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Ciliary Dynein Conformational Changes As Evidenced by the Extrinsic Fluorescent Probe 8-Anilino-1-naphthalenesulfonate†

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ABSTRACT: The binding of 8-anilino-1-naphthalenesulfonate (ANS) to ciliary dynein ATPase leads to a marked increase in the dye's fluorescence intensity, accompanied by a blue shift in the observed fluorescence emission maximum. We found that dynein has 37 ± 3 ANS binding sites and that experimentally applied ANS concentrations failed to alter enzyme activity. The fluorescence properties of the enzyme–dye complex were used to learn more about the binding characteristics of dynein substrates and effectors and to probe for possible conformational changes of the enzyme. The fluorescence of the dynein–ANS complex is increased by a number of substrates, including ATP, GTP, and UTP. The transfer of excitation energy from dynein chromophores to adsorbed ANS was also investigated. Our findings indicate that dynein appears to undergo a localized conformational change in its interaction with ATP. Native dynein was also found to be conformationally different from heat-activated or NEM-modified enzyme as evidenced by the emission and excitation spectra of the various enzyme–ANS complexes.

For a number of protein systems, the fluorescent dye 8-anilino-1-naphthalenesulfonate has been used successfully as a probe in the investigation of hydrophobic regions and conformational changes. ANS[1] as a probe of protein hydrophobic sites was suggested by Stryer (1965), who studied its binding to apomyoglobin and apohemoglobin. In some instances, the binding of the ANS occurs at specific sites on the protein. Thus, for bovine serum albumin, the ANS may bind at the fatty acid binding site (Brand, 1970); for transaldolase, it may bind at the fructose 6-phosphate site (Daniel & Weber, 1966); for apohemoglobin and apomyoglobin, it may bind at the heme site (Stryer, 1965). An alternative use of the dye is to investigate the effector–protein interactions that may be associated with the changes in the fluorescence of the protein–ANS complex (Bloxham, 1973; Cheung, 1969). For example, several studies have reported the use of ANS binding to monitor glutamate dehydrogenase conformational changes (Thompson, 1967). The utility of ANS for studying the conformational state of myosin has been demonstrated in several reports, particularly that by Cheung & Morales (1969). They demonstrated that the fluorescence of myosin was quenched by its interaction with ANS and that this quenching resulted from a transfer of the excitation energy from myosin chromophores to the absorbed dye. They also found that this quenching was influenced by chemical modification of the enzyme; indeed, the decrease in transfer of excitation energy was attributed to changes in myosin conformation resulting from the amino acid side-chain modifications.

Dynein conformational changes at the active sites or at the location of dynein–microtubule interactions are important because such changes might relate to the origin of mechanical thrust in cell motility. Yet, virtually nothing is known about these anticipated conformational changes, and we investigated the fluorescent properties of the ciliary dynein ATPase interactions with ANS. As will be reported below, we found that dynein ATPase displays a large number of ANS binding sites, and this is an unlikely property for ANS binding strictly at the nucleotide hydrolytic site. The fluorescent properties of the complex appear to be a useful indicator of conformational changes that may be induced in the protein by different substrates and/or effectors of the enzyme. The transfer of excitation energy from dynein chromophores to the adsorbed ANS was also investigated. Because transfer of excitation energy results from dipolar interactions, transfer efficiency is

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate magnesium salt; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; NEM, *N*-ethylmaleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; PEP, phosphoenolpyruvate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMPPCH$_2$P, adenosine 5′-(β,γ-methylenetriphosphate).

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governed by, among other factors, donor to acceptor distance. Thus, the efficiency of energy transfer could be a sensitive indicator of structural changes of dynein attending ligand interaction. Our findings indicate that dynein undergoes a localized conformational change during its catalytic interaction with ATP. Native dynein was also found to be conformationally different from heat-activated or NEM-modified dynein.

