Steady-State Kinetic Study of Vanadate-Induced Inhibition of Ciliary Dynein Adenosinetriphosphatase Activity from *Tetrahymena*†

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ABSTRACT: Inhibitory action of vanadate (orthovanadate and metavanadate) on ciliary dynein adenosinetriphosphatase (ATPase) from *Tetrahymena* was investigated. The apparent concentrations of vanadate giving half-maximal inhibition of Mg-ATPase activity of various dynein fractions were as follows: the axoneme-bound form of dynein at 100 nM, solubilized crude dynein at 50 nM, 14S dynein at 5 μM, and 30S dynein at 20 nM. The Ca-ATPase of 30S dynein was more than 30-fold less sensitive than its Mg-ATPase, and still less sensitive was the Ca-ATPase of 14S dynein. The Mg-ATPase of 30S dynein was most sensitive to vanadate at neutral pH, and the addition of KCl or NaCl into the assay mixture reduced its sensitivity. Varying the assay temperature between 0 and 37 °C affected the sensitivity to a slight extent. Metavanadate was as much a potent inhibitor of dynein ATPase as orthovanadate, but vanadium pentoxide was less potent. When the dynein ATPase activity was reciprocally plotted against the concentration of vanadate (the Dixon plot), the inhibition was proved to be biphasic. At lower concentrations of vanadate, the inhibition was more significant. Therefore, the Dixon plot had a downward bent. Reexamination of the Lineweaver–Burk plot of 30S dynein Mg-ATPase showed a downward bent, which indicates that 30S dynein may have at least two K_m values, ca. 1 μM and 3 μM; or otherwise, 30S dynein might possibly have a negatively cooperative nature (Hill coefficient 0.67). The vanadate-induced inhibition of 30S dynein Mg-ATPase was noncompetitive in the entire range of ATP concentration examined. Since the vanadate-induced inhibition of 30S dynein Mg-ATPase could be classified into "tight-binding inhibition", we could estimate the dissociation constant of vanadate and the molecular weight per enzymatic active site according to the kinetics of tight-binding inhibition with several assumptions. Thus, the dissociation constant was 10–15 nM, depending on the ATPase assay condition, while the molecular weight per enzymatic active site was 420 000–480 000, independent of the assay condition with the assumption that the present 30S dynein preparation is totally pure. This value would be reduced about 20% when the purity was taken into consideration.

In 1966, Gibbons reported that only Mg-ATPase[1] of ciliary dynein of *Tetrahymena* was inhibited by an unknown factor which probably contaminated certain batches of commercial ATP. Later, ATP prepared from skeletal muscle was proved to contain an anionic substance which was responsible for the potent inhibition of dynein ATPase (Nagata & Flavin, 1978). This substance was shown to inhibit Na,K-ATPase more strongly and was determined to be orthovanadate, VO_4^{3-} (Cantley et al., 1977). Further, metavanadate (VO_3^-) was shown to inhibit dynein ATPase as potently as orthovanadate (Gibbons et al., 1978). These anions are known to be converted to the same form (HVO_4^{2-}) in a dilute aqueous solution.

Recent studies revealed that various kinds of membrane ATPases (O'Neal et al., 1979; Wang et al., 1979; Bowman & Slayman, 1979) as well as some phosphatases (Lopez et al., 1976; Seargeant & Stinson, 1979) and other enzyme (Velours et al., 1975) could be inhibited by vanadate. Skeletal muscle myosin was also shown to be inhibited by this anion (Goodno, 1979). The inhibition of some of these enzymes was demonstrated to be competitive (Seargeant & Stinson, 1979; Goodno, 1979; Hansen, 1979; Jorgensen & Anner, 1979): vanadate is likely to compete for the phosphate site.

In this study, we investigated vanadate-induced inhibition of ciliary dynein from *Tetrahymena*. It was 30S dynein Mg-ATPase that was most potently inhibited by vanadate. The mode of inhibition of this enzyme was shown to be noncompetitive. The inhibition of 30S dynein Mg-ATPase by vanadate was so potent that the kinetics of tight-binding inhibition could be applicable to the present system. Thus, the molecular weight per enzymatic active site of 30S dynein was estimated with several assumptions.

Experimental Procedures

Preparative methods for the ciliary axoneme, the crude dynein fraction, the outer fiber fraction (extracted residues), 14S dynein, and 30S dynein were described previously (Shimizu & Kimura, 1974). The polypeptide compositions of 14S and 30S dynein appearing on $NaDodSO_4$–polyacrylamide gel electrophoresis were almost the same as already described (Mabuchi & Shimizu, 1974; Shimizu et al., 1979).

The ATPase assay was performed in a standard assay mixture consisting of 30 mM Mops–NaOH (pH 7.0), 3 mM $MgCl_2$ or $CaCl_2$, 1 mM ATP, and an appropriate concentration of the enzyme (usually 5–30 μg/mL for 14S or 30S dynein) unless otherwise specified. The concentration of the enzyme affected the vanadate-induced inhibition when a very low concentration of vanadate was used due to high sensitivity of the enzyme. Therefore, when necessary, the enzyme was diluted so as not to affect the results significantly. The detailed kinetical study concerning the enzyme concentration, however, is one of the main concerns of this paper.

The ATPase reaction was started by addition of the enzyme fraction. The order of addition of the constituents might be important in some cases of tight-binding inhibition systems (Williams & Morrison, 1979) but was shown not to be crucial in the present system. The vanadate-induced inhibition of

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[1] Abbreviations used: Mg-ATPase, Mg^{2+}-dependent adenosinetriphosphatase; Ca-ATPase, Ca^{2+}-dependent adenosinetriphosphatase; Na,K-ATPase, sodium- plus potassium-dependent adenosinetriphosphatase; Mops, 3-(*N*-morpholino)propanesulfonic acid; PEP, phosphoenolpyruvate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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dynein ATPase was apparent from the early phase of enzyme reaction, which is consistent with the observation by Gibbons et al. (1978). The ATPase reaction was developed for 5–30 min at 25 °C and terminated by adding trichloroacetic acid to a final concentration of 5%. Inorganic phosphate liberated was determined by the method of Fiske & SubbaRow (1925). Since vanadate of more than 20 μM interferes with this method of phosphate determination, the correction was made to obtain true values.

