A novel adenosine triphosphate analog with a heavy atom to target the nucleotide binding site of proteins

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Abstract

We have synthesized 2'-deoxy-2'-iodoadenosine-5'-triphosphate (2'-IATP), a heavy-atom analog of adenosine-5'-triphosphate. This compound was made for X-ray structural studies to target the nucleotide site of ATP binding proteins. It was diffused successfully into crystals of the microtubule-based motor proteins ncd (non-claret disjunctional protein from *Drosophila melanogaster*) and kinesin. With ncd, the nucleotide binding site was 70% occupied and the crystals were able to diffract X-rays to 2.5 Å. The iodo-analog provided a useful isomorphous derivative with overall phasing power 1.89 in the range of 25.0–2.5 Å. With kinesin, 2'-IATP co-crystallized with the protein. The crystals diffracted to at least 2.8 Å with a phasing power of 1.73 in the range of 20.0–5.0 Å. The analog was also found to be a substrate for all of the enzymes tested, including creatine kinase, pyruvate kinase, hexokinase, and myosin, with values of K_m and V_{max} that were within a factor of 10 of those for ATP. The analog supported muscle contraction, relaxing fibers, and producing active tension with values not statistically different from those obtained with ATP. These results all suggest that this analog should be useful for providing a heavy-atom derivative for crystals of enzymes that bind ATP.

Keywords: 2'-deoxy-2'-iodoadenosine-5'-triphosphate; nucleotide analogs; motor proteins; X-ray crystallography

Numerous nucleoside and nucleotide analogs have been synthesized in efforts to enhance their chemotherapeutic activity. These compounds have been modified on the base, the phosphate, and the carbohydrate moiety to achieve new functional activities. Modifications of the carbohydrate portion of purine nucleosides and nucleotides have been investigated for numerous biological and biochemical reasons (Yount, 1975; Srivastava et al., 1988; Chladek, 1994). Ribose-modified moieties are of considerable interest because the ribose ring provides reactive sites for the introduction of specific groups. In addition, the specificity of some enzymes is less restrictive for this part of the ATP molecule. Thus, these sites have proven to be useful for preparation of affinity labels and affinity chromatographic columns (Yount, 1975). Compounds with the aminoacyl ester bond on the 2'- or 3'-position of the ribose have special importance because of aminoacyl transfer ribonucleic acid (aa-tRNA), the key intermediate in the biosynthesis of proteins (Chladek, 1994).

In spite of the wide interest in nucleotide analogs, one group, the metallonucleosides, has received little attention. Although likely to be of only limited potential as chemotherapeutic agents, the covalently bound, heavy metal atom could potentially produce a useful tool for structural studies, such as electron microscopy and protein X-ray crystallography. Metallonucleosides were first reported by Dale and coworkers (1973), who introduced mercury into the 5-position of the bases of uridine triphosphate and cytidine triphosphate and into the 7-position of deazaATP. The conversion of a hydroxyl group on the glycosyl moiety of purine nucleosides and nucleotides to a halogen has been of interest because of the synthetic utility of these halo sugar derivatives and because of an interest in their biological properties (Srivastava et al., 1988). The iodo derivatives have received less attention due to their instability and the difficulty of their synthesis. Todd and Ulbricht (1960) have reported the synthesis of 3'-deoxy-3'-iodoadenosine with the D-xylo configuration as an intermediate in the synthesis of cordycepin, without structural proof.

In the present study, we synthesized 2'-deoxy-2'-iodoadenosine-5'-triphosphate (2'-IATP). The motivation for the synthe-

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sis was to produce a compound that would allow us to target the nucleotide site of ATP binding proteins for use in X-ray crystallographic studies. The 2'-position of the ribose moiety was chosen for modification because we have determined previously that the motor proteins, myosin and the kinesin family, are tolerant to substrate modification at this position. The motor proteins myosin, non-claret disjunctional protein (*ncd*) from *Drosophila melanogaster*, and kinesin have been shown to bind several spin label analogs of ATP with the spin probe moiety on the 2'- or 3'-positions of the ribose ring (Crowder & Cooke, 1989; Alessi et al., 1992; Naber & Cooke, 1995). The substrates are hydrolyzed by the proteins, and they support translation of the appropriate microfilament (actin for myosin, microtubules for *ncd* and kinesin) with in vitro assay protocols. Additional support for these observations comes from the recent X-ray crystallographic structure of myosin subfragment 1 (S1) with bound nucleotide complex (Fisher et al., 1995). Here it is seen that the hydroxyl groups on the 2'- and 3'-positions of the ribose point toward the opening of the nucleotide cleft, exposed to solvent, indicating little interaction with the protein surface.

