Characterization of Nucleotide-Free Uncoating ATPase and Its Binding to ATP, ADP, and ATP Analogues

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ABSTRACT: The interactions of the 70-kDa heat-shock proteins (hsp70s) with their protein substrates appear to be regulated by bound nucleotide. Previous work has shown that the nucleotide binding site of the bovine brain uncoating ATPase, a constitutive member of the hsp70 family, crystallographically resembles the nucleotide binding site of actin and, like actin, the uncoating ATPase has a strongly bound ADP which cannot be removed by dialysis or treatment with ethylenediaminetetraacetic acid (EDTA). This suggests that, like the bound nucleotide of actin, it may be required for the enzyme to retain its native structure. In this study, the strongly bound ADP was removed by first replacing it with 5′-adenylyl imidodiphosphate (AMP-PNP) and then removing the bound AMP-PNP by dialysis. Following this treatment, more than 95% of the uncoating ATPase becomes nucleotide-free. The nucleotide-free uncoating ATPase retains its ability to bind and hydrolyze ATP and to uncoat clathrin-coated vesicles, even after 10 days of storage at 4 °C. Therefore, in contrast to actin, the bound nucleotide of the uncoating ATPase is not required to prevent denaturation of the enzyme. Using nucleotide-free uncoating ATPase, we were able to accurately measure the dissociation constants of ATP, ADP, and the nucleotide analogues AMP-PNP and 2′-deoxyadenosine 5′-triphosphate (dATP). The dissociation constants of both ATP and ADP are about 10^{-8} M, more than 1–2 orders of magnitude stronger than previously reported, while AMP-PNP and dATP bind 2–3 orders of magnitude more weakly than ATP.

The hsp70s[1] are one of the most intensively studied families of major heat-shock proteins because of their unique properties and important cellular functions. These proteins are so well conserved that hsp70s isolated from all organisms have similar protein structures and share similar properties [for review see Craig (1985), Lindquist (1986), and Nover and Scharf (1991)]. While some hsp70s are produced only under stress conditions, such as heat shock, others are expressed constitutively. Studies show that these constitutive hsp70s are involved in many fundamental cellular processes, such as protein translocation across membranes of cell organelles (Chirico et al., 1988; Deshaies et al., 1988; Shi & Thomas, 1992; Imamoto et al., 1992), nascent protein folding multiunit-protein assembly (Beckmann et al., 1990; Bole et al., 1986; Flynn et al., 1989), antigen presentation (Vanbuskirk et al., 1989), protein degradation in the lysosome (Chiang et al., 1989; Terlecky et al., 1992), and uncoating of clathrin-coated vesicles (Schlossman et al., 1984; Greene & Eisenberg, 1990; Gao et al., 1991). Interestingly, there is evidence that almost all of these processes are affected by the nucleotide bound to hsp70.

We previously demonstrated that bovine brain uncoating ATPase, one of the constitutively expressed members of the hsp70 family, has a single nucleotide-binding site which, following the usual preparation method, is saturated by a strongly bound ADP. Although it exchanges rapidly with free nucleotide, this bound ADP cannot be removed by extensive dialysis (Gao et al., 1993) suggesting that it is bound very tightly to the enzyme. Even the addition of EDTA did not remove this nucleotide (Schmid et al., 1985), suggesting that, like the bound nucleotide of actin or tubulin (Korn, 1982), it might be required for the enzyme to retain its normal structure and function. This possibility is particularly intriguing since the nucleotide binding site of the uncoating ATPase has been found to be crystallographically similar to the nucleotide binding site of actin (Flaherty et al., 1991). In addition, like actin, the uncoating ATPase is able to polymerize to dimers, trimers, and perhaps higher-level polymers (Schlossman et al., 1984).

In the present study we investigated whether the uncoating ATPase requires a bound nucleotide to retain its enzymatic properties. Although conventional dialysis cannot be used to remove the bound nucleotide, we found that, by replacing bound ADP with AMP-PNP and then removing the bound AMP-PNP by dialysis, we were able to prepare enzyme which was more than 95% nucleotide-free. Using this nucleotide-free uncoating ATPase, we found that, in contrast to G-actin, the uncoating ATPase does not denature following removal of its bound nucleotide; it retains its enzymatic functions, including binding and hydrolyzing ATP and uncoating clathrin-coated vesicles, even after 10 days of storage at 4 °C.

Removal of the bound nucleotide of the uncoating ATPase permits investigation of numerous properties of the enzyme which cannot be undertaken as long as a tightly bound nucleotide is present. One of the most important of these properties is accurately determining the binding strength of ATP, ADP, and various nucleotide analogous which have been used in many studies on the hsp70s. In the present study we were able to show that the dissociation constants of ATP and ADP are both about 10^{-8} M, 1–2 orders of magnitude stronger than previously reported (Schmid et al., 1985; Palleros et al.,

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[1] Abbreviations: hsp70, 70-kDa heat shock protein; AMP-PNP, 5′-adenylyl imidodiphosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; P_i, sodium phosphate, monobasic; SDS, sodium dodecyl sulfate.