When the concentration of ATP in the assay mixture was very low, an assay system coupled with pyruvate kinase and PEP was adopted. In this case, 15 mM KCl, 0.5 mM PEP, and 50–150 μg/mL pyruvate kinase were further added to the standard assay mixture, and the amount of released pyruvate was determined by the method of Reynard et al. (1961). The concentration of pyruvate kinase was determined to give the most satisfactory values under the conditions employed. Inclusion of pyruvate kinase in the assay mixture containing vanadate was confirmed not to interfere with the vanadate action on dynein ATPase (Kobayashi et al., 1978).

[γ-^{32}P]ITP was synthesized by the method of Glynn & Chappell (1964). The Mg-ITPase activity of 30S dynein was determined by using the standard assay mixture containing 2 mM ITP instead of 1 mM ATP. The reaction was started and developed in the same manner as the ATPase reaction but was terminated by the addition of perchloric acid (to a final concentration of 5%) containing carrier P_i (final concentration 0.1 mM). After charcoal treatment, a portion of the supernatant was transferred into a vial, and the radioactivity was measured by the method of Cerenkov counting.

One unit of the enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 μmol of P_i or ADP (pyruvate) per min under the above condition.

The concentrations of vanadium compounds in aqueous solutions were determined by using a Hitachi 170-70 Zeeman Effect atomic absorption spectrophotometer. The distinction of the starting material, namely ortho- or metavanadate, was made when necessary since a little difference of the effectiveness was observed in some cases, depending on the starting material. The term vanadate was used when the distinction seemed to be unnecessary.

The concentration of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. $NaDodSO_4$–polyacrylamide gel electrophoresis and scanning of gels were performed as described previously (Shimizu, 1978).

Water was deionized and distilled. ATP was obtained from Kyowa Hakko Co. and was confirmed to be substantially vanadium free. Chemicals of the highest purity available were the same as already described (Shimizu & Kimura, 1977). Pyruvate kinase was obtained from Sigma or from Boehringer. It was also purified from rabbit skeletal muscle by the method of Tietz & Ochoa (1958), but the purity was not sufficient (Inoue & Tonomura, 1976). We purified it further by using a Sephadex G-200 and hydroxylapatite columns to obtain the enzyme fraction showing a single band on $NaDodSO_4$ gels.

Results

Inhibition by Orthovanadate of Mg-ATPase Activities of Axoneme-Bound Dynein, Solubilized Crude Dynein, and Residual Outer Fiber Fraction. The Mg-ATPase activity of dynein bound to the axoneme was strongly inhibited by orthovanadate (Figure 1) as previously reported (Kobayashi et al., 1978). The half-maximal inhibition was observed at around 100 nM, which was lower than that reported by Kobayashi et al. (1978).

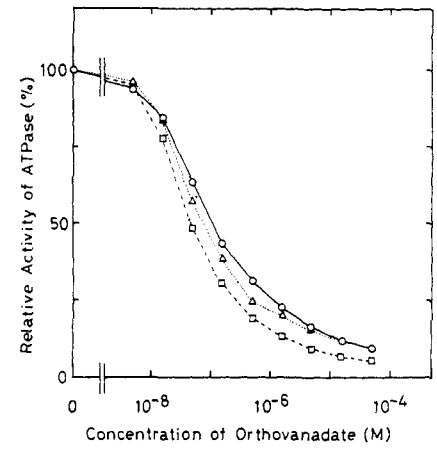

FIGURE 1: Inhibition by orthovanadate of Mg-ATPase activities of axoneme-bound dynein, solubilized crude dynein, and residual outer fiber fraction. The Mg-ATPase assay was carried out as described under Experimental Procedures in the standard assay mixture containing the indicated concentrations of orthovanadate. The specific activities of the axonemal dynein (○), solubilized crude dynein (□), and the outer fiber fraction (△) in the absence of orthovanadate were 0.125, 0.351, and 0.0403 unit/mg of protein, respectively.

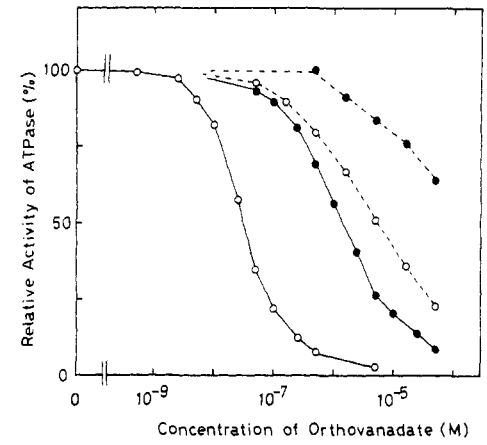

FIGURE 2: Inhibition by orthovanadate of 14S and 30S dynein Mg- or Ca-ATPase activities. The ATPase assay was carried out as described under Experimental Procedures in the standard assay mixture containing the indicated concentrations of orthovanadate. The specific activities of 14S dynein Mg-ATPase (--○--), Ca-ATPase (--●--), 30S dynein Mg-ATPase (—○—), and Ca-ATPase (—●—) in the absence of orthovanadate were 0.961, 0.433, 0.745, and 0.518 unit/mg of protein, respectively.

Solubilization of dynein from the axoneme by extraction with Tris–EDTA solution [1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA] affected the sensitivity to orthovanadate (Figure 1); the half-maximal inhibition was observed at 50 nM. Dithiothreitol (0.1 mM) which was supplemented to the Tris–EDTA solution did not affect the results.