Experimental Procedures

Materials. ATP (orthovanadate free), UTP, GTP, NEM, ANS (magnesium salt), and phosphoenolpyruvate were purchased from Sigma. P^1,P^5-Bis(5′-adenosyl)pentaphosphate, NADH, pyruvate kinase, and lactate dehydrogenase were obtained from P-L Biochemicals. *erythro*-9-(2-Hydroxy-3-nonyl)adenine (EHNA) was purchased from Burroughs Wellcome (Research Triangle Park, NC). For isolation of dynein, axonemes of *Tetrahymena thermophila* (strain SB-255, obtained from Professor Eduardo Orias, University of California, Santa Barbara) were prepared as described elsewhere (Haimo et al., 1979). Axonemes were resuspended in extraction buffer (pH 7.4, containing 0.5 M NaCl, 10 mM HEPES, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL PMSF, 1.0 μg/mL leupeptin), extracted at 0 °C for 30 min, and centrifuged at 39000*g* for 20 min. Where indicated, the supernatant fluid containing the crude dynein (about 3.0 mg/mL) was diluted with 7 volumes of 10 mM Tris-HCl, pH 8.0, and 4 mM $MgCl_2$ and immediately applied to a DEAE-Sephacel column (2-mL bed volume) that had been previously equilibrated with 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 4 mM $MgCl_2$, as described elsewhere (Johnson & Wall, 1983). Dynein-containing fractions eluted upon addition of the same buffer containing 0.2 M NaCl.

For sucrose density centrifugation, a 5-mL linear gradient from 5 to 20% sucrose (w/v) was prepared in the dynein extraction buffer (described above). Samples of dynein (200 μL) containing 500–600 μg of protein were layered onto gradients. In companion runs, gradients with catalase (11.2 S) served as sedimentation standards. All samples were centrifuged for 5 h at 4 °C in cellulose nitrate tubes in a Beckman SW 50.1 rotor at 50000 rpm. Seven-drop fractions were manually collected from the bottom of each pierced tube.

Fluorescence Measurements. A Spex Fluorolog II spectrofluorometer, equipped with a 150-W xenon source, was used to record fluorescence intensities (uncorrected). A four-position variable-temperature accessory was used in all experiments to keep the sample temperature constant at 10 °C. The ANS–dynein complex was excited at 380 nm. Fluorescence emission of the complex was observed at 470 nm. All fluorescent measurements used a 10-mm path-length cell that was magnetically stirred. Dye concentration was calculated from the molar absorption coefficient of 4.95×10^3 (Christian & Janetszko, 1971). All experiments were corrected for fluorescence of dye, enzyme, and reagents. Titrations with the ANS were manually made by the addition of small volumes of a concentrated ANS solution to a standard solution of dynein in 10 mM Tris-HCl (pH 8.0) and 4 mM $MgCl_2$. The extent of dilution never exceeded 5% of the starting volume.

Enzymatic Assays. The ATPase was assayed with the pyruvate kinase–lactate dehydrogenase coupled assay originally described by Pullman et al. (1960) and modified by White et al. (1980). Reactions were monitored on a Cary 210 recording spectrophotometer equipped with a five-sample rotating turret maintained at 25 °C. The reaction was initiated by the addition of dynein to the assay system. Adenosine diphosphate production was followed by the loss in 340-nm absorbance accompanying the oxidation of NADH.

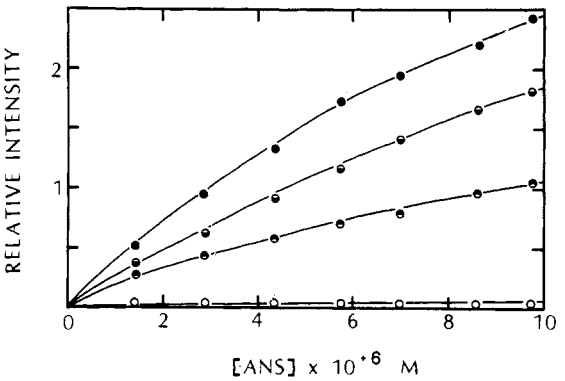

FIGURE 1: Fluorescence titration of *Tetrahymena* dynein ATPase with ANS. Equilibrium mixtures of dye–protein complex are (○) no protein and (⊖) 50, (⊖) 100, and (●) 150 μg of protein/mL. The titrations were carried out in a total volume of 2.5 mL of buffer [10 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$] and ANS in the concentration range from 0 to 10^{-5} M.

Results

Characterization of Fluorescence of the Dynein–ANS Complex. To assess the potential utility of ANS as a probe of dynein–ligand interactions, we carried out a series of experiments on the excitation behavior of ANS in the absence and presence of dynein. We observed that the excitation of ANS (20 μM) at 380 nm resulted in a low fluorescence emission with a maximum 545 nm. Addition of dynein ATPase (24 nM final) resulted in a large increase in the fluorescence intensity, which was accompanied by a blue shift in the emission spectrum to a broad maximum of 470 nm. These data indicate that dynein readily binds ANS, but the quantitative interactions remained to be assessed. Under similar conditions, the bovine serum albumin–ANS complex had an emission maximum of 468 nm.