1991; Huang et al., 1993) while AMP-PNP and dATP bind 2–3 orders of magnitude more weakly than ATP.

MATERIALS AND METHODS

Purification of Bovine Brain Clathrin-Coated Vesicles and Uncoating ATPase. Clathrin-coated vesicles were prepared from fresh calf brains according to the procedure of Nandi et al. (1982). Purified coated vesicles were stored at 4 °C at a clathrin concentration of 15 μM. Bovine brain uncoating ATPase was purified by the method of Schlossman et al. (1984) with modifications described by Green and Eisenberg (1990). Purified uncoating ATPase was stored as ammonium sulfate pellets at 4 °C and dialyzed in buffer A (20 mM imidazole, 25 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium acetate, and 1 mM dithiothreitol, at pH 7.0) for 20 h at 4 °C before use. Protein concentrations of clathrin and uncoating ATPase were determined using extinction coefficients reported previously ($\in^{1\%}_{280}$ = 12 for clathrin and 6.2 for uncoating ATPase; Unanue et al., 1981; Greene & Eisenberg, 1990).

Preparation of Uncoating ATPase with Bound ^{14}C-ADP or ^{14}C-ATP. Uncoating ATPase with bound ^{14}C-ADP or ^{14}C-ATP was prepared as described by Gao et al. (1993). Briefly, 10 μM purified uncoating ATPase (with 0.8 mol of ADP bound/mol of enzyme) was incubated with 10 μM ^{14}C-ADP (50–60 mCi/mmol) at 25 °C for 60 min before being dialyzed in buffer A at 4 °C for 20 h to remove free ADP. The uncoating ATPase with bound ^{14}C-ATP was prepared by incubating ^{14}C-ADP-bound uncoating ATPase with 30 units/mL creatine kinase and 10 mM creatine phosphate at 25 °C for 30 min before use. Specific activity of the bound nucleotide was 30 mCi/mmol as determined by HPLC after the bound ^{14}C-nucleotide was extracted with perchloric acid and by liquid scintillation counting of the nucleotide peaks collected from HPLC. Results of specific activity determination suggest that ^{14}C-nucleotide binds equally as well as nonlabeled nucleotide.

Removing Free Nucleotide by Desalting Columns. Free nucleotide was removed by spinning samples through Sephadex G-50 columns as described by Penefsky (1977). Generally, samples of 50–100 μL were loaded onto 1-mL columns and centrifuged at 200*g* for 2 min in a Sorvall RT 6000D centrifuge. To minimize nonspecific binding of proteins, 50–100 μL of BSA (1 mg/mL) was spun through the columns before samples were loaded. Results showed that more than 80% of the protein and less than 0.1% of the free nucleotide were recovered after the desalting columns.

Nucleotide Content Analysis by HPLC. Procedures for nucleotide extraction and HPLC analysis have been described previously (Gao et al., 1993). All samples were treated with 0.6 M perchloric acid, and denatured protein was removed by centrifugation. Then the supernatant was neutralized with KOH and clarified by another centrifugation before loading onto HPLC columns.

Assay for ATPase Activity. ATPase activity of uncoating ATPase was determined by a slightly modified method of Chock and Eisenberg (1979). Uncoating ATPase (4–5 μM) was incubated at 25 °C with 100 μM [γ-^{32}P]ATP, (3000 Ci/mmol, New England Nuclear, catalogue no. NEG-002H, diluted to 300 mCi/mmol with unlabeled ATP, Sigma, catalogue no. A5394). Inorganic phosphate was extracted by adding successively 1.5 M HCl and 1.5 mM P_i, 1.5 M H_2SO_4 and 1.8% silicotungstic acid, 54% isobutyl alcohol/toluene (1:1), and 0.6% ammonium molybdate. After mixing and a brief centrifugation, the top organic phase of the extract was removed for scintillation counting.

Uncoating of Clathrin-Coated Vesicles. Uncoating of coated vesicles by uncoating ATPase was measured according to the procedure described by Greene and Eisenberg (1990). Reaction mixtures of 0.5 μM uncoating ATPase and coated vesicles containing 0.5 μM clathrin triskelion were incubated with 5 mM ATP-Mg^{2+} and ATP -regenerating system (30 units/mL creatine kinase and 10 mM creatine phosphate) at 25 °C for 20 min. After centrifugation, clathrin released in the supernatant was analyzed by SDS gels and quantified by densitometric scanning.