Results and discussion

In this work, we synthesized an ATP analog in which the 2'-hydroxyl group is replaced by an iodo moiety (Fig. 1). Starting with ara-adenosine (where the hydroxyl groups at the 2' and 3' positions are in a *trans* configuration), the 3' and 5' hydroxyls were simultaneously protected using 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane, a reagent that is specific for the pro-

tection of the 3'- and 5'-hydroxyl function of ribonucleosides (Markiewicz et al., 1980). The iodo moiety was then introduced to the 2'-position of the ribose by a series of two chemical reactions, replacing the OH group with an inversion of configuration at the 2'-position (Fukukawa et al., 1983). The protecting group was subsequently removed and the resulting iodo nucleoside phosphorylated. In some ribose-substituted compounds (e.g., those with an ester linkage where a carbonyl group is attached to the hydroxyl oxygen at the 2'- or 3'-position), an isomerization between the 2'- and 3'-positions has been observed due to the hydrolysis of the ester bond in solution. In the present case, however, the iodide at the 2'-position will not exchange with the OH group at the 3'-position because the C-I bond is a strong bond, requiring severe reaction conditions to break. In addition, the Patterson map described below shows a single peak. If some iodide had migrated to the 3'-position, we would see two peaks. Hence we conclude that our modification in the analog is restricted to the 2'-position only.

The resulting 2'-IATP has a similar structure to that of ATP. The iodo moiety is attached directly to the ribose replacing the hydroxyl group and has the same configuration as the hydroxyl. It is important to have the correct configuration, because we found that ara-ATP and ara-spin label ATP analogs with the spin label attached to the 2'-hydroxyl group of ara-ATP did not bind to myosin, *ncd*, or kinesin (Naber & Cooke, 1995). We show below that the 2'-IATP was able to diffuse into crystals of the motor proteins *ncd* and kinesin, that it binds to myosin, and that it is a substrate for several enzymes.

Incorporation of 2'-IATP into *ncd* and kinesin crystals

A key step in solving X-ray crystal structures is to provide phases for the experimentally observed X-ray reflections. The multiple isomorphous replacement (MIR) method of phasing proteins is the oldest and often the only possible way of phase evaluation for new macromolecular structures. This technique involves the trial-and-error preparation of heavy-atom derivatives and it is frequently limited by lack of isomorphism.

2'-IATP can provide a more rational mechanism for the design of heavy-atom isomorphous derivatives for ATP-binding proteins. As was shown for the *ncd* and kinesin crystals, the endogenous ADP could be replaced by 2'-IATP either by soaking the *ncd* crystals in a solution containing 5 mM 2'-IATP or by co-crystallizing kinesin in the presence of 5 mM 2'-IATP. The incorporation of 2'-IATP into the crystals and the presence of a single heavy-atom site was proved by solving the isomorphous difference Patterson map for the 2'-IATP derivative of both proteins (Fig. 2).

The *ncd* crystals with incorporated 2'-IATP diffracted X-rays to at least 2.5 Å and provided a useful isomorphous derivative with overall phasing power of 1.89 in the range of 25.0–2.5 Å (E.P. Sablin & R.J. Fletterick, unpubl. results). The coordinates of the iodine atom also allowed us to determine the location of the nucleotide binding site within the *ncd* motor domain. The 2'-IATP kinesin crystals diffract to at least 2.8 Å with a phasing power of 1.73 in the range of 20.0–5.0 Å (J. Kull & R.J. Fletterick, unpubl. results). These results (Table 1) demonstrate that heavy-atom nucleotide analogs such as 2'-IATP are extremely useful for obtaining phase information to aid in the structure solution of proteins of at least 40 kDa molecular weight.

Fig. 1. Synthesis of the ATP analog 2'-deoxy-2'-iodoadenosine-5'-triphosphate (see text for details).