The extraction with Tris–EDTA solution caused a majority of the axonemal ATPase to be solubilized, but the remaining 10–20% of the ATPase activity of the axoneme persisted to be bound to the residual outer fiber fraction. This residual ATPase could be inhibited by orthovanadate as strongly as the axonemal ATPase (Figure 1). $NaDodSO_4$–polyacrylamide gel electrophoresis of the outer fiber fraction showed the existence of a small amount of the protein bands peculiar to dyneins (Mabuchi & Shimizu, 1974). Therefore, the residual ATPase activity appeared to derive from remaining dynein molecules.

Inhibition of 14S and 30S Dynein ATPase Activities by Orthovanadate. Orthovanadate inhibited Mg- and Ca-ATPase activities of both 14S and 30S dynein, but the sensitivities were different from one another (Figure 2); 30S dynein Mg-ATPase

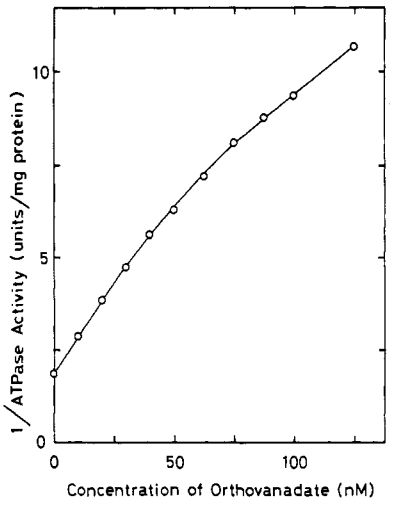

FIGURE 3: Dixon plot of the Mg-ATPase activity of 30S dynein as a function of orthovanadate concentration. The Mg-ATPase assay was carried out as described under Experimental Procedures in the standard assay mixture containing the indicated concentrations of orthovanadate. The reciprocal of the Mg-ATPase activity was plotted against concentrations of orthovanadate.

Table I: Apparent K_i Values of Vanadium Compounds for Dynein[a]

expt no.	dynein	divalent cation added	vanadium compd	apparent K_i value (nM) of first phase	second phase
1	14 S	Mg	VO_4^{3-}	1800	18000
2	30 S	Mg	VO_4^{3-}	20	65
3	30 S	Ca	VO_4^{3-}	1200	6000
4[b]	30 S	Mg	VO_4^{3-}	600	4000
5	30 S	Mg	VO_3^-	15	41
6	30 S	Mg	V_2O_5	50	90

[a] The Mg- or Ca-ATPase assay was performed as described under Experimental Procedures in the standard assay mixture containing various concentrations of each vanadium compound. Apparent K_i values, defined as half-maximally inhibitory concentrations, were determined by the Dixon plots. [b] In the presence of 0.6 M KCl.

was the most sensitive. The apparent half-maximal inhibition occurred at an orthovanadate concentration of 20 nM. This value will be further discussed from kinetic considerations in the following. In contrast, the Mg-ATPase of 14S dynein was less sensitive, as suggested by Nagata & Flavin (1978). The Ca-ATPase activity of 14S or 30S dynein was far less sensitive than the Mg-ATPase activity of each enzyme, which was in accord with earlier observations by Gibbons (1966) and by Kobayashi et al. (1978).

The dose–response curves shown in Figure 2 seemed to be simple, but the Dixon plot of the data revealed a downward bent, as seen in Figure 3 with respect to the Mg-ATPase of 30S dynein; the inhibition was not monophasic but biphasic, at least. At lower concentrations of orthovanadate (the first phase), the inhibition was more significant, giving smaller apparent K_i values (an apparent K_i value was defined as a half-maximally inhibitory concentration of an inhibitor); at higher concentrations of the inhibitor (the second phase), the inhibitory action was less potent. The biphasic inhibition was observed in almost all cases examined. The apparent K_i values of both phases under various conditions are listed in Table I.

Since the Mg-dependent ATPase activity of 30S dynein seems to be physiologically important in ciliary motility and since it was most sensitive to this kind of inhibition, we investigated 30S dynein Mg-ATPase with regard to the mode

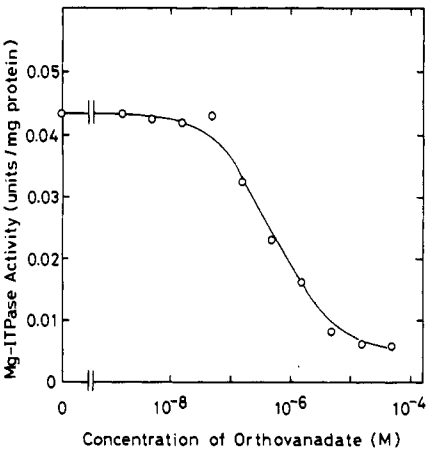

FIGURE 4: Inhibition by orthovanadate of 30S dynein Mg-ITPase activity. The Mg-ITPase assay was carried out as described under Experimental Procedures in the standard assay mixture containing 2 mM ITP instead of 1 mM ATP in the presence of the indicated concentrations of orthovanadate.

of inhibition more precisely under various conditions.

(*i*) *pH.* The inhibition was most prominent at neutral pH (data not shown). At an acidic or at an alkaline pH, the enzymatic activity was high, but the sensitivity to orthovanadate was low. The following experiments were, therefore, carried out at pH 7.0.

(*ii*) *Ionic Strength.* Addition of KCl to the assay mixture reduced the sensitivity of the 30S dynein Mg-ATPase to orthovanadate, as reported by Kobayashi et al. (1978). Substitution of NaCl for KCl gave the same result, suggesting that species of cation is not crucial but ionic strength is.

(*iii*) *Temperature.* Raising the assay temperature from 25 to 37 °C or lowering to 0 °C affected the sensitivity to a slight extent. At higher concentrations of orthovanadate, the inhibition was a little more significant at 37 °C. On the other hand, the sensitivity at lower concentrations was higher at 0 °C.

Inhibition of 30S Dynein Mg-ITPase by Orthovanadate. Dyneins are known to have a considerably strict substract specificity: nucleoside triphosphates other than ATP or dATP are hydrolyzed at a rate of several percent of the ATP hydrolysis (Gibbons, 1966; Ogawa & Mohri, 1972; Shimizu & Kimura, 1974; Takahashi & Tonomura, 1979). We investigated whether vanadate would inhibit the nucleoside triphosphatase activity of 30S dynein by using Mg-ITP as a substrate.