Equilibrium Dialysis. The method for equilibrium dialysis has been described previously (Gao et al., 1993). For measurement of AMP-PNP binding, 3–4 μM nucleotide-free uncoating ATPase was equilibrium-dialyzed with a concentration series of 0.5–9.6 μM [α-^{32}P]AMP-PNP (50 Ci/mmol, ICN Biomedicals, Inc., catalogue no. 37002, diluted to 200 mCi/mmol with unlabeled AMP-PNP, Sigma, catalogue no. A4794) added to both sides of the dialysis membrane. For measurement of dATP binding, 9–10 μM nucleotide-free uncoating ATPase was dialyzed with 1–56 μM [α-^{32}P]dATP (25 Ci/mmol, ICN Biochemicals, Inc., catalogue no. 33002, diluted to 250 mCi/mmol with unlabeled dATP, Sigma, catalogue no. D6500) added to both sides of the dialysis membrane. For nucleotide-binding competition experiments, 1 or 2 μM uncoating ATPase with 0.8 or 1.7 μM of bound ^{14}C-ADP or ^{14}C-ATP (30 mCi/mmol) was equilibrium-dialyzed with 0–8 mM dATP or 0–4 mM AMP-PNP added to both sides of the dialysis membrane. The competition binding was carried out in the presence of either 30 units/mL creatine kinase and 10 mM creatine phosphate (when ^{14}C-ATP was used) or 50 units/mL hexokinase and 10 mM glucose (when ^{14}C-ADP was used). The nucleotides and analogues were quantified by liquid scintillation counting after a 30-h dialysis at 4 °C.

RESULTS

Preparation of Nucleotide-Free Uncoating ATPase. Earlier observations suggested that dissociation constants for ATP and ADP were in the nanomolar range (Gao et al., 1993). Therefore, our inability to remove the bound nucleotide by dialysis was due to the fact that dissociation of a very small percentage of the bound nucleotide provides enough free nucleotide to prevent further dissociation. This is more likely to occur when the enzyme is dialyzed at high concentration, as we did previously using 120 μM uncoating ATPase. Therefore, we attempted to make nucleotide-free enzyme by dialyzing the uncoating ATPase at a concentration of 10 μM. As shown in Figure 1a, even after 2 days of dialysis during which the dialysis buffer was changed 5 times, more than 40% of the enzyme still retained ^{14}C-ADP. Although this was a greater reduction in bound nucleotide than we observed at higher enzyme concentration, we still had no obtained uncoating ATPase with negligible bound nucleotide. When we reduced the enzyme concentration further to facilitate the dialysis of the bound nucleotide, a significant amount of the protein denatured.

In contrast to the model of Schmid et al. (1985), which proposed that ATP analogues and ADP bind at different sites, our previous data suggested that the uncoating ATPase has only one nucleotide-binding site. On this basis, since ATP analogues often bind to enzymes more weakly than ATP and ADP, we reasoned that it might be possible to prepare nucleotide-free enzyme by replacing the bound ADP with AMP-PNP and then removing the bound AMP-PNP by dialysis. To test this idea, we incubated the uncoating ATPase

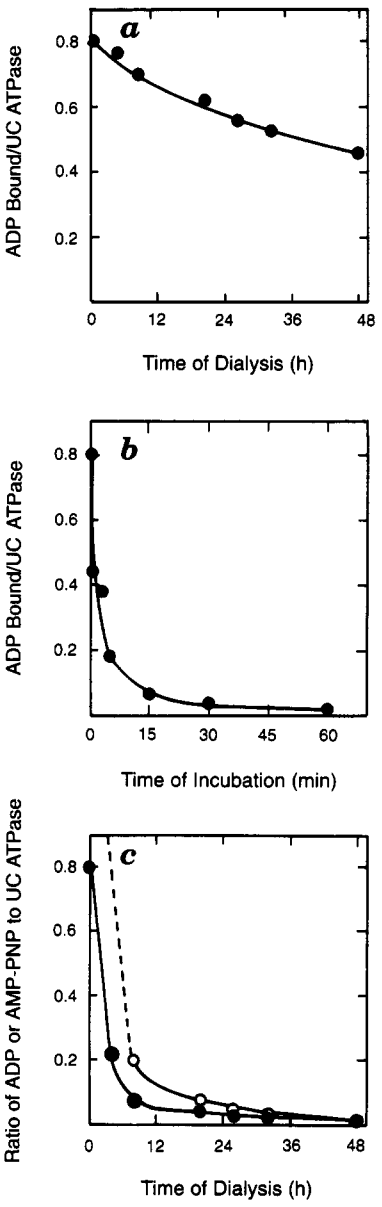

FIGURE 1: Removing bound nucleotide from purified uncoating ATPase. Uncoating ATPase (10 μM) with 8 μM bound ^{14}C-ADP (30 mCi/mmol) was dialyzed in 1000 volumes of buffer A (panel a) or was incubated with 5 mM AMP-PNP at 25 °C for 60 min and then spun through a desalting column (panel b; see Materials and Methods) or was incubated with 5 mM [α-^{32}P]AMP-PNP (2.8 mCi/mmol) at 25 °C for 60 min and then dialyzed in 1000 volumes of buffer A (panel c). Dialysis buffer was changed at each data point in panels a and c, except for the first and last data points. ADP (●) and AMP-PNP (○) contents were determined by liquid scintillation counting.