Fig. 2. 2'-IATP derivative Patterson map from the ncd motor domain. The three Harker sections ($u = 0$, $v = 0$, $w = 0$) of an isomorphous derivative Patterson map are shown for a 2'-IATP derivative of the ncd motor domain. Fourier amplitudes used in calculating this map are $(FP - FPH)^2$. The three prominent peaks show the positions of three Harker vectors. The dashed lines illustrate how these peaks line up when the sections are aligned as shown. Contours are drawn at 0.5σ intervals. The lowest contour level shown is at 2σ . Only data between 10.0 and 3.0 Å resolution was used in this Fourier calculation. This Patterson map shows no features on the non-Harker sections and can be explained by a single heavy-atom site.

2'-IATP as an enzyme substrate

The 2'-IATP was found to be a substrate for all of the enzymes that were tested. Each of the three enzymes produced linear double reciprocal plots of $1/\text{velocity}$ versus $1/[\text{substrate}]$ as shown in Figure 3. The values of K_m and V_{max} determined from least-squares fits to the kinetic data are given in Table 2 and discussed

in more detail below. Prior to the assay of creatine and pyruvate kinases, 2'-IATP was hydrolyzed to the diphosphate form with myosin and separated from the myosin.

The analog 2'-IADP was a reasonable substrate for creatine kinase, with a resulting K_m of 43 μM (see Table 2), indicating that 2'-IADP binds about a factor of 10 more tightly to creatine kinase than does the natural substrate, ADP. In contrast, the

Table 1. Data processing statistics for crystalline *ncd*-ATP and *ncd*-2'-IATP

	<i>ncd</i> -ATP	<i>ncd</i> -2'-IATP
Resolution (Å)	2.5	2.5
No. of measured reflections	16,690	16,216
% Observed	89.4	87.0
R_{sym}^a	0.061	0.058
R_{scale}^b		0.14

$$^a R_{\text{sym}} = \sum [I(h)i - \langle I(h) \rangle] / \langle I(h) \rangle.$$

$$^b R_{\text{scale}} = \sum |FP_h - FP| / \sum FP.$$

maximum rate of the reaction for the iodo analog was about a factor of 10 less than that for ADP. These results are in agreement with those of Watts (1973), who reported that limited variation of the sugar moiety has a relatively small effect on the activity of creatine kinase. Both these results can be explained if the iodo moiety enhances the stability of the initial enzyme-substrate complex without perturbing the transition state, as discussed below.

Substitution at the 2'- and 3'-position of the ribose ring has been shown to inhibit the activity of rabbit muscle pyruvate kinase, indicating that the ribose ring plays a significant role in the binding of ADP to the enzyme (Hohnadel & Cooper, 1973). Even the simple replacement of the 2'- or the 3'-hydroxyl groups of ADP by a hydrogen to give deoxy-ADP decreased the V_{max} by 80–90% for the rabbit enzyme (Hohnadel & Cooper, 1973). Inversion of either the 2'- or 3'-hydroxyl groups of ADP led to a larger reduction in V_{max} . However, the rat muscle enzyme is less specific at this site and the activity of 2'-*O*-methyl-ADP is comparable to ADP itself (Hai et al., 1982). We found that with the rabbit muscle enzyme, the rate of the enzymatic reaction was slightly higher and the value of K_m was slightly lower for the iodo analog than for ADP (Table 2). These results indicate that 2'-IADP is a very good substrate analog for pyruvate kinase and could be useful for different types of studies on this enzyme.

Hexokinases are highly restrictive as regards substrate specificity, with ATP giving by far the highest activity (Grossbard & Schimke, 1966). We tested 2'-IATP as a substrate for the yeast hexokinase. Our results indicated that 2'-IATP binds to hexokinase with a K_m of 146 μM compared with a K_m of 96 μM for

Table 2. Enzyme substrate kinetic data^a

Enzyme	Substrate	K_m (μM)	V_{max} ($\mu\text{mol/min mg}$)
Creatine kinase	2'-IADP	43 $\pm$ 4.7	16 $\pm$ 1.3
	ADP	523 $\pm$ 43	152 $\pm$ 17
Pyruvate kinase	2'-IADP	374 $\pm$ 60	565 $\pm$ 73
	ADP	282 $\pm$ 39	278 $\pm$ 21
Hexokinase	2'-IATP	146 $\pm$ 8	5.2 $\pm$ 0.1
	ATP	96 $\pm$ 9.4	37 $\pm$ 2.6

^a K_m and V_{max} values ($\pm$ SE) obtained from the least-squares linear fits to the kinetic data of Figure 3 using ADP and 2'-IADP or the triphosphate forms for the above kinases.