The apparent K_m value for Mg-ITP of rabbit skeletal muscle myosin was reported to be similar to that for Mg-ATP (Inoue et al., 1975). In contrast, the apparent K_m for Mg-ITP of 30S dynein was determined to be 0.8 mM, being much higher than that for Mg-ATP (Takahashi & Tonomura, 1978; see later). Within the Mg-ITP concentration range 0.08–1.0 mM, the Lineweaver–Burk plot was straight. The V_{max} value of Mg-ITPase of 30S dynein was less than one-tenth that of the Mg-ATPase.

The orthovanadate-induced inhibition of 30S dynein Mg-ITPase was less significant than that of Mg-ATPase (Figure 4). The half-maximally inhibitory concentration was about 400 nM, 20-fold higher than that of the Mg-ATPase.

Inhibition of 14S or 30S Dynein ATPase Activity by Metavanadate or Vanadium Pentoxide. The potency of inhibitory action of vanadium compounds other than orthovanadate with five valencies was investigated by using metavanadate and vanadium pentoxide. Metavanadate exhibited an inhibitory effect on 30S dynein Mg-ATPase as potently as orthovanadate, supporting that both vanadates might be converted to the same

anion as described above. However, when the concentration was in the micromolar range, the metavanadate-induced inhibition was slightly more potent than the orthovanadate-induced one, which could be seen in the inhibition of 14S dynein Mg-ATPase; the apparent half-maximally inhibitory concentrations of ortho- and metavanadate were 5×10^{-6} M and 5×10^{-7} M, respectively. The metavanadate-induced inhibition was also proved to be biphasic by the Dixon plot (Table I). The potent inhibitory effect of metavanadate on dynein Mg-ATPase was also reported by Gibbons et al. (1978). On the other hand, the inhibition by vanadium pentoxide was less potent than that by ortho- or metavanadate (Table I). Metavanadate or vanadium pentoxide showed weaker inhibitory action on the Ca-ATPase of 14S or 30S dynein than on the Mg-ATPase of each enzyme, as in the case of the orthovanadate-induced inhibition (Figure 2).

Inhibition by Metavanadate of 30S Dynein Mg-ATPase Activity at Low Concentrations of ATP. On the basis of the above results, we tried to determine the mode of inhibitory action of vanadate on 30S dynein Mg-ATPase under the condition under which the enzyme is most sensitive to vanadate. Because of low concentrations of ATP, we adopted a coupled assay system as described under Experimental Procedures. In this section, metavanadate was used instead of orthovanadate.

In an earlier study (Shimizu & Kimura, 1974), we presented a K_m value of about 10 μM for native 30S dynein Mg-ATPase by using the method of Fiske & SubbaRow (1925), but because of the low sensitivity of this method for phosphate determination, our previous result inevitably required reevaluation. In fact, several investigators (Gibbons & Gibbons, 1974; Takahashi & Tonomura, 1978; Shimizu et al., 1979; Sale & Gibbons, 1979; Blum & Hayes, 1979; Okuno, 1980) reported that dyneins have a high-affinity site for ATP as low as 1 μM and that the K_m value is in this range.

In the present study, it was confirmed that 30S dynein Mg-ATPase has a K_m of 0.8–1.2 μM, depending on the preparation of the enzyme (Figure 5A). Further, it showed another K_m of around 3 μM. It appeared that about two-thirds of the activity was due to one enzyme with a lower K_m and the rest, due to another enzyme with a higher K_m.

In the presence of several different concentrations of metavanadate, the K_m values did not change but V_{max} values decreased. Apparently, the inhibition was noncompetitive in both lower and higher ranges of ATP concentration.

As mentioned above, 30S dynein Mg-ATPase seems to have two K_m's, and the double-reciprocal plot shows a downward bent (Figure 5A). When the reciprocal of the enzyme activity was plotted against the reciprocal of $[ATP]^{0.67}$, straight lines were obtained both in the presence and absence of metavanadate, and the intercepts on the abscissa were identical (2.7 μM). Therefore, this suggests another possibility that 30S dynein Mg-ATPase might exhibit negative cooperativity. Since 30S dynein is by no means completely pure and there might be various forms of 30S dynein ATPase, it is still obscure that this enzyme really has a cooperative characteristic. Anyway, we replotted the data according to the Hill equation (Figure 5B). A straight line which gives a Hill coefficient of $\sim$0.67 was obtained. This relation was held exactly true even in the presence of 27.5 or 88 nM metavanadate. The reciprocal plot of the enzyme activity against the reciprocal of $[ATP]^{0.67}$ gave a linear relationship as far as examined independent of the preparation of 30S dynein. The aging of 30S dynein fraction up to 2 weeks in an ice bath did not alter this characteristic.