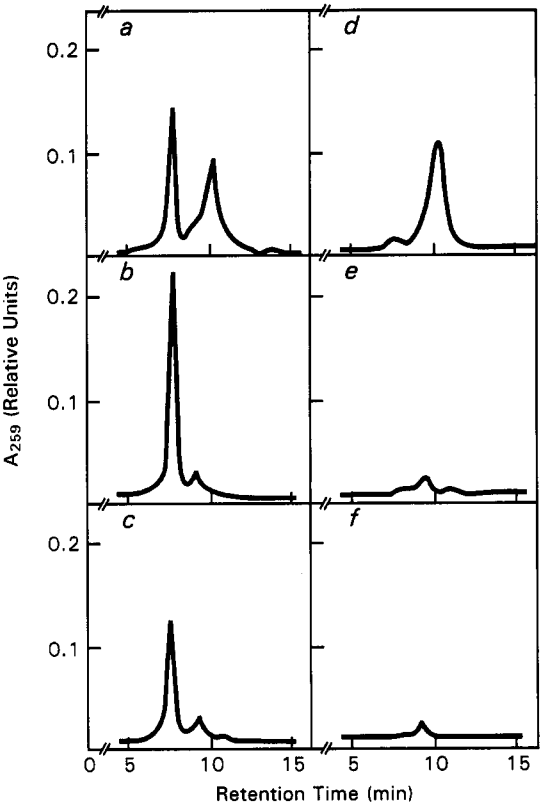

FIGURE 2: Nucleotide analysis of uncoating ATPase preparations by HPLC. HPLC profiles of nucleotide standards containing 0.5 μM ADP and 0.5 μM AMP-PNP (panel a); nucleotide extracted from 1 μM purified uncoating ATPase before dialysis (panel b); after 48 h of dialysis (panel c); after incubation with AMP-PNP for 60 min and removal of free ADP and AMP-PNP by desalting columns (panel d); after AMP-PNP treatment and 48 h of dialysis (panel e); and the buffer alone after undergoing nucleotide extraction procedure (panel f).

with a 600-fold excess of AMP-PNP relative to the bound ADP, to see if the bound ADP could be completely replaced. As shown in Figure 1b, nearly all of the originally bound ^{14}C-ADP was removable by a desalting column after 10 min of incubation. These data show that the bound ADP was indeed rapidly replaced by AMP-PNP, confirming that ADP and AMP-PNP bind to the same binding site on the uncoating ATPase.

We next determined if we could remove the bound AMP-PNP as well as the free AMP-PNP and ADP by dialysis. Uncoating ATPase with bound ^{14}C-ADP was incubated with [α-^{32}P]AMP-PNP for 60 min at 25 °C. The mixture was then dialyzed against 1000 volumes of buffer A for 2 days with frequent buffer changes to remove free ADP and free AMP-PNP. After 4–5 buffer changes, the uncoating ATPase contained only about 1% ADP (Figure 1c, closed circles) and 1% AMP-PNP (Figure 1c, open circles). Unlike the result in Figure 1a, ADP was easily removed by dialysis because it was no longer bound to the uncoating ATPase. Since, in contrast to bound ADP, the bound AMP-PNP was also removable by dialysis, this result shows that AMP-PNP indeed binds to the uncoating ATPase much more weakly than ADP.

To confirm these results, the nucleotide bound to uncoating ATPase following dialysis was extracted with perchloric acid and analyzed by HPLC. Figure 2b shows that purified uncoating ATPase after being dialyzed overnight contains about 0.8 mol of ADP/mol of enzyme. After extended dialysis under the conditions described in Figure 1a, the ADP content of the uncoating ATPase was reduced to about 50% (Figure 2c). After the bound ADP was replaced by AMP-PNP (as in Figure 1b) and free ADP and AMP-PNP were removed by spinning samples through desalting columns, the enzyme-bound nucleotide was extracted and analyzed by HPLC (Figure 2d). The result indicated that most of the bound nucleotide was AMP-PNP. Only when the enzyme was first treated with AMP-PNP and then dialyzed as described in Figure 1c did we obtain uncoating ATPase with no detectable bound ADP or AMP-PNP (Figure 2e). It should be noted that the minor peak occurring at 9 min is an artifact which occurs in all samples which undergo nucleotide extraction, including buffer A alone (Figure 2f).

To further confirm these results, we obtained spectrum absorbance of the samples described in Figure 2b,d,e after

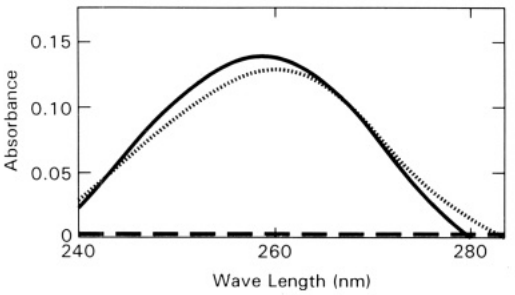

FIGURE 3: Nucleotide analysis by spectrophotometry. Spectrum absorbance of the supernatants of perchloric acid-extracted 10 μM uncoating ATPase (solid line), 10 μM uncoating ATPase after AMP-PNP treatment and desalting columns (dotted line), and 10 μM uncoating ATPase after AMP-PNP treatment and 48 h of dialysis (dashed line). Buffer A alone that underwent the same extraction procedure was used as blank.