Fig. 3. Double-reciprocal plots of 1/velocity versus 1/[substrate] from enzymatic activity assays for (A) creatine kinase, (B) pyruvate kinase, and (C) hexokinase, using the physiological substrate (Δ) and the 2'-iodo analog ($\circ$) as substrate. Solid lines are least-squares linear fits to the data, with the vertical intercept and slope used to determine V_{max} and K_m .

ATP, and a V_{max} that is about 14% of that of ATP (Table 2). Thus 2'-IATP appears to be a more effective substrate for hexokinase than any other nucleotide analogs that have been investigated.

The above results indicate that exchanging the 2'-hydroxyl group of ATP for an iodo does not greatly inhibit the ability of the resulting analog to act as a substrate for the enzymes tested. The modest perturbations produced can be analyzed in terms of simple Michaelis-Menten kinetics. An analog that stabilizes an initial substrate-enzyme complex, but does not perturb other interactions, should decrease both K_m and V_{max} by the same factor (see Fersht [1985] for discussion). This is exactly what is observed for creatine kinase, suggesting that the iodo-ADP binds more tightly to the enzyme by about 6 kJ/mol than does ADP. The opposite effect is observed for pyruvate kinase, with both V_{max} and K_m increasing, suggesting that the initial enzyme-substrate complex has been destabilized by about 1 kJ/mol. The data suggest that the free energy of the transition state is not affected for either enzyme. The changes observed in the activity of hexokinase are more complex suggesting that both the initial complex and the transition state are destabilized, by about 1 and 6 kJ/mol, respectively. Together these results suggest that the iodo analog provides a useful perturbation of the interaction between enzyme and the ribose of the substrate. Most probably the iodo group eliminates hydrogen bonds at the 2'-position and also introduces a modest steric perturbation. The value of 6 kJ/mol is in the range observed for the stabilization provided by a hydrogen bond. The data for creatine kinase, in which the analog binds more tightly than does ADP, could be an indication that the enzyme does not form a hydrogen bond with the 2' position in the initial complex. The analog will then bind more tightly, because the iodo eliminates a hydrogen bond with water in the free nucleotide that would stabilize the free over the bound nucleotide for ADP. Thus, analysis of the data can help define the role of interactions of the 2'-position in the energetics of specific states. In addition, the data show that the presence of an iodo group does not produce a perturbation that is too great to prevent its use as a heavy atom for structural studies of ATP binding proteins.

Fiber mechanics

2'-IATP was found to be a good substrate for the myosin ATPase activity. Samples of both ATP and 2'-IATP were hy-

drolyzed separately by myosin under the same conditions as described in the Materials and methods. The results showed that 2'-IATP is hydrolyzed to the diphosphate form at approximately double the rate of ATP hydrolysis. Myosin has evolved to have a minimal ATPase activity by itself, and a more rapid turnover rate has been found previously for a number of ATP analogs (Sekiya, 1973). A measure of how well the analog binds to and functions in the active site in a manner analogous to MgATP can be made by using fiber mechanics as described below.

We compared the ability of 2'-IATP and the physiological substrate, ATP, to support muscle contraction using glycerinated rabbit psoas fibers. As shown in Table 3, 2'-IATP relaxes fibers and supports active tension and stiffness, with values not statistically different from those obtained with ATP. The values in Table 3 with ATP as substrate are similar to values we have obtained previously using similar experimental conditions (Wang et al., 1993). Figure 4 shows the accumulated force-velocity data and Hill fits for the determination of maximum shortening velocity. Values for the maximum shortening velocity are again comparable, with 2'-IATP having a value that is only 13% less than ATP. The Hill fit for 2'-IATP has a slightly higher curvature than that obtained with ATP. The correspondence between the mechanical parameters provides evidence that 2'-IATP binds to and resides in the myosin nucleotide site in a manner analogous to that of ATP. This supports the conclusion that the 2'-hydroxyl of bound nucleotides does not interact strongly with the protein, as shown by structural studies (Fisher et al., 1995).