For our purposes, the absolute quantum yield of the fluorescence of the ANS bound to dynein was not directly determined; however, it was shown that, at pH 8, dynein caused a 40-fold increase in the relative quantum yield compared to the ANS free in solution. At the same molar protein concentration and ANS concentration, the bovine serum albumin–ANS complex had a relative quantum yield that was 3.5 times greater than that observed with the dynein–ANS complex.

We next established a titration of dynein with varying levels of ANS. The increase in fluorescence at the emission maximum of 470 nm was plotted as a function of increasing ANS concentrations. The titration curves for the ciliary dynein ATPase are shown in Figure 1 with data obtained at four different enzyme concentrations. All fluorescence intensity measurements were taken within 30 s after the addition of small increments of concentrated ANS solutions. Nonetheless, we have found that the same behavior is observed at sampling times as short as a few seconds or as long as 1 h.

The number (n) of dye binding sites on dynein ATPase and the dissociation constant for the dye–protein complex (K_d) were determined from the equation originally developed by Klotz et al. (1946):

$$d^{-1} = \frac{K_d}{n(1 - X)[ANS_0]} + n^{-1}$$

where d is the number of moles of bound ANS per mole of protein, n is the number of binding sites per protein molecule, K_d is the dissociation constant, $[ANS_0]$ is the total ANS

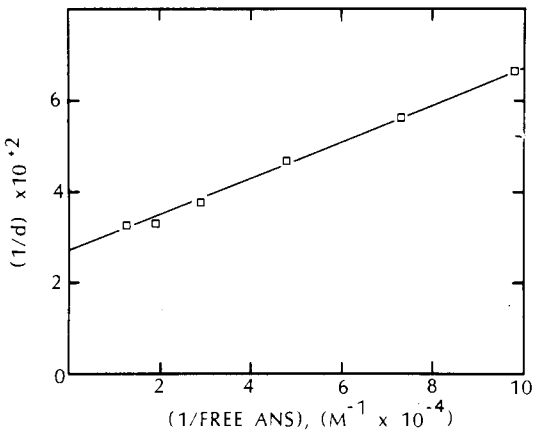

FIGURE 2: Klotz plot ($1/d$ vs. $1/$[free ANS]) for the determination of the number of ANS binding sites on dynein ATPase. Experiments were performed as described in the text with an enzyme concentration of 2.2×10^{-7} M. The theory line is fitted to points by least-squares analysis.

concentration, and X is the fraction of ANS bound. In these calculations the assumption is made that ANS binding to each site is independent of the dye occupancy of other sites and that all binding sites are identical in character. The data were fitted by a least-squares method. For each concentration of ANS, the fraction of dye bound (X) was determined from the relationship

$$X = (F_{obsd} - F_{dye})/(F_{max} - F_{dye})$$

where F_{obsd} is the observed fluorescence, F_{max} is the fluorescence at infinite dynein concentration, and F_{dye} is the fluorescence of the dye free in solution. The concentration of unbound ANS was determined by multiplying [ANS_0] by $1 - X$. F_{max} was obtained at a given ANS concentration by measuring the fluorescence at various dynein concentrations. The linear double-reciprocal plots (i.e., F_{obsd}^{-1} vs. [dynein]$^{-1}$) gave F_{max} values by extrapolation to infinite enzyme concentration; such values agreed with companion experiments in which linear plots of F_{obsd}^{-1} vs. [ANS]$^{-1}$, at fixed dynein concentration, were used to estimate F_{max}.

A typical Klotz plot for ciliary dynein ATPase is shown in Figure 2. We found that the DEAE-treated enzyme (using a value of 1.7×10^6 daltons per dynein outer arm) had 37 binding sites and an average protein–dye dissociation constant of 1.4×10^{-5} M. The number of binding sites for the ANS is in excess of the number of ATP and tubule binding sites estimated for the enzyme (Johnson & Wall, 1983); so, it seems unlikely that ANS binds exclusively to the catalytic or tubule sites on dynein. Further indication that interactions among ANS molecules and dynein are independent of ATP binding was obtained from enzyme activity measurements. The results of one such experiments are shown in Figure 3, and they indicate that dynein's enzymatic properties over a range of substrate concentrations were unaffected by ANS (50 μM). In a companion experiment, it was also demonstrated that ANS had no effect on the EHNA inhibition pattern of the ciliary dynein ATPase. The significance of this observation will be discussed below.