Table 1: Binding of Nucleotide to Nucleotide-Free Uncoating ATPase[a]

		control E[b] (mol/mol)	NFE[c] (mol/mol)
bound ATP	fresh	0.85 ± 0.02	0.85 ± 0.04
	10 days	0.80 ± 0.02	0.76 ± 0.04
bound ADP	fresh	0.82 ± 0.02	0.80 ± 0.02
	10 days	0.78 ± 0.02	0.73 ± 0.02

[a] Values are averages of at least three independent experiments in which 5 μM uncoating ATPase was equilibrium dialyzed with 5 μM ^{14}C-nucleotide added on both sides of the membrane. Binding of ATP was determined in the presence of the ATP-regenerating system, and binding of ADP was determined in the presence of hexokinase and glucose (see Materials and Methods). [b] Regular purified uncoating ATPase containing 0.80–0.85 bound nucleotide as determined by HPLC. [c] Nucleotide-free uncoating ATPase.

perchloric acid extraction but before HPLC. Figure 3 shows that after AMP-PNP treatment and 48 h of dialysis the uncoating ATPase has no detectable bound nucleotide, while purified uncoating ATPase and uncoating ATPase treated with AMP-PNP and desalting columns have about 0.8 and 0.7 mol of nucleotide bound/mol of enzyme, respectively. Similar results were obtained when we extracted these samples with trichloroacetic acid (data not shown). All of these results demonstrate that, after incubation with AMP-PNP and dialysis, the uncoating ATPase is essentially nucleotide-free. Free ADP, free AMP-PNP, and most of the bound AMP-PNP could also be removed by column chromatography rather than dialysis. However, we routinely used dialysis to remove the nucleotide and analogue in preparing nucleotide-free uncoating ATPase to avoid dilution of the enzyme during column chromatography.

Characterization of the Nucleotide-Free Uncoating ATPase. Having made nucleotide-free uncoating ATPase, we next examined whether it still retains its enzymatic activity. The properties of the nucleotide-free uncoating ATPase were compared to that of regular purified uncoating ATPase, which contains about 1 bound nucleotide/enzyme molecule. Equilibrium dialysis in excess ATP or ADP showed that the nucleotide-free uncoating ATPase is able to rebind about 0.8 mol of nucleotide/mol of uncoating ATPase, virtually the same amount as is bound to the regular purified uncoating ATPase. Furthermore, this nucleotide-binding ability is retained by the nucleotide-free uncoating ATPase when it is stored for 10 days at 4 °C (Table 1).

The nucleotide-free uncoating ATPase also retained its ATPase activity and the ability to uncoat clathrin-coated vesicles. The ATPase activity of the nucleotide-free enzyme was the same as that obtained with the regular purified

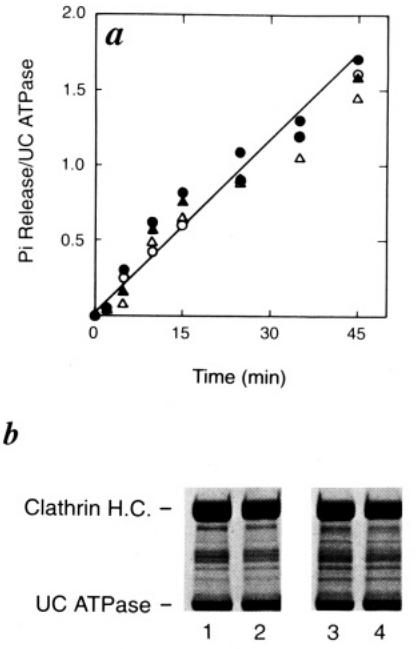

FIGURE 4: ATPase and uncoating activities of nucleotide-free uncoating ATPase. (Panel a) ATPase activities of fresh uncoating ATPase control (●), fresh nucleotide-free uncoating ATP (○), 10-day-old uncoating ATPase control (▲), and 10-day-old nucleotide-free uncoating ATPase (△) were determined at 25 °C with 4–5 μM uncoating ATPase and 100 μM [γ-^{32}P]ATP (300 mCi/mmol). (Panel b) The uncoating reaction mixture (0.5 μM uncoating ATPase, coated vesicles containing 0.5 μM clathrin triskelion, 5 mM ATP-Mg^{2+}, and the ATP-regenerating system) was incubated at 25 °C for 20 min before centrifugation. The supernatant containing clathrin released from coated vesicles and free uncoating ATPase was analyzed by SDS–PAGE. Lane 1, fresh uncoating ATPase control; lane 2, fresh nucleotide-free uncoating ATPase; lane 3, 10-day-old uncoating ATPase control; and lane 4, 10-day-old nucleotide-free uncoating ATPase.