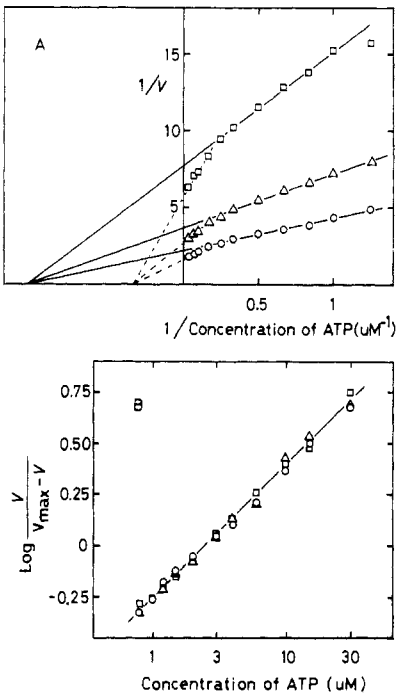

FIGURE 5: Mode of inhibition of 30S dynein Mg-ATPase by metavanadate. (A) Double-reciprocal plot of 30S dynein Mg-ATPase activity in the presence or absence of metavanadate. The ATPase assay was carried out as described under Experimental Procedures, using a coupled assay system. The assay mixture contained 30 mM Mops–NaOH (pH 7.0), 3 mM $MgCl_2$, 15 mM KCl, 0.5 mM PEP, 8.1 μg/mL 30S dynein, 0.1 mg/mL pyruvate kinase, and the indicated concentrations of ATP. The concentration of metavanadate was 0 (○), 27.5 nM (△), or 88 nM (□). V in the ordinate indicates the Mg-ATPase activity (units/mg of protein). (B) Hill plot of 30S dynein Mg-ATPase activity in the presence or absence of metavanadate. The data presented in (A) were replotted according to the Hill equation. V_{max}'s of the control (○) and those in the presence of 27.5 nM (△) and 88 nM (□) metavanadate were estimated to be 0.645, 0.392, and 0.186 unit/mg of protein, respectively, by extraporation of the double-reciprocal plot (Figure 5A). V indicates the Mg-ATPase activity (units/mg of protein).

Recently, it was shown that an appreciable amount of Al^{3+} contaminates some batches of ATP and that the resultant Al–ATP complex inhibited certain hexokinases to make the Lineweaver–Burk plot convex (Kosow & Rose, 1971; Womack & Colowick, 1979; Viola et al., 1980). This type of inhibition and nonlinearity were released by addition of citrate to the assay mixture; citrate strongly chelates Al^{3+} (Womack & Colowick, 1979; Viola et al., 1980). Therefore, we investigated the effect of citrate (final concentration of 1 mM) on the Lineweaver–Burk plot of 30S dynein Mg-ATPase and found that citrate did not have any effect either on the ATPase activity or on the double-reciprocal plot.

On the other hand, Figure 6 shows the Dixon plot in the presence of 1 μM ATP, which was almost linear. Under the condition described in the legend, the apparent half-maximally inhibitory concentration was about 30 nM.

Estimation of Molecular Weight per Enzymatic Active Site of 30S Dynein by means of Kinetics of Tight-Binding Inhibition. As described above, the vanadate-induced inhibition of 30S dynein Mg-ATPase was very potent so that the apparent inhibition constant, K_i, was likely to be comparable to the enzyme concentration. In fact, the apparent inhibition constant varied with the enzyme concentration. Thus, the vanadate-induced inhibition of 30S dynein Mg-ATPase would be classified into the category of tight-binding inhibition. According to this type of kinetics (Morrison, 1969; Henderson, 1972; Dixon, 1972; Williams & Morrison, 1979), the molec-

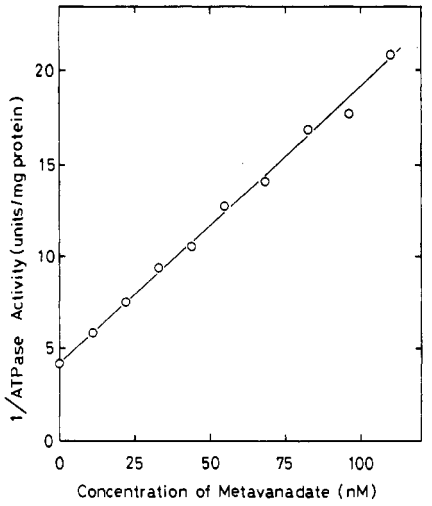

FIGURE 6: Dixon plot of 30S dynein Mg-ATPase activity in the presence of 1 μM ATP. The Mg-ATPase assay was carried out as described under Experimental Procedures by using a coupled assay system. The assay mixture contained 30 mM Mops–NaOH (pH 7.0), 3 mM $MgCl_2$, 15 mM KCl, 0.5 mM PEP, 10.4 μg/mL 30S dynein, 64 μg/mL pyruvate kinase, and the indicated concentrations of metavanadate.

ular weight per an enzymatic active site could be obtained by means of

$$I_t = K[(v_0/v_i) - 1] + E_t\frac{(v_0/v_i) - 1}{v_0/v_i} \quad (1)$$

if the following assumptions are made: (i) the inhibition is noncompetitive, (ii) all the catalytic unit of the enzyme can bind a single molecule of the inhibitor and (iii) the inhibition is reversible. I_t, E_t, K, v_i, and v_0 denote the total concentration of the inhibitor, that of the enzyme, the dissociation constant of the inhibitor, the enzyme activity in the presence of inhibitor and that in the absence, respectively. Assumption i was proved to be the case, as shown in the preceding section. If the inhibition is extraordinarily potent, that is, for example, $E_t/K > 100$, competitive inhibition becomes similar to a noncompetitive one with regard to the Lineweaver–Burk plot (Henderson, 1972). However, as seen later, vanadate-induced inhibition of 30S dynein Mg-ATPase was likely not to be "extraordinarily" potent. Assumption iii was likely to be the case for the following reason: after starting the enzyme reaction in the presence of a certain concentration of metavanadate, the reaction mixture was diluted 5-fold, with the reaction medium devoid of the enzyme and the inhibitor; that is, only the concentrations of the enzyme and the inhibitor were lowered. After dilution, the time course of the enzyme reaction was linear, and the reaction rate afterward coincided with that of the system consisting of the same concentrations of the enzyme and inhibitor from the start. The reversibility of vanadate-induced inhibition was also reported by Gibbons et al. (1978) and by Kobayashi et al. (1978). In addition, it should be noted that the inhibition of 30S dynein ATPase by vanadate seemed to appear without any detectable lag phase, unlike that of Na,K-ATPase (Gibbons et al., 1978). The equilibrium seemed to be attained rather rapidly.

(*i*) *Concentration of ATP in the Assay Mixture 1 mM.* The concentration of 30S dynein was varied from about 2 to about 40 μg/mL. The inhibition was dependent on the concentration of the enzyme: when it was higher, the inhibition was less potent. This agrees qualitatively with kinetics described above (Figure 7A).