In summary, the above results suggest that the analog, 2'-IATP, should provide a useful method of phasing small molecules that bind adenine nucleotides. In addition, the replacement of the 2'-hydroxyl group by an iodo perturbs enzyme function. This provides a measure of the importance of this position in the binding of the nucleotide, which could be useful in studies of enzyme kinetics.

Materials and methods

The sources of commercial compounds were as follows: adenine 9- β -D-arabinofuranosyl-adenine (ara-adenosine), tri-*n*-butylammonium pyrophosphate, creatine phosphokinase type I from rabbit muscle, pyruvate kinase type III from rabbit muscle, hexokinase type VI from Bakers yeast, L-lactic dehydrogenase type II from rabbit muscle, glucose-6-phosphate dehydrogenase type IX from

Table 3. Mechanical parameters of muscle fiber contraction^a

Parameter	Substrate	
	ATP	2'-IATP
Relaxed tension (N/mm ²)	0.0026 $\pm$ 0.0006	0.0025 $\pm$ 0.0007
Relaxed stiffness (N/mm ²)	0.41 $\pm$ 0.023	0.45 $\pm$ 0.10
Active tension (N/mm ²)	0.16 $\pm$ 0.01	0.17 $\pm$ 0.02
Active stiffness (N/mm ²)	12.0 $\pm$ 0.83	11.8 $\pm$ 1.76
Maximum shortening velocity (lengths/s)	0.94 $\pm$ 0.03	0.82 $\pm$ 0.04

^a Tension, stiffness, and maximum shortening velocity for fibers using 2 mM MgATP and 2 mM Mg2'-IATP as substrates. Errors are $\pm$ SE for P_0 , $\pm$ asymptotic 95% confidence limits from the Hill fit for the maximum contraction velocity. Each table entry represents data from three to six fibers.

Fig. 4. Accumulated force-velocity data for fibers contracting with 2 mM MgATP (○) and 2 mM Mg2'-IATP (△) as substrate. Solid lines are least-squares, hyperbolic Hill fits to the force-velocity data. Tensions (horizontal axis) are normalized with respect to the isometric value.

Bakers yeast, Q-sepharose (Sigma); 1,3-dichloro-1,1,3,3-tetra-isopropylidisiloxane, trifluoromethanesulfonyl chloride, lithium iodide, tetrabutylammonium fluoride, 4-dimethylaminopyridine, hexamethylphosphoric triamide, 1-naphthol, diacetyl (Aldrich). Thin-layer chromatography (TLC) was carried out using Baker-Flex silica gel plates with fluorescent indicator (254 nm). Flash chromatography was performed on Baker Silica gel for flash chromatography purchased from VWR. The mass spectra were measured on a Kratos-MS50 instrument at the UCSF Mass Spectroscopy Facilities. Enzyme assays were measured on a Beckman, DU-40 spectrophotometer. The NMR spectra were measured on a GE 300-MHz spectrometer.

Synthesis of 2'-IATP

3',5'-O-(Tetra-isopropylidisiloxane-1,3-diyl)ara-adenosine (Compound 1)

Ara-adenosine (1.33 g, 5 mmol) and imidazole (1.5 g, 22 mmol) were dissolved in 15 mL of dimethylformamide. 1,3-Dichloro-1,1,3,3-tetra-isopropylidisiloxane (1.75 g, 5.6 mmol) was then added to the solution and the mixture was stirred at room temperature for 45 min. The reaction was quenched with 10 mL of ice water and the product was extracted with dichloromethane. The organic layer was evaporated to dryness and the resulting oil was purified on a silica gel flash chromatography column using a mixture of dichloromethane/methanol (19/1). The product is a colorless oil. Yield: 1.97 g, 3.88 mmol, 76%; r_f : 0.38 dichloromethane/methanol, 19/1; MS m/e : 509 (M^+), 466 ($M^+ - \text{iso-Pr}$).