Table 2: Enzymatic Activities of Nucleotide-Free Uncoating ATPase[a]

		control E	NFE[b]
ATPase activity ($\times 10^{-4}$ s^{-1})	fresh	6.1 ± 0.3	5.8 ± 0.3
	10 days	6.3 ± 0.3	5.5 ± 0.4
uncoating activity[c]	fresh	0.40 ± 0.02	0.36 ± 0.02
	10 days	0.38 ± 0.02	0.33 ± 0.02

[a] Values are averages of at least three independent experiments. [b] Nucleotide-free uncoating ATPase. [c] Clathrin released per uncoating ATPase as quantified by gel scanning.

uncoating ATPase (Figure 4a). The first two lines of Table 2 show that the results from several experiments all gave hydrolysis rates of about 6×10^{-4} s^{-1} for the different uncoating ATPase preparations. Furthermore, the ATPase activity of nucleotide-free uncoating ATPase remained the same after 10 days of storage at 4 °C. Figure 4b shows that the nucleotide-free uncoating ATPase also retains its ability to uncoat coated vesicles. The last two lines in Table 2 quantify the extent of uncoating by different enzyme preparations. In all cases, 0.5 μM uncoating ATPase uncoated about 0.2 μM clathrin, which is in agreement with the previous observation that three uncoating ATPase molecules are needed to uncoat one clathrin triskelion (Greene & Eisenberg, 1990). As observed with nucleotide-binding and ATPase activities, the uncoating activity of the nucleotide-free enzyme was retained after storage for 10 days at 4 °C. Therefore, the nucleotide-free uncoating ATPase retains its full activity and is stable upon storage.

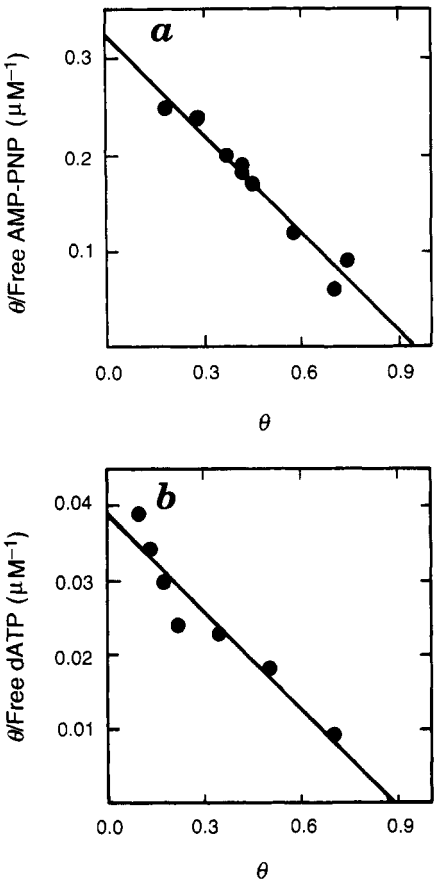

FIGURE 5: Binding of ATP analogues to nucleotide-free uncoating ATPase. Binding of AMP-PNP to nucleotide-free uncoating ATPase (panel a) was determined by equilibrium dialysis using 0.5–9.6 μM [α-^{32}P]AMP-PNP (200 mCi/mmol) and 3–4 μM nucleotide-free uncoating ATPase. Binding of dATP to nucleotide-free uncoating ATPase (panel b) was determined by equilibrium dialysis using 1–56 μM [α-^{32}P]dATP (250 mCi/mmol) and 9–10 μM nucleotide-free uncoating ATPase. The amounts of bound and free AMP-PNP or dATP were determined by scintillation counting after 30 h of dialysis. θ represents the number of moles of nucleotide analogues bound per mole of uncoating ATPase.

Measurement of Nucleotide-Binding Strength. Using nucleotide-free uncoating ATPase, the binding affinities of ATP analogues could be determined by simple equilibrium dialysis. Panels a and b of Figure 5 are Scatchard plots for the binding of AMP-PNP and dATP, respectively, to nucleotide-free uncoating ATPase. With both of these analogues, we obtained linear plots showing independent binding of nucleotide to the uncoating ATPase. The abscissa intercept extrapolates to 1, indicating 1 mol of analogue bound/mol of uncoating ATPase. The dissociation constants for AMP-PNP and dATP were determined to be 2.8×10^{-6} and 2.3×10^{-5} M, respectively. We also tried to measure the binding of ATPγS to nucleotide-free uncoating ATPase. However, we had problems with both ADP contamination in commercially available ATPγS and slight hydrolysis of the ATPγS by our uncoating ATPase preparations.

Having obtained the dissociation constants for two ATP analogues, we determined dissociation constants for ATP and ADP by measuring the concentration of analogues required to displace bound ^{14}C-ATP or ^{14}C-ADP on the uncoating ATPase. Figure 6a shows that it takes more than a 100-fold excess of free AMP-PNP over free ATP or ADP to displace 50% of the bound ATP or ADP from the uncoating ATPase, indicating that these latter nucleotides bound to the uncoating ATPase more than 2 orders of magnitudes more strongly than

