A High-Affinity ATP-Binding Site on 30S Dynein

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The enhancing effect of low concentrations (eg, 8 μM) of bis(4-fluoro-3-nitrophenyl)sulfone (FNS) on 30S dynein ATPase activity is increased when 1 mM dithiothreitol (DTT) is present. The effect of FNS + DTT is optimal at pH 7.5. Activation of the latent ATPase activity of 30S dynein by FNS + DTT is partially prevented by 1–3 μM ATP. Adenylylimidodiphosphate (AMP-PNP) is less effective than ATP, while β,γ-methylene-adenosine triphosphate (AMP-PCP), though a much stronger inhibitor of ATPase activity than AMP-PNP, does not protect against enhancement. These results demonstrate the presence of a high-affinity ATP-binding site on 30S dynein.

In previous papers [1–3] it was shown that FNS caused an enhancement of the ATPase activity of demembranated cilia and of extracted 30S dynein, and that this reaction was about 10^4 times slower than the rate of enhancement by the sulfhydryl reagent PPDM. It was also reported that the ATPase activity of 14S dynein could be increased upon reaction with FNS, but that this effect was not observed if the reaction was "stopped" by adding DTT before the addition of ATP. The finding that 14S dynein has latency is of interest because it suggests a possible mechanochemical role of this dynein in cilia. The finding that addition of DTT altered the rate of enhancement of 14S dynein by FNS suggested that it was the addition of ATP that was stopping the reaction, and led us to investigate the effects of ATP on the reaction of FNS + DTT on 30S dynein. It will be shown that concentrations of ATP as low as 1 μM give noticeable protection against the enhancing effects of FNS + DTT on 30S dynein, thus demonstrating the presence of a high-affinity binding site for ATP on 30S dynein.

MATERIALS AND METHODS

Demembranated axonemes, 14S and 30S dyneins, and twice-extracted axonemes of Tetrahymena pyriformis, strain HSM, were prepared as described elsewhere [1], which also presents the procedure for performing the ATPase assays. In this work, all preincubations were at $0°$. After the desired time at $0°$, 0.1 ml 10 mM ATP was added, bringing the volume to 1.0 ml, and the tubes were immediately transferred to a $25°$ bath for 20 min and the reaction terminated by addition of trichloroacetic acid (TCA) as described [1].

Preparation of Reagents

FNS was dissolved in acetone and diluted in acetone as desired. PPDM and OPDM were dissolved in acetone and diluted in buffer. When these reagents were used, they were always as 0.02 ml, control tubes receiving 0.02 ml of the appropriate concentrations of acetone. Solutions of FNS, PPDM, OPDM, and DTT were made fresh daily and kept at $0°$.

Protein Determination

The protein content of axonemes and of pellet II was determined as described earlier [3]. Protein content of dynein solutions was measured by our previously described procedure [3] except that the Coomassie blue reagent was made as described by Spector [4].

Reagents

N,N'-o-phenylenedimaleimide was purchased from Aldrich Chemical Co.; AMP-PNP and TNBS from Sigma; AMP-PCP, from P-L Biochemicals. The source of all other reagents is as earlier reported [1].

RESULTS

We have earlier reported that incubation of 30S dynein with FNS caused enhancement of the ATPase activity, whereas 14S dynein ATPase activity was inhibited [3]. In experiments in which the reaction was "stopped" by addition of DTT just before the addition of ATP and transfer of the tubes to $25°$ for the 20-min ATPase assay, it was found that the presence of DTT appeared to change the inhibitory effect of FNS to an enhancing effect. Because the DTT was present in considerable excess of the FNS, it appeared that the DTT might somehow be participating in the reaction. A series of experiments were undertaken to further clarify the role of DTT in this system. It was found that preincubation of 30S dynein with 1 mM DTT plus 8.7 μM FNS at $0°$ for 90 min could cause up to a sixfold enhancement of ATPase activity, whereas with 14S dynein a twofold enhancement was the maximum obtained. The optimal concentration for DTT was about 1 mM, and β-mercaptoethanol was very much less effective than DTT (Blum and Hayes, unpublished data). In addition to calling into question the assumption [3] that FNS was acting as an SH reagent, these experiments clearly demonstrated that instead of stopping the reaction with FNS, the presence of DTT increased the rate of enhancement. This, in turn, suggested that the addition of ATP might have been responsible for preventing further enhancement from occurring during the 20-min ATPase assay, especially since ATP protects against the enhancing effect of NEM [5, 6], SLM [7], PPDM [3], p-chloromercuribenzoate [5], and p-chloromercuriphenylsulfonate [8]. Figure 1 shows that both at pH 7.5 and at pH 8.6 ATP is a very potent protector against the enhancing effect of FNS +