Next, I_{tr}, the total concentration of metavanadate when $v_0/v_i = r$, was determined from Figure 7A and was plotted against

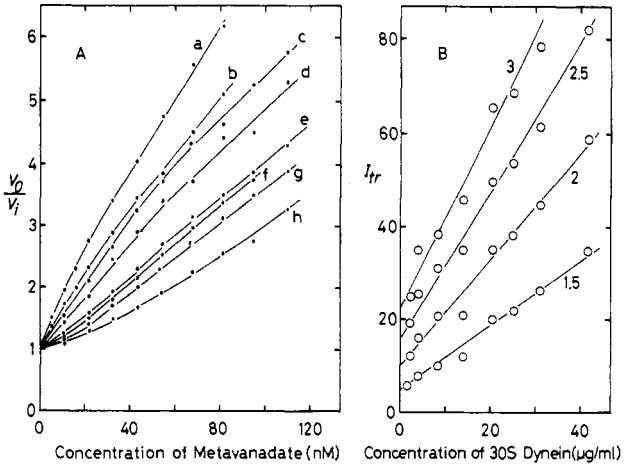

FIGURE 7: Effect of the concentration of 30S dynein on metavanadate-induced inhibition. (A) Dixon plots of 30S dynein Mg-ATPase activity against metavanadate concentration at 1 mM ATP. v_0/v_i (defined in the text) obtained experimentally was plotted against metavanadate concentration. The ATPase assay mixture contained 30 mM Mops–NaOH (pH 7.0), 3 mM $MgCl_2$, 1 mM ATP, and (a) 2.2, (b) 4.2, (c) 8.3, (d) 13.9, (e) 20.9, (f) 25.0, (g) 31.3, or (h) 41.7 μg/mL 30S dynein. The concentration of metavanadate in the assay mixture is indicated on the abscissa. (B) Estimation of the dissociation constant (K) and the enzyme concentration term (E_t). According to eq 1, I_{tr} was plotted against the enzyme concentration, using the data in (A). The numbers in the figure indicate the value r, namely, v_0/v_i.

Table II: Estimation of the Dissociation Constant (K) and the Enzyme Concentration Term (E_t) [a]

	K (nM)		E_t (nM for 10 μg/mL 30S dynein)	
r	1 mM [b]	1 μM [b]	1 mM [b]	1 μM [b]
1.5	10	15	21	22
2	10	16	23	24
2.5	11	17	26	28
3	11	16	28	34

[a] According to eq 1, values for K and E_t were estimated from Figure 7 and from the data of the same experiments as those described in Figure 7 except for the ATP concentration (1 μM) and using a coupled assay system for determination of the enzyme activity. The value for E_t is expressed as nM for 10 μg/mL 30S dynein. [b] The concentration of ATP in the assay mixture.

the enzyme concentration (Figure 7B) according to eq 1. As seen in the figure, straight lines were obtained. Thus, in Table II, K was determined to be about 10–11 nM, while the estimation of the enzyme concentration term (E_t) depended upon the value r. When r was chosen to be 1.5, the enzyme concentration term was 21 nM for 10 μg/mL, but when a larger value was chosen for r, such as 3, the enzyme concentration term became larger, 28 nM for 10 μg/mL. From the reliability of the data, it seems better to choose a smaller value for r, such as 1.5 or 2. The enzyme concentration term, however, always increased with the value r, which will be discussed later. At any rate, the enzyme concentration term thus obtained corresponded to the molecular weight per enzymatic active site, which turned out to be 420000–480000. This figure will be discussed later in relation to the purity of the enzyme.

(*ii*) *Concentration of ATP in the Assay Mixture 1 μM.* Some experiments similar to those described above were performed under the condition that the ATP concentration was lowered to 1 μM, and the determination of the ATPase activity was performed by using the coupled assay system. According to eq 1, the concentration of substrate would not affect the results. With regard to enzyme concentration term, this held true (Table II). A concentration of 10 μg/mL 30S

dynein was estimated to be 22, 24, and 28 nM when r was chosen to be 1.5, 2, and 2.5, respectively. These values are in good accord with those obtained at 1 mM ATP, and furthermore, the figure for the enzyme concentration term also increased with r.

On the other hand, the dissociation constant, K, was rather different: 15–17 nM was obtained instead of 10–11 nM (Table II). This variation, however, might be due to the variation of constituents in the assay mixture, such as addition of 15 mM KCl. In fact, when the coupled assay system was adopted at 1 mM ATP, the inhibition by metavanadate was less potent.

Discussion

General Properties of Vanadate-Induced Inhibition of Dynein ATPase. Vanadate strongly inhibited the Mg-ATPase activity of dynein, as reported earlier (Gibbons et al., 1978; Kobayashi et al., 1978). The apparent half-maximally inhibitory concentration for 30S dynein Mg-ATPase was shown to be around 20 nM, which was lower than that described previously (Gibbons et al., 1978; Kobayashi et al., 1978) but will be discussed later. Whereas vanadium pentoxide showed less potent inhibitory action than orthovanadate, metavanadate was proved to be an inhibitor as potent as orthovanadate when the concentration was less than 1 μM. The present result supported the inorganic chemical knowledge that ortho- and metavanadate in very dilute solutions are converted to an identical ionic form, probably HVO_4^{2-}, which would be responsible for the potent inhibition. In solutions of slightly higher concentrations, however, metavanadate was more potently inhibitory than orthovanadate so that the predominant ionic form of vanadates might be different to each other. A solution of vanadium pentoxide is acidic so that an anionic substance(s) is (are) considered to be formed, which might be identical with that described above and, therefore responsible for the inhibitory action of vanadium pentoxide.

The Mg-ATPase remaining in the outer fiber fraction was as sensitive as axonemal Mg-ATPase. Together with the observation that axonemal Mg-ATPase was not affected by ouabain (K. Kaji, personal communication), it is plausible that the nonextractable Mg-ATPase in the outer fiber fraction is a dynein-like enzyme. The location and the reason it could not be solubilized by repeated dialysis against Tris–EDTA solution remain to be solved.