Influence of Substrates and Effectors of Dynein ATPase on the Enhancement of Fluorescence. In Figure 4, we show the effect of dynein substrates and effectors on the fluorescence of the enzyme–ANS complex. Of all the compounds tested, the most potent agent for increasing the fluorescence was ATP, followed by ADP, GTP, and ATPγS. AMPPCH$_2$P and UTP caused only a slight increase in fluorescence, whereas EHNA was virtually without effect. To assess the significance of the

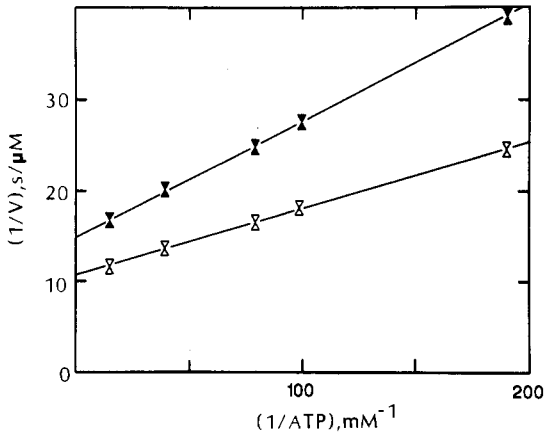

FIGURE 3: Plot of the reciprocal of the initial velocity (v) vs. the reciprocal of the millimolar concentration of MgATP, in the absence and presence of 50 μM ANS. Final enzyme concentration in the assay was 30 μg/mL, and ATP was varied in the concentration range from 5 to 50 μM. (Lower line) Activity observed in the absence (▽) and presence (△) of 50 μM ANS; (upper line) inhibition of *Tetrahymena* dynein ATPase by EHNA (180 μM final concentration) in the absence (▼) and presence (▲) of 50 μM ANS.

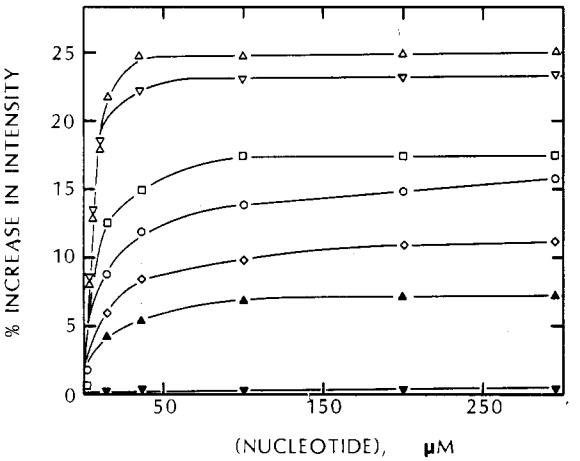

FIGURE 4: Influence of substrates and effectors on the fluorescence of the dynein–ANS complex plotted as the percentage increase in the corrected fluorescence intensity vs. ligand concentration. Fluorescence of the ANS (30 μM) was recorded in 10 mM Tris-HCl (pH 8.0) and 4 mM $MgCl_2$ in the presence of dynein ATPase (100 μg/mL) and plotted concentrations of the following effectors: (△) ATP; (▽) ADP; (□) GTP; (○) ATPγS; (◇) AMPPCH$_2$P; (▲) UTP; (<<*ds) EHNA.

increase in fluorescence in the presence of nucleotides, the fluorescence properties of the dynein–ANS complex were investigated under conditions where the enzyme was denatured. In 6 M guanidine hydrochloride, the fluorescence intensity of the ANS (30 μM) in the presence of dynein ATPase increased slowly upon combination, reaching a maximum after about 30 min. The relative fluorescence intensity was decreased in the presence of guanidine hydrochloride. In the absence of the denaturing reagent, maximal fluorescence was always promptly attained. Under denaturing conditions, the addition of ATP (100 μM), ADP (100 μM), GTP (300 μM), or UTP (300 μM) had no effect on the fluorescence, and this observation suggests that the increase of fluorescence caused by the nucleotides is dependent on the structural integrity of dynein.