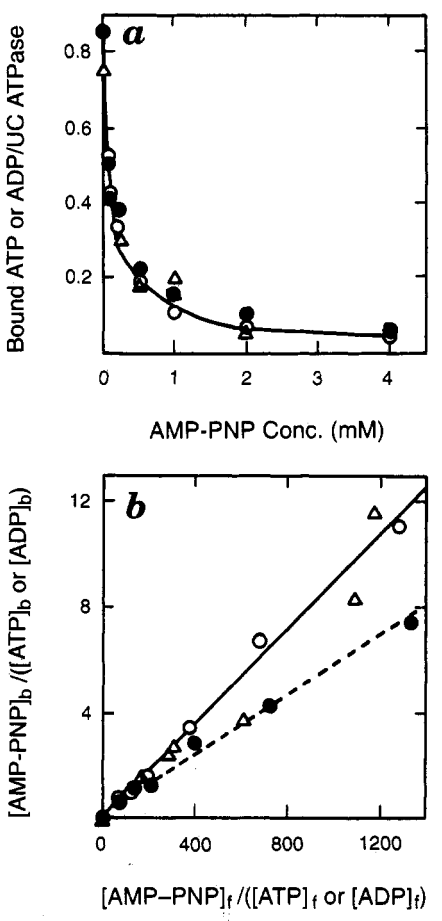

FIGURE 6: Competition binding of AMP-PNP with uncoating ATPase-bound ATP or ADP. Increasing amounts of AMP-PNP (0–4 mM) were equilibrium-dialyzed with 2 μM uncoating ATPase containing 1.7 μM bound ^{14}C-ATP (●) or ^{14}C-ADP (○), prepared from purified uncoating ATPase (see Materials and Methods). The amounts bound and free ^{14}C-ATP and ^{14}C-ADP (30 mCi/mmol) were determined by liquid scintillation counting at the end of dialysis. Data of competition binding using ^{14}C-ADP-bound uncoating ATPase prepared from nucleotide-free uncoating ATPase are indicated by open triangles (△). Panel b is a replot of the data in panel a. $[\ ]_b$ and $[\ ]_f$ represent concentrations of the bound and free nucleotide (or analogue), respectively. The solid line is for competition with ADP, and the dashed line is for competition with ATP.

AMP-PNP. By replotting the data in Figure 6a according to

$$\frac{[\text{analogue}]_{\text{bound}}}{[\text{ATP or ADP}]_{\text{bound}}} = \frac{K_{\text{d[ATP or ADP)}}\,[\text{analogue}]_{\text{free}}}{K_{\text{d(analogue)}}\,[\text{ATP or ADP}]_{\text{free}}}$$

it was found that ADP and ATP bind 110 and 170 times more strongly than AMP-PNP, respectively (Figure 6b). As the dissociation constant for AMP-PNP is 2.8×10^{-6} M, this competition study shows that dissociation constants are about 2×10^{-8} M for both ATP and ADP.

Since the analogue binding experiments were carried out with nucleotide-free enzyme while the competition experiments were carried out with normally purified uncoating ATPase, we made certain that ^{14}C-ADP-bound enzyme prepared by incubating nucleotide-free uncoating ATPase with ^{14}C-ADP acts the same way as regular ^{14}C-ADP-bound enzyme. Figure 6 (open triangles) shows that this is indeed the case. Therefore, the relative binding affinities of ADP and AMP-PNP to uncoating ATPase are not affected by prior removal of the strongly bound nucleotide.

Similar competition experiments were performed using

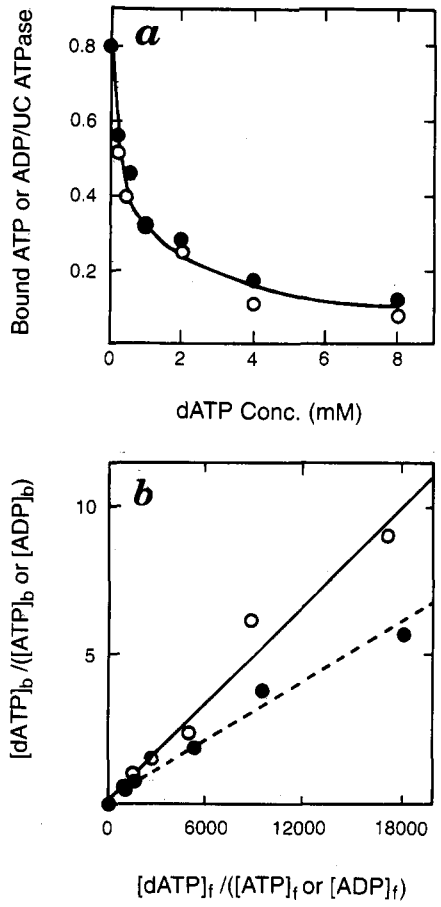

FIGURE 7: Competition binding of dATP with uncoating ATPase-bound ATP or ADP. Increasing amounts of dATP (0–8 mM) were equilibrium-dialyzed with 1 μM uncoating ATPase containing 0.8 μM bound ^{14}C-ATP (●) or ^{14}C-ADP (○). The amounts of bound and free ^{14}C-ATP and ^{14}C-ADP were determined by scintillation counting after 30 h of dialysis. Panel b is a replot of the data in panel a. $[\ ]_b$ and $[\ ]_f$ represent concentrations of the bound and free nucleotide (or analogue), respectively. The solid line is for competition with ADP, and the dashed line is for competition with ATP.