DTT on 30S dynein ATPase; ~ 1 μM ATP and ~ 3 μM ATP gave 50% protection at pH 8.6 and 7.5, respectively. Similar results (data not shown) were obtained with 14S dynein, but the relatively small enhancement obtained with 14S dynein even in the absence of ATP precludes any firm conclusions from being drawn as to the potency of ATP in preventing the enhancement of 14S dynein by FNS.

Since previous studies of the protective effects of ATP against enhancement by NEM had shown that high concentrations of ATP were required [6], an experiment was performed to test the potency of ATP as an inhibitor of the enhancing effect of PPDM. Figure 1 shows that 12.5 μM ATP gave only a small protection against the enhancing effect of PPDM, whereas 0.63 mM ATP gave almost complete protection [3]. Figure 1 (and un-published data) also shows that OPDM is as potent an activator of 30S dynein as is PPDM and that only a small degree of protection against its enhancing effect is afforded by 12.5 μM ATP. That OPDM is as potent as PPDM supports the view [3] that PPDM is not acting as a divalent SH reagent on 30S dynein.

Protection by AMP-PNP Against the FNS + DTT Enhancement of 30S Dynein ATPase

Because ATP was so potent a protector against the enhancement of 30S dynein ATPase activity, it was of interest to examine the effects of the ATP analogues AMP-PNP and AMP-PCP. Figure 2 shows the effect of incubating 30S dynein with 8.7 μM FNS + 1.11 mM DTT and varying concentrations of AMP-PNP. At the highest concentration of AMP-PNP used (0.56 mM) there was practically no inhibiting effect of AMP-PNP on
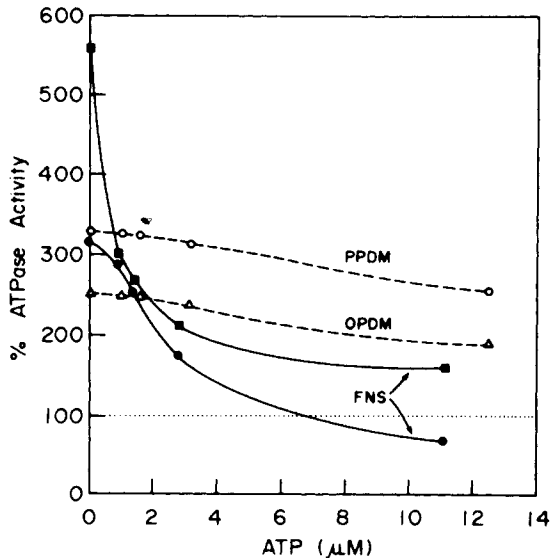