2'-O-Triflyl-3',5'-O-(tetra-isopropylidisiloxane-1,3-diyl)ara-adenosine (Compound 2)

Compound 1 (1.95 g, 3.83 mmol) was dissolved in 45 mL of dichloromethane. Triethylamine (0.59 mL, 4.21 mmol) and 4-dimethylaminopyridine (0.47 g, 3.83 mmol) were added and

the mixture was cooled in an ice bath. Trifluoromethanesulfonyl chloride (0.45 mL, 4.21 mmol) was added and the mixture was stirred under nitrogen at room temperature for 20 min. The reaction was quenched with 40 mL of ice-cold water and extracted with dichloromethane several times. The organic layer was evaporated to dryness and the resulting oil was purified on a silica gel flash column using a mixture of dichloromethane/methanol 15/1. The product is a white crystalline compound. Yield: 2.05 g, 3.2 mmol, 83%; r_f : 0.48 dichloromethane/methanol, 19/1; r_f : 0.38 ethyl acetate/hexane, 1/1; MS m/e : 641 (M^+), 598 ($M^+ - \text{iso-Pr}$).

2'-Deoxy-2'-iodo-3',5'-O-(tetra-isopropylidisiloxane-1,3-diyl)adenosine (Compound 3)

Compound 2 (1.02 g, 1.6 mmol), hexamethylphosphoric triamide (12 mL), and lithium iodide (4.8 mmol, 640 mg) were mixed and the solution was allowed to stir at room temperature for 2 days. The progress of the reaction was monitored by TLC. The reaction was quenched with 10 mL of ice water and dichloromethane was added to dissolve the gummy precipitate. The aqueous layer was extracted with dichloromethane and the combined organic layer was evaporated to dryness. The product was purified on a silica gel flash chromatography column using a mixture of dichloromethane/methanol, 19/1. The product is a white solid, m.p. (152, dec.); yield: 0.79 g, 1.28 mmol, 80%; r_f : 0.52 dichloromethane/methanol, 19/1; r_f : 0.28 hexane/ethyl acetate, 1/1; MS m/e : 619 (M^+), 576 ($M^+ - \text{iso-Pr}$).

2'-Deoxy-2'-iodoadenosine (Compound 4)

Compound 3 (1.11 g, 1.8 mmol), tetra-*n*-butylammonium fluoride (3.6 mL of a 1 M solution in tetrahydrofuran, 3.6 mmol) and tetrahydrofuran (22 mL) were mixed and stirred at room temperature for 10 min. The resulting mixture was evaporated to dryness and the residue was dissolved in a minimum amount of dichloromethane and purified on a silica gel flash column using a mixture of dichloromethane/methanol, 18/1. Yield: 0.54 g, 1.44 mmol; r_f : 0.19 dichloromethane/methanol, 18/1; MS m/e : 377 (M^+), 250 ($M^+ - \text{I}$).

2'-IATP (Compound 5)

Compound 4 was phosphorylated following the method of Ludwig (1983). The product was purified on a Q-sepharose column using a triethylammonium bicarbonate gradient of 0.15–0.5 M. Fractions from the column were checked on TLC with *n*-propanol/ammonia/water (3/6/1) containing 0.5 g/L EDTA. Fractions containing the triphosphate were combined and evaporated to dryness at 30 °C. A few milliliters of methanol were added and evaporated to dryness twice. The product was then dissolved in water and stored at –30 °C in small fractions of concentrations 15–40 mM. Yield, 50%; MS m/e : 617 (M^+), 616 ($M-H$), 490 ($M-I$); ^1H NMR (D_2O): δ 8.51 (1H, s, H-8), 8.25 (1H, s, H-2), 6.50 (1H, d, H-1'), 5.05 (1H, m, H-3'), 4.49 (1H, dd, H-2'), 4.34 (1H, m, H-4'), 4.31 (2H, m, H-5'); UV $\lambda_{\text{max}}(\text{H}_2\text{O})$ 260 nm.

The sodium salt of 2'-IATP was prepared as follows: 2 mL of a 10 mM aqueous solution of the triethylammonium salt of 2'-IATP was evaporated to dryness under vacuum. Two milliliters of methanol was added and evaporated again. The resulting solid was dissolved in 0.3 mL of methanol, and 3 mL of a 10% solution of sodium perchlorate was added. The precipitated

sodium salt was centrifuged, washed twice with acetone, air dried, and stored at -30°C .