To understand further the nucleotide binding effects on dynein–ANS fluorescence, it was necessary to investigate substrate specificity and kinetic properties of *Tetrahymena* dynein ATPase. We found that the ligands exhibited the following order of effectiveness in enhancing ANS fluorescence: ATP (100), ADP (94), GTP (53), ATPγS (36), AMPPCH$_2$P (26), UTP (18), and EHNA (1). (The nu-

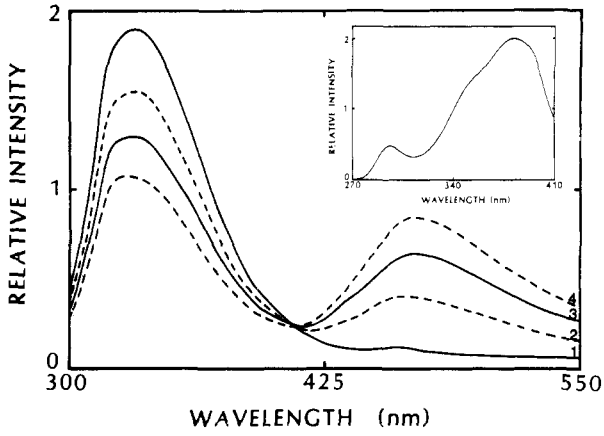

FIGURE 5: Fluorescence emission spectra of dynein–ANS. The dynein concentration was 2.1×10^{-7} M, and excitation was at 289 nm. Spectra were obtained for dynein alone (curve 1), dynein plus 1×10^{-5} M ANS (curve 2), dynein plus 3×10^{-5} M ANS (curve 3), and dynein plus 6×10^{-5} M ANS (curve 4). (Insert) Excitation spectum of native dynein–ANS complex: 2×10^{-7} M dynein, 2×10^{-5} M ANS, 10 °C. The wavelength of the emitted light was preset at 470 nm, and excitation data were collected by scanning the excitation monochromator from 270 to 410 nm.

cleotide concentrations were each 10 μM, and the number in parentheses refers to the percentage attainment of the ATP-induced enhancement.) Fluorescence of the enzyme–ANS complex parellels the nucleoside 5′-triphosphate substrate specificity of dynein ATPase as reported by S. A. Anderson et al. (unpublished results).

Energy Transfer of the Dynein–ANS Complex. Other important considerations in our study were the energy-transfer properties of the protein-bound dye. In Figure 5, the emission spectra of dynein and the dynein–ANS complex, obtained by exciting at 289 nm, are shown. In the absence of 8-anilino-1-naphthalenesulfonate, the fluorescence peak at 334 nm arises largely from excitation of the tryptophan residues. Upon ANS addition, the tryptophan residues are quenched, and a second peak appears in the 470-nm region. This peak at 470 nm is the fluorescence of the adsorbed dye (Cheung & Morales, 1969). With increasing dye concentrations, the tryptophan fluorescence is progressively quenched. This transfer of energy from intrinsic chromophores on a protein to an adsorbed dye has been previously shown for the myosin complex with 8-anilino-1-naphthalenesulfonate (Cheung & Morales, 1969). The energy transfer is also demonstrated from the excitation spectrum (Figure 5, insert). From the equation developed by Cheung & Morales (1969), the efficiency of energy transfer for the native dynein is estimated at 27%.

Modification of 30S Dynein by N-Ethylmaleimide and Heat Treatment. Blum & Hayes (1977, 1978) reported that treatment of 30S dynein with NEM (10 μM) or elevated temperature exposure (37 °C for 20 min) increases the ATPase activity. Their studies indicated that the enhancement of *Tetrahymena* ciliary dynein ATPase activity by mild heat treatment and by incubation with low concentrations of NEM or a spin-labeled analogue of NEM resulted in a similar conformational change.