Fig. 1. Protection by ATP against the enhancing effect of FNS and dithiothreitol on 30S dynein ATPase. Tubes containing 8.9 μg of 30S dynein were preincubated at pH 8.6 (■——■) and at pH 7.5 (●——●) with 69 μM FNS and 1.1 mM DTT for 90 min with the concentrations of ATP indicated on the abscissa. At the end of the preincubation, 0.1 ml of 10 mM ATP was added and the ATPase activity was assayed as described in Methods. The experiments with PPDM (o- - -o) and OPDM (△- - -△) were performed similarly, at pH 7.5, but the preincubation volume was 0.8 ml and the concentrations were 0.63 μM. The ATPase assay was initiated by addition of 0.2 ml of 5 mM ATP + 5 mM DTT and immediate transferral of the tubes to 25°. 100% ATPase activity was 0.60 μmole/min·mg at pH 7.5 and 0.63 μmole/min·mg at pH 8.6.
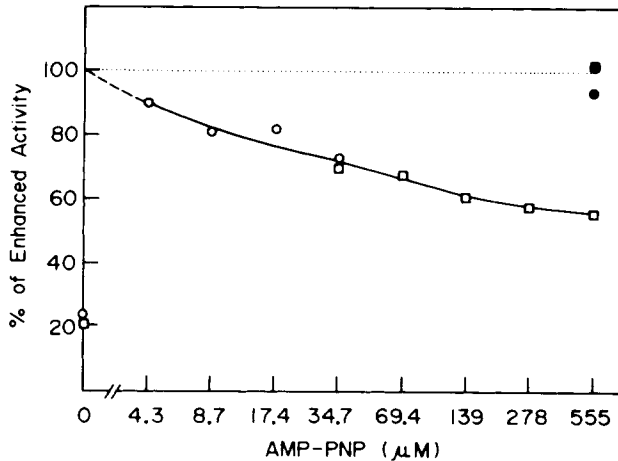
Fig. 2. Protection by AMP-PNP against the ATPase-enhancing effect of FNS + DTT. Tubes containing 7.6 μg of 30S dynein were preincubated at pH 7.5 with 8.7 μM FNS + 1.11 mM DTT for 90 min at 0° with the concentrations of AMP-PNP indicated on the abscissa. The ATPase assay was then initiated by addition of 0.1 ml 10 mM ATP and transferral of the tubes to 25°. The experiment was performed in two parts, one for AMP-PNP concentrations ranging from 34.7 to 555 μM (○, ●), and one from 4.3 to 34.7 μM (□, ■). In each case, the activity attained in the absence of AMP-PNP was taken as 100%; for the points indicated with circles and squares, this was 1.80 and 1.69 μmole/min·mg, respectively, corresponding to enhancement by a factor of 4.7 and by 4.2. The filled symbols (●, ■) show the effect of 0.55 mM AMP-PNP added to the fully enhanced dynein. The open symbols on the ordinate (○, □) show the unenhanced activity for each experiment.

control axonemes, which is in agreement with the findings of Shimizu and Kimura [6]. However, when this concentration of AMP-PNP was present during the 90-min incubation, less than half of the enhancement with FNS + DTT alone was obtained. The concentration dependence of this protection is remarkably broad; even 4.3 μM AMP-PNP gave a noticeable protection against the enhancement. Thus AMP-PNP affords partial protection against the enhancing effect of FNS + DTT, but it is less potent than ATP and does not behave as if it were a simple inhibitor of the enhancement reaction.

Experiment A of Table I shows that if 0.56 mM AMP-PCP is present during a 90-min preincubation with FNS + DTT, the ATPase activity increases to about the same extent as in the absence of AMP-PCP, whereas the presence of AMP-PNP prevented the enhancement. The effect of AMP-PCP in this experiment is difficult to interpret, however, since even if the AMP-PCP did not prevent the enhancement, one might have expected to observe about 24% of the enhanced activity, ie, 0.24 × 237 = 57% of the original activity. This suggested that AMP-PCP lost its inhibitory potency after reaction of 30S dynein with FNS + DTT, and and this interpretation is confirmed in experiment B of Table I. Loss of inhibitory potency of AMP-PCP is not observed, however, after enhancement by FNS alone (data not shown).

