₆ and α"E₇ (unlabeled and, hence, not visible). α"E₈ appears to be further cleaved to yield α"E₉, b, map showing the location of the E₁ and E₂ sites and the origin of the various fragments.

Fig. 7. Location of phosphorylation sites within the α DHC. A map of the α DHC (adapted from King and Witman, 1988) showing the locations of the sites of vanadate-mediated photolysis (large arrows labeled V₁, V₂a, V₂b, and V₂c), elastase (E) and tryptic (T) cleavage sites (small arrows), the sections containing the epitopes recognized by monoclonal antibodies 18aA and 18aB, and the regions that are phosphorylated in vivo (hatched bars) is shown. The scale of the diagram is also shown. Map distances were derived from the mobility of fragments in polyacrylamide gels (King and Witman, 1988). Molecular cloning of other DHCs suggests that the value of ~480 kDa used for the mass of the α DHC is an underestimate by ~30-40 kDa (i.e. by <10%).

Note:⁴ One axonemal polypeptide (Mₙ ~20,000) was phosphorylated in the presence of cAMP (and, to a much lesser extent, cGMP) but not in its absence. This polypeptide and its associated kinase were not extracted from the axoneme under high salt conditions.
Fig. 8. Phosphorylation of the α DHC in vitro. Washed axonemes were incubated with [γ-32P]ATP for 10 min. The reaction was stopped by the addition of SDS-sample buffer, and the components were separated in a 3–5% acrylamide gradient gel. The high molecular weight regions of the silver-stained gel (SS) and the corresponding autoradiograph (AR) are shown.

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To both the ATP hydrolytic site and also to a 30-amino acid region that is similar to the ATP-sensitive microtubule-binding site of kinesin (Wilkinson et al., 1994). This region also is relatively close to the predicted location of the junction between the head and stem domains of the particle (see Witman et al. (1994) for review). The second region is between the proteolytic cut site T1 and the C terminus. Within this 90-kDa region, at least one site is found in the ~2-kDa section between the T1 and E1 sites. Although this region of the α DHC has yet to be cloned and sequenced (see Mitchell and Brown (1994) and Wilkerson et al. (1994)), these sites are close to regions that in other DHCs are predicted to adopt an α-helical coiled-coil structure (Gibbons et al., 1991; Ogawa, 1991; Koonce et al., 1992; Mikami et al., 1993; Mitchell and Brown, 1994; Wilkerson et al., 1994).

Both of the above regions are labeled during 10-min pulses, indicating that at least some of the sites in both regions turn over rapidly in vivo. This suggests that these sites may be involved in the regulation of outer arm activity. In cells labeled to steady state, relatively more label is found in the α DHC region N-terminal to the V1 site. This indicates that some modifications in this region also are turned over more slowly. Indeed, acylation at certain of these sites may occur either within the cell body or during transport and/or assembly of the outer arm into the axoneme.

When purified axonemes were incubated with ATP, the α DHC and some inner arm DHCs were phosphorylated. Importantly, neither the β DHC nor tubulin (the major axonal component) were modified in situ; both were readily acylated by endogenous kinases following extraction from the axoneme. This strongly suggests that the labeling observed in situ is specific; similar criteria for the specificity of in vitro labeling reactions utilizing endogenous kinases were employed by Segal and Luck (1985) in a study of axonemal phosphoproteins that mediate the Ca++-dependent photophobic response. As the axoneme is a solid-state system, the kinase(s) responsible for these acylations must be located close to their targets in the axonemal superstructure. Possibly, they are associated with the wall of the B-tubule of the adjacent outer doublet. Alternatively, as the heads of one outer arm overlap the base of the next (Goodenough and Heuser, 1982), they may reside at the base of the arm.

The α DHC contains at least 6 phosphorylated residues. However, the stoichiometry of phosphorylation at each site is unknown. Therefore, in the event of substoichiometric levels of modification at one or more of these sites, the actual number of acylated residues may be somewhat higher. Purification and sequence analysis of individual phosphopeptides from cells labeled to steady state will be required to answer this important point.

In Paramecium, CAMP stimulates the phosphorylation of several axonemal polypeptides, including a 29-kDa protein that copurified in sucrose gradients with 22 S dynein, and a high molecular weight protein that sedimented in the 19 S region and may be a DHC (Bonini and Nelson, 1990). It has been reported that CAMP-dependent thiophosphorylation of the 29-kDa polypeptide affects the rate of microtubule translocation by dynein in vitro (Hamasaki et al., 1991). However, the origin of this peptide is uncertain. Purified 22 S dynein contained only 0.3 mol of the 29-kDa polypeptide/mol of 22 S dynein. Furthermore, only ~2% of the 29-kDa molecules were modified, and it is unclear how phosphorylation of such a small fraction of molecules could affect microtubule gliding rates in vitro. By analogy with Chlamydomonas (Pfister et al., 1982; Piperno and Luck, 1979) and Tetrahymena (Porter and Johnson, 1983) dyneins, most of the Paramecium 22 S dynein (Larsen et al., 1991; Schroeder et al., 1990) probably represents outer arm dynein. However, it is possible that an inner arm dynein species also sediments at ~22 S in sucrose gradients (as is the case for Chlamydomonas (Piperno et al., 1990)), that this inner arm species is responsible for the microtubule translocation observed in the in vitro assays of Hamasaki et al. (1991), and that the 29-kDa polypeptide is a regulatory light chain of inner arm dynein.

In another study (Chilcote and Johnson, 1990), it was reported that Tetrahymena 22 S dynein was phosphorylated in vivo, primarily on 78-, 76-, 47-, and 23-kDa polypeptides and, to a lesser extent, on a 30-kDa polypeptide and a DHC. The stoichiometries of these chains relative to known outer arm components was not reported. Again, further characterization of ciliary dyneins will be necessary to determine whether these polypeptides represent inner or outer arm components. In any event, it is unlikely that the above results from ciliates are relevant to the situation for the Chlamydomonas outer arm dynein, which appears to phosphorylated in vivo only on the α DHC.

Cyclic AMP-dependent phosphorylation also has been observed in dyneins from molluscan cilia and flagella (Stephens and Prior, 1992). Interestingly, two dynein light chains were labeled in gill cilia, whereas in sperm flagella, incorporation of label was found solely into the α DHC. In both cases, phosphorylation was correlated with the stimulation of motility by serotonin but did not affect steady-state ATPase activity in vitro. Analysis of the α DHC from sperm dynein showed that the modification occurred within the N-terminal V1 fragment. Thus, this phosphorylation site may be analogous to that which is located in the 20-kDa section between T1 and the V1 site of the Chlamydomonas α DHC.

In conclusion, we have identified sites of phosphorylation within the α DHC. These sites are located close to regions of functional or structural significance and turn over rapidly in vivo. The data presented here provide the necessary framework to identify each modified residue. By engineering α DHC genes so that the encoded polypeptides cannot be phosphorylated at these sites and then introducing the altered genes into the null mutant tad1 by transformation (Kindle et al., 1989), it will be possible to determine the functional significance of...
the individual phosphorylation sites on this DHC.

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