Emission and excitation spectra of dynein–ANS complexes were obtained with dynein samples that had been modified by treatment with NEM or by mild heating. In Figure 6, we compared the emission spectrum of native vs. NEM-modified dynein, and this reveals that the energy transfer in the modified sample is greater than that observed with the native sample. This difference is also represented in the excitation spectrum (Figure 6, insert). At levels of NEM known to activate 30S

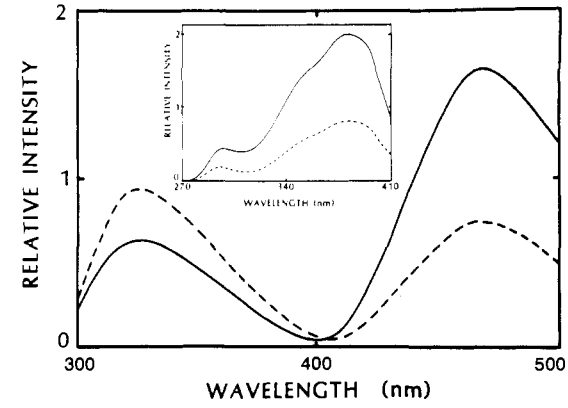

FIGURE 6: Comparison of emission spectra for native and NEM-modified dynein–ANS complex. One sample of dynein (—) was incubated with 10 μM NEM for 24 h at 0 °C, and unmodified dynein (– –) was also analyzed at the same final enzyme concentration (24 nM). (Insert) Comparison of excitation spectra for native and NEM-modified dynein–ANS complex. NEM-modified dynein (—), prepared as described above, and native dynein (– –) were analyzed at 24 nM final protein concentration. Emission spectra were obtained by excitation at 289 nm; excitation spectra were obtained by fixing the emission wavelength at 470 nm.

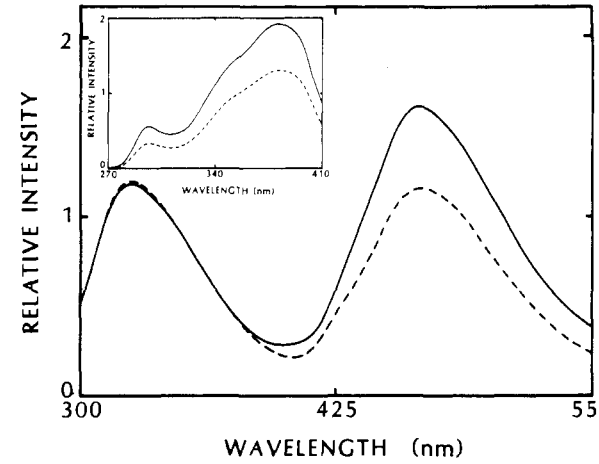

FIGURE 7: Comparison of emission spectra for native and heat-activated dynein–ANS complex. One sample of dynein was incubated at 37 °C for 20 min and then placed on ice for 10 min. The final enzyme concentration for the heat-activated (—) and the native dynein (– –) was 24 nM. Data were obtained at 25 °C. (Insert) Comparison of the excitation spectra for native and heat-activated dynein ATPase. The ANS concentration was 30 μM; native dynein (– –) and heat-activated dynein (—) final concentration was 24 nM. Emission spectra were obtained by excitation at 289 nm; excitation spectra were obtained by fixing the emission wavelength at 470 nm.

dynein ATPase, the efficiency of energy transfer is calculated to be about 57%.

We also obtained and compared the excitation and emission spectra of dynein ATPase after activation of its latent form by heat treatment. As shown from Figure 7, the efficiency of energy transfer is comparable to that found with the NEM-modified dynein (i.e., about 50%).

Finally, ANS binds to both the 30S and 14S ciliary dynein ATPases (data not shown). This was demonstrated in several experiments with 30S and 14S forms isolated on sucrose gradients.

Discussion

8-Anilino-1-naphthalenesulfonate has proven to be a valuable extrinsic fluorescent probe of protein conformational changes. In principle, its utility can be quite impressive, and the immediate microenvironment of the probe can be characterized by interpretation of excitation and emission spectra,

fluorescence quantum yields, fluorescence polarization, energy-transfer efficiency, and even fluorescence lifetime data.