Steady-State Kinetical Characteristics of 30S Dynein Mg-ATPase and the Effect of Metavanadate on Them. The Mg-ATPase of 30S dynein, which seems to be physiologically important for ciliary motility of *Tetrahymena*, appears to have at least two K_m's. It is possible that the 30S dynein fraction contains at least two different ATPases with different K_m values. Even if the enzyme fraction consisted of a single species of ATPase, it is still possible that the enzymatic properties of some of them might be altered since ATPase activity of 30S dynein, like that of other dyneins (Mabuchi et al., 1976; Gibbons & Fronk, 1979), is sensitive to various kinds of treatment and could be changed (Shimizu & Kimura, 1974; Blum & Hayes, 1974, 1977; Shimizu, 1979). Furthermore, we could not exclude the possibility that dynein might undergo some proteolytic hydrolysis during preparation. Therefore, there might be various forms of the enzyme in a single preparation, which might cause the nonlinearity of the double-reciprocal plot.

However, there is another possibility that explains the downward bent in the double-reciprocal plot, negative cooperativity. Although negative cooperativity should be determined only after an enzyme is completely purified to be homogeneous (Levitzki & Koshland, 1976), the idea that 30S dynein would have a cooperative nature is attractive since the Hill plot (Figure 5B) or the reciprocal plot of the activity against the reciprocal of $[ATP]^{0.67}$ gives linear relationships independent of the 30S dynein preparation. It might be difficult to get linear relationships in these plots when the enzyme fraction consists of a simple mixture of different enzymes. As shown by Gibbons & Rowe (1965), 30S dynein has a molecular weight of more than 5 000 000 and probably consists of several subunit enzymes. Therefore, it seems likely that the 30S dynein molecule has several active sites for ATPase so that the site–site interaction(s) is (are) possible to occur. It is of great interest to know whether the Lineweaver–Burk plot would be affected by the alteration of an assay condition such as pH, temperature, divalent cation, or ionic strength as well as by chemical modification of 30S dynein. In this connection, it would also be interesting to investigate the kinetical characteristics of 30S dynein dATPase since dATP is likely to be the only other good substrate of dynein (Gibbons, 1966; Takahashi & Tonomura, 1979).

We described that the molecular form of dynein, which seemed to be physiologically important, was not the 11–13S form but the 20S form for flagellar dynein of starfish spermatozoa (Mabuchi et al., 1976). Recently Gibbons & Fronk (1979), Gibbons & Gibbons (1979), and Yano & Miki-Noumura (1980) confirmed our results more clearly by using flagellar dynein of sea urchin spermatozoa. Therefore, it seems to be a prerequisite that dynein molecules are coordinated to each other to compose a large, possibly ordered form, such as the 20S form of echinoderm flagellar dynein and the 30S form of *Tetrahymena* ciliary dynein. This form may be the native state of dynein ATPase and may be important for the function. Thus, it is of much interest to know whether 20S dynein of echinoderm spermatozoa also exhibits a nonlinear relationship in the Lineweaver–Burk plot.

Kobayashi et al. (1978) reported that dynein was inhibited by vanadate uncompetitively, using a dynein preparation with a high K_m value (about 30 μM). Gibbons et al. (1978) showed that vanadate-induced inhibition was not competitive. Recently, Okuno (1980) showed that flagellar beat frequency of Triton models of spermatozoa was inhibited uncompetitively. It was, however, demonstrated in this study that metavanadate did not affect the K_m value(s) of 30S dynein Mg-ATPase, reducing the V_{max} value only. Apparently, the inhibition was noncompetitive. It might be possible that ATPase activity is inhibited noncompetitively but beat frequency, uncompetitively.

Vanadate is known to be an inhibitor of several kinds of phosphate-related enzymes. The mode of inhibition of Na,K-ATPase was reported to be competitive (Hansen, 1979; Jorgensen & Anner, 1979), although K-stimulated phosphatase activity of Na,K-ATPase was inhibited noncompetitively (Nieder et al., 1979). Other membrane ATPases were inhibited by vanadate, but the mode of inhibition has not been studied. Certain phosphatases were shown to be inhibited competitively (Kobayashi et al., 1978; Seargeant & Stinson, 1979). Myosin or actomyosin ATPase was reported to be rather insensitive to vanadate in earlier papers (Gibbons et al., 1978; Kobayashi et al., 1978). In a recent report (Goodno, 1979), myosin ATPase was also shown to be an enzyme inhibited potently by vanadate in a competitive manner. Myosin active site·ADP·vanadate complex was considered to be formed instead of the ordinal myosin active site·ADP·P_i complex. As judged from the above-mentioned observations, vanadate action on dynein ATPase is rather unique.

Our present hypothesis on the inhibitory effect of vanadate on dynein ATPase seems to agree well with the possible

mechanism presented by Sale & Gibbons (1979), which was based on the effects of vanadate on rigor waves of Triton models of the sea urchin spermatozoa and on the ATP-induced extrusion of doublet microtubules from the trypsin-treated axonemes.

Application of Tight-Binding Inhibition Kinetics. According to the tight-binding inhibition kinetics (Morrison, 1969; Henderson, 1972; Dixon, 1972; Williams & Morrison, 1979), the dissociation constant, K, of the metavanadate-induced inhibition of 30S dynein Mg-ATPase activity was determined to be 10–11 nM when the concentration of ATP was 1 mM. This value, 10–11 nM, was lower than that presented in Table I because the enzyme concentration was not taken into consideration in Table I. This might be the lowest value among data reported for vanadate-induced inhibition of enzymes (Cantley et al., 1977; Kobayashi et al., 1978; Gibbons et al., 1978; O'Neal et al., 1979; Seargeant & Stinson, 1979). However, Na,K-ATPase was shown to bind vanadate with a dissociation constant of 4–5 nM (Cantley et al., 1978; Jorgensen & Anner, 1979), although the half-maximal inhibition of this enzyme occurred at 35–50 nM (Cantley et al., 1977). When the concentration of ATP was lowered to 1 μM, near the K_m value for 30S dynein Mg-ATPase, the inhibition constant increased to 15–17 nM. A possible reason for such a variation was described under Results.