Incorporation of 2'-IATP into ncd and kinesin crystals

The native ncd motor domain was crystallized in the presence of MgATP (Sablin & Fletterick, 1995). The crystals belong to the orthorhombic space group I222 with unit cell dimensions $a = 127.1 \text{ \AA}$, $b = 122.3 \text{ \AA}$, $c = 68.0 \text{ \AA}$, and there is one ncd molecule per asymmetric unit. An iodo derivative was made by soaking the crystals in a 5 mM solution of 2'-IATP for 2 days. Data on the native and the derivative ncd were collected on an R-AXIS IIC image plate detector. X-ray data were reduced with the R-AXIS IIC software programs. Scaling, difference Patterson map calculation, and heavy-atom parameters refinement were carried out using the programs of the Crystallographic Computing Package CCP4. Data processing statistics on ncd are given in Table 1. The soaking method was ineffective for introducing the 2'-IATP into kinesin crystals. Co-crystallization was used to produce isomorphous derivative crystals that diffracted as well as the native crystals (J. Kull & R.J. Fletterick, unpubl. results).

Enzyme assays

2'-IATP and ATP (or the diphosphate forms) were tested as substrates for creatine phosphokinase, hexokinase, and pyruvate kinase. Enzymatic activity assays were carried out at room temperature. Values of K_m and V_{max} were calculated from least-squares linear fits to double-reciprocal plots of $1/(\text{enzyme activity})$ versus $1/[\text{substrate}]$. All steps were carried out at room temperature ($20\text{--}22^{\circ}\text{C}$).

The diphosphate form of the ionodonucleotide was obtained by enzymatic hydrolysis. Rabbit muscle myosin was obtained following the methods of Tonomura et al. (1966). A 3 mM solution of 2'-IATP was added to a 6-mg/mL myosin suspension in buffer containing 50 mM potassium chloride, 5 mM magnesium chloride, 1 mM EGTA, and 20 mM MES, pH 7.0. The progress of the hydrolysis to the diphosphate form was followed by TLC using *n*-propanol/ammonia/water (6:3:1, v/v), containing 0.5 g/L EDTA. The hydrolysis was completed in approximately 2 h at room temperature.

The activity of hexokinase was measured spectrophotometrically ($\lambda = 340 \text{ nm}$) using a 1-mL reaction mixture containing 100 mM TES, 1 mM glucose, 0.48 mM NADP, 0.6 U hexokinase, 25 U glucose-6-phosphate dehydrogenase, pH 7.6.

Creatine kinase activity was measured using the diphosphate as a substrate. The assay is based on the formation of a colored complex between the creatine released from phosphocreatine and α -naphthol-diacetyl reagent. This complex is measured spectrophotometrically ($\lambda = 535 \text{ nm}$) as described by Morrison et al. (1961).

Pyruvate kinase activity was measured spectrophotometrically ($\lambda = 340 \text{ nm}$) in a 1-mL reaction mixture containing a 100 mM TES buffer containing 1.0 mM magnesium chloride, 0.7 mM phosphoenolpyruvate, 0.16 mM NADH, 10 units of lactic dehydrogenase, and 0.5 units of pyruvate kinase, pH 7.5.

Fiber mechanics

Rabbit psoas muscles were harvested and chemically skinned as described previously (Cooke et al., 1988). Single fibers were

mounted between a solid-state force transducer and an arm connected to a rapid motor for changing muscle length. The fiber was then washed for 1 min in a rigor buffer containing 0.16 M potassium acetate, 9.0 mM magnesium acetate, 40 mM MOPS, 1 mM EGTA, 10°C , pH 7.0. The fiber was then relaxed in the above rigor buffer, additionally containing 2.2 mM 2'-IATP or ATP. This yields a calculated concentration of 2 mM [MgATP] or [Mg2'-IATP] (Pate et al., 1991). Relaxed stiffness and tension were measured and the fiber activated by addition of 1.1 mM calcium chloride. Final ionic strength was 200 mM. Active tension and stiffness were determined and a series of isotonic releases performed to determine shortening velocity as a function of tension. Stiffness was determined from the change in tension following a small (0.1%), rapid (90% complete in 0.5 ms) extension of the fiber length. Tension and stiffness were normalized with respect to fiber cross-sectional area. Velocity was determined from a least-squares linear fit to the position of the motor arm during the period 10–40 ms following initiation of an isotonic release. Accumulated force-velocity data were fit to the Hill equation (Hill, 1938), $V = V_{max}(a/P_0)(1 - P/P_0)/(a/P_0 + P/P_0)$. Extrapolated velocity at zero tension was taken as V_{max} . The experimental apparatus and fiber protocols are described in greater detail in Cooke et al. (1988) and Pate et al. (1991).

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