With dynein, we have observed a rather high stoichiometry of ANS binding that approaches 37 ± 3 sites per dynein outer arm. This corresponds to about 12 ± 1 sites per dynein subunit if one uses the trimer model of Shimizu & Johnson (1983). Unlike the unit stoichiometry of ANS binding to myosin (Cheung & Morales, 1969) or chymotrypsin (Johnson et al., 1979), the high stoichiometry of ANS binding to dynein limits the range of approaches one may apply. For example, fluorescence polarization of the ANS probe at a single site of chymotrypsin has been used to characterize the correspondence of solution and crystal structures of the protease–ANS complex by referring to X-ray structural data on the complex (Johnson et al., 1979). On the other hand, polarization studies with dynein–ANS complexes would yield data that may not readily admit to structural inferences about the microenvironments around each of the dozen binding sites per subunit. Specific binding of an extrinsic fluorescent probe at a single site still may or may not have value in serving as a reporter group for sensing gross or global conformational changes in protein conformation. Everything depends on the observed sensitivity of the dye's microenvironment to changes in the protein's conformation. Thus, high-stoichiometry ANS complexes increases the chance that a fraction of the sites may be sensitive to protein-shape changes. Bloxham et al. (1973), for instance, observed that rabbit muscle phosphofructokinase also had about 40 binding sites, and this oligomeric protein of M_r 380 000 is at least 4 times smaller than dynein. In spite of the large number of extrinsic reporter groups, they observed that ligands such as ATP, ADP, AMP, and fructose 6-phosphate can decrease the fluorescence of the kinase–sulfonate complex. These findings were interpreted as indicative of a substantial conformational change upon binding adenine nucleotides (or hexose phosphate) and a decrease in the fraction of dye bound to the kinase.

In our experiments, we observe that dynein–ANS interactions with ligands actually result in increased fluorescence intensity. This behavior is opposite to that noted for phosphofructokinase, and two explanations could account for these findings. The number of ANS sites may increase upon exposure to the ligands (or treatments experimentally imposed), and/or a substantial fraction of the bound ANS molecules may be transferred to a more hydrophobic environment. Reaching a distinct choice between these possibilities is difficult for protein–ANS complexes of high stoichiometry, because both can be true for different classes of ANS sites. In any case, we were more concerned with the value of the extrinsic probe as a reporter group for conformational alterations in dynein. We have indeed obtained strong evidence for dynein-shape changes, and these observations may ultimately afford a more direct means for evaluating dynein–ligand interactions.

We are also cognizant that it is still somewhat speculative to assume that ligand binding to the dynein–ANS complex is identical with ligand binding to dynein in the absence of bound fluorescent dye. There is, however, a strong correlation between the observed nucleotide substrate preference (S. A. Anderson et al., unpublished results) and the ability of such ligands to induce conformation changes in the dynein–ANS complex. We also found that ANS binding did not change the kinetic parameters of the ATPase reaction (Figure 3) in the absence or presence of EHNA, a known inhibitor of dynein (Bouchard et al., 1981). Together, these findings suggest that ANS interactions with dynein can be valuable for probing the enzyme's reactions with other ligands.

Although there are many parallelisms between the action of myosin and dynein (e.g., the likely participation of sliding-filament mechanisms based upon the cross-bridging steps linked to the energetics of ATP binding, hydrolysis, and release), we do note that the ANS binding behavior is significantly different. ANS binds to a single site on myosin with a dissociation constant at 20 °C of 4.9×10^{-5} M (Cheung & Morales, 1969), while ANS binds to 37 sites per dynein outer arm with an average dissociation constant of 1.4×10^{-5} M. Ligand binding to and chemical modification of myosin decrease the observed fluorescence of bound ANS (Cheung & Morales, 1969); yet, we find that corresponding treatments increase the observed fluorescence of dynein interactions with ANS. Interestingly, we note that the emission spectra of both are generally similar in terms of observed band shapes and the isoemissive point at 416 nm. A common isoemissive point at high stoichiometry of ANS binding suggests that each ANS molecule is bound in a rather similar hydrophobic locale. The absence of any significant dye–dye interactions is also suggested by the linearity of the Klotz plot (see Figure 2) over the experimentally applied range of protein and ANS concentrations.

Finally, these studies foreshadow the application of rapid-mixing experiments with substrates and enzyme–ANS complex to gain information about transients in enzyme conformation attending nucleotide substrate binding and release steps. Such dynamic information on dynein action would complement work already taking place in a number of laboratories.

ACKNOWLEDGMENTS

We are pleased to thank Michael Saucier for his expert help in computer programming and data analysis.

Registry No. ATPase, 9000-83-3; ANS, 82-76-8; ATP, 56-65-5; ADP, 58-64-0; GTP, 86-01-1; ATPγS, 35094-46-3; $AMPPCH_2P$, 87562-48-9; UTP, 63-39-8; NEM, 128-53-0.

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