From the enzyme concentration term (E_t), we could get a minimal molecular weight ranging from 420 000 to 480 000 per metavanadate-binding site or enzymatic active site when r was 1.5 or 2. If we assume that only the prominent polypeptide band on the $NaDodSO_4$ gel is responsible for the ATPase activity, and the others are mere contaminants [cf. Gibbons & Fronk (1979) and Piperno & Luck (1979)], the molecular weight would be reduced to 330 000–380 000 because the purity is around 80% (Shimizu et al., 1979). This value is in good agreement with the estimation of the chain weight of the prominent band (Warner et al., 1977; Gibbons & Fronk, 1979; Hayashi & Takahashi, 1979), although it is smaller than our earlier evaluation (Mabuchi & Shimizu, 1974) which needs reexamination. We previously reported (Shimizu et al., 1979) that 30S dynein exhibited the initial burst of phosphate liberation, the size of which was 1.2–1.9 mol of P_i/1 000 000 g of protein. This corresponds to 1 mol of P_i/420 000–670 000 g of protein of the prominent band. Since the presumptive intermediate D·(ADP)·P_i, which is responsible for the phosphate burst, might be in a state of equilibrium with D·ATP, as myosin active site·ADP·P_i is with myosin active site·ATP (Bashaw & Trentham, 1974; Arata et al., 1975), the size of the initial burst would depend upon the equilibrium constant. Therefore, our previous value might be reduced to a slight extent. Furthermore, our method to determine the size of the phosphate burst was not good enough to exclude slight error. The precise determination of the burst size should be made by using a quench-flow apparatus, which will give a reasonable value for the molecular weight agreeable with that obtained by the present or other methods.

As a matter of fact, however, the situation concerning the enzyme concentration term (E_t) is rather complicated. When the value r was chosen to be 1.5 or 2, the enzyme concentration term obtained was 21–24 nM for 10 μg/mL 30S dynein irrespective of ATP concentration. However, we could not explain that the value always tends to increase with the value r. This fact corresponds to the biphasic appearance of the Dixon plot. In the case of 1 μM ATP, where the Dixon plot seemed almost straight, the situation with regard to the enzyme concentration term is the same, and the theoretical curve calculated from K and E_t values would not agree well at higher concentrations of metavanadate (unpublished results). Apparently, the real situation is more complex than our simplest noncompetitive scheme.

In this connection, there are several problems: (a) Is the ATPase activity really inhibited by association of a single vanadate ion with a single dynein subunit particle? (b) Does 30S dynein have another site for vanadate anion? We assume that the inhibition is simply noncompetitive, so that the enzyme–substrate–inhibitor ternary complex (ESI complex) will not dissociate to give rise to EI and product. Is that true? (d) The interaction between the enzymatic active sites is neglected, although 30S dynein is most likely to be composed of several subunits (Warner et al., 1977). (e) Are all enzyme units catalytically active as well as competent for vanadate binding? (f) The 30S dynein fraction might contain catalytically inactive but vanadate-binding protein.

These problems are not answered yet and rather serious. For instance, the Dixon plot exhibited a downward bent, and the enzyme concentration term is rather variable. A preliminary attempt is now being carried out to modify our simple noncompetitive scheme, such as introducing a concept that the ESI complex does dissociate to give rise to EI and product. In this case, the K and/or E_t seems to be reduced to smaller values to some extent.

At any rate, the simple noncompetitive scheme seems to hold well if the metavanadate-induced inhibition is in the range of moderate inhibition, and further modification of the simple scheme would be necessary when the inhibition is profound, such as that of more than half-maximal. Therefore, the K and E_t values described above seem reasonable.

Acknowledgments

I wish to express my thanks to Professor H. Sakai (the University of Tokyo) and to Drs. T. Kobayashi (Jikeikai Medical College), M. Iwakura, and Y. Tanaka (our Institute) for their valuable discussions.

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Self-Association of Insulin and the Role of Hydrophobic Bonding: A Thermodynamic Model of Insulin Dimerization†

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ABSTRACT: The association constant for insulin dimerization calculated from concentration-dependent circular dichroic (CD) spectra of insulin, $K_{12} = 7.5 \times 10^5$ M^{-1}, is used along with other association constants (K_{24}, K_{46}, and K_{26}) in an attempt to decipher the complex association behavior in solution and in crystal of this protein hormone. The free-energy change associated with dimerization, $-RT \ln K_{12}$, is -8.01 kcal mol^{-1}, a value which is used to test a semiquantitative thermodynamic model of the process based in part on the X-ray crystallographic data of insulin. By delineation of the hydrophobic core on the surface of insulin, which is implicated in the dimer formation, the free energy of association, $\Delta G°_{assoc}$, is estimated as -8.27 kcal mol^{-1} by using the thermodynamic parameters of Némethy & Scheraga [Némethy, G., & Scheraga, H. A. (1962) *J. Phys. Chem. 66*, 1773-1789] and as -10.27 kcal mol^{-1} by using the values of Nozaki & Tanford [Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211-2217]. The role of hydrophobic bonding in the dimerization of insulin is discussed, and the calculated values of free energy of association are compared with the experimental value. The importance of this thermodynamic model is delineated in regard to both hormone-hormone and hormone-receptor interactions.

Insulin exhibits a complex association behavior in solution and in crystal (Jeffrey & Coates, 1966; Pekar & Frank, 1972; Lord et al., 1973; Goldman & Carpenter, 1974; Pocker & Biswas, 1980). A monomer-dimer-tetramer-hexamer (M-D-T-H)[1] equilibria for insulin solutions were proposed by

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[1] Abbreviations used: CD, circular dichroism; M-D-T-H, monomer-dimer-tetramer-hexamer; M-D-H, monomer-dimer-hexamer; assoc, association; ent, entropic.

0006-2960/81/0420-4354$01.25/0