DISCUSSION

The discovery that even 1 μM ATP confers appreciable protection against the en-hancing effect of FNS + DTT on 30S dynein shows that there is a very high affinity ATP-

TABLE I. Effect of AMP-PNP and AMP-PCP on Enhancement of 30S Dynein by FNS + DTT

		FNS	
	Inhibitor	−	+
A. Inhibitors present during 90 min			
incubation with FNS + DTT	None	100	237
	AMP-PCP	24	223
	AMP-PNP	72	76
B. Inhibitors added after 17 h			
incubation with FNS + DTT	None	84	268
	AMP-PCP	27	242
	AMP-PNP	73	262

In experiment A, 30S dynein was preincubated with 35 μM FNS and 1.1 mM DTT at $0°$, pH 7.5, for 90 min in the presence of 0.56 mM AMP-PNP or AMP-PCP, as indicated. In experiment B, 30S dynein was preincubated with 22 μM FNS and 1.4 mM DTT for 17 h at $0°$, pH 7.5, and then AMP-PNP and AMP-PCP were added to bring the volume to 0.9 ml and the concentrations of AMP-PNP and AMP-PCP to 0.56 mM. In each experiment, the volume was then brought to 1.0 ml by addition of 0.1 ml 10 mM ATP, and the ATPase activity was assayed as described in Methods. 100% activity corresponds to 0.43 μmole/min·mg and 0.74 μmole/min·mg for experiments A and B, respectively.

binding site on 30S dynein. Although all previous studies on polymeric dyneins or on dyneins in situ have reported K_m values greater than 11 μM (eg, Gibbons [9]), the ability of 1.5 μM ATP to relax rigor waves on sea urchin sperm [10] and Chlamydomonas flagella [11] is consistent with the presence of a high-affinity site in the axoneme. Takahashi and Tonomura [12] have recently shown that a 1/V versus 1/[ATP] plot for Tetrahymena axonemes was composed of two straight lines, from which values of 1.0 and 12.7 μM were estimated. The dependence of the turbidity decrease of axonemal suspensions on ATP concentration led these workers to identify the high-affinity site with the ability to cause the arms to dissociate from the B-subfiber. The report [13] that millimolar concentrations of AMP-PNP, but not AMP-PCP, can relax the rigor waves on sea urchin sperm flagella further supports the view that ATP binding, at concentrations of 3–9 μM, releases cross-bridges from their attachment sites on the B-subfiber. The ability of high concentrations of AMP-PNP to cause relaxation of rigor waves cannot, however, merely be a matter of its binding to the high-affinity (for ATP) site. Our results show that AMP-PCP is a much stronger inhibitor of 30S dynein ATPase than is AMP-PNP, yet high concentrations of AMP-PCP did not protect against the enhancing effects of FNS + DTT, whereas very low concentrations of AMP-PNP — much lower than the minimum found necessary to cause relaxation of rigor waves [13] — gave noticeable protection. We are led, therefore, to postulate that low concentrations of AMP-PNP, which scarcely inhibit the ATPase activity of 30S dynein, interact with the high-affinity site and, like ATP, cause a transition to a state in which binding to the B-subfiber is weakened and an (FNS + DTT)-reactive group is rendered inaccessible.

The observation that AMP-PCP loses potency as an inhibitor of 30S dynein ATPase after enhancement by FNS + DTT suggests that this reagent causes a change in the structure of the 30S dynein that primarily influences the low-affinity ATP-binding site, but further studies will be required to assess the full implications of this observation.

ACKNOWLEDGMENTS

REFERENCES

1. Blum JJ, Hayes A: J Supramol Struct 6:155, 1977.
2. Gibbons BH, Fronk E, Gibbons IR: J Cell Biol 70:49a, 1976.
3. Blum JJ, Hayes A: J Supramol Struct 8:153, 1978.
4. Spector T: Anal Biochem 86:142, 1978.
5. Blum JJ, Hayes A: Arch Biochem Biophys 161:239, 1974.
6. Shimizu T, Kimura I: J Biochem 82:165, 1977.
7. Blum JJ, Hayes A, Whisnant CC, Rosen G: Biochemistry 16:1937, 1977.
8. Shimizu T, Kaji K, Kimura I: J Biochem 82:1145, 1977.
9. Gibbons IR: J Biol Chem 241:5590, 1966.
10. Gobbons BH, Gibbons IR: J Cell Biol 63:970, 1974.
11. Peningroth SM, Witman GB: J Cell Biol 79:295a, 1978.
12. Takahashi M, Tonomura Y: J Biochem 84:1339, 1978.
13. Peningroth SM, Witman GB: J Cell Biol 79:827, 1978.