Table 3: Binding of Nucleotide and Analogues to Uncoating ATPase[a]

	K_d (M)
ATP	1.2×10^{-8}
ADP	1.8×10^{-8}
AMP-PNP	2.8×10^{-6}
dATP	2.3×10^{-5}

[a] Dissociation constants (K_ds) of ATP and ADP were averages of the values obtained from competition binding experiments with AMP-PNP and dATP; K_ds of AMP-PNP and dATP were determined by direct binding to the nucleotide-free uncoating ATPase.

dATP to compete with bound ^{14}C-ATP or -ADP (Figure 7). Results show that ADP and ATP bind about 2000–3000-fold more strongly than dATP. As the dissociation constants for dATP is 2.3×10^{-5}, the calculated the dissociation constants for both ATP and ADP are about 10^{-8} M, in good agreement with the values obtained from competition with AMP-PNP. The dissociation constants for the nucleotides and analogues determined in this study are listed in Table 3.

To confirm our finding that ATP and ADP bind with about the same affinity to the uncoating ATPase, direct competition experiments between ATP and ADP were carried out in both the presence and absence of P_i. Table 4 shows that ATP and ADP bind with about equal affinity to the uncoating ATPase in both the presence and absence of P_i, although there is an

Table 4: Competition Binding of ATP and ADP to Uncoating ATPase[a]

	% of total bound nucleotide	
	without P_i	1 mM P_i
ATP	59 ± 1	45 ± 1
ADP	41 ± 1	55 ± 1

[a] Uncoating ATPase (12–15 μM, with 0.8 nucleotide bound/enzyme) was incubated with 1 mM ATP and 1 mM ADP, with or without 1 mM P_i at 25 °C for 60 min. Free nucleotides were then removed by spinning samples through desalting columns (see Materials and Methods). The bound nucleotides were extracted and analyzed by HPLC. Values are averages of three independent determinations.

indication that 1 mM P_i slightly increases the affinity of ADP to the uncoating ATPase.

DISCUSSION

In the present study, we succeeded in preparing nucleotide-free uncoating ATPase by replacing the tightly bound ADP with AMP-PNP and then removing the weakly bound AMP-PNP by dialysis. The preparation of the nucleotide-free uncoating ATPase made it possible to investigate if the bound nucleotide may be required to prevent denaturation of the enzyme. This is particularly important as studies have shown that the ATPase fragment of the uncoating ATPase has a three-dimensional structure similar to that of G-actin (Flaherty et al., 1991; Bork et al., 1992), which rapidly denatures when its bound nucleotide is removed (Korn, 1982). Furthermore, we previously found that during the preparation of the uncoating ATPase, the small fraction of enzyme which lost its bound nucleotide appeared denatured (Gao et al., 1993). However, in fact, we found that for at least 10 days at 4 °C the nucleotide-free uncoating ATPase retains essentially the same enzymatic activities as the normally purified enzyme. Therefore, in contrast to G-actin, it appears that a bound nucleotide is not required for the uncoating ATPase to maintain its native conformation.

Using nucleotide-free uncoating ATPase, we have examined the binding of ATP, ADP, and ATP analogues to the uncoating ATPase. Our previous results indicated that there was only one nucleotide-binding site. The results in the present study further support this view, in contrast to the model of Schmid et al. (1985), which proposed two nucleotide-binding sites, a catalytic and a hydrolytic site, on the uncoating ATPase. They proposed that the catalytic site binds ATP and ATP analogues but not ADP, whereas the hydrolytic site binds ATP and ADP but not ATP analogues. Therefore, their model predicts that AMP-PNP and dATP should not replace bound ADP on the uncoating ATPase, in contrast to the results of our binding studies. Their model also predicts that the addition of ATP analogues should replace only half of the bound ATP, i.e., the ATP at the catalytic site, again in contrast to the results of our binding studies.

One observation that was used as a basis for their model was that, although dATP could not substitute for ATP in supporting the uncoating reaction, it did facilitate the binding of uncoating ATPase to clathrin. However, it is not uncommon for a nucleotide analogue to affect one aspect of an interaction between two proteins without supporting the full reaction. For example, AMP-PNP greatly weakens the binding of actin to myosin but does not support muscle contraction (Eisenberg & Hill, 1985). Another observation that supported the model of Schmid et al. was that a 40-fold excess of dATP over ATP inhibited neither the uncoating reaction nor the accompanying ATP hydrolysis by the enzyme (Braell et al., 1984). However,

our competition studies show that it would take a 1000-fold excess of dATP over ATP to replace half of the ATP bound to the enzyme with dATP, which may explain why they did not see an effect of dATP.

Previous studies on the binding of ATP and ADP to the uncoating ATPase yielded much weaker binding constants than we have obtained in this study. Schmid et al. (1985) obtained dissociation constants of 7×10^{-7} and 1.4×10^{-6} M for ATP and ADP, respectively. More recently, Polleros et al. (1991) reported dissociation constants for ATP and ADP of 9.5×10^{-6} and 1.6×10^{-6} M, respectively, and Huang et al. (1993) reported a dissociation constant of 3×10^{-7} M for ATP. One difficulty with these latter experiments is that it is not clear how much tightly bound ADP was already present on the uncoating ATPase prior to the binding experiments. In this regard, Schmid et al. reported that their enzyme was 68% saturated with ADP prior to their binding study.

It should be noted that, given the relative weak binding affinities reported by these workers, ATP or ADP should have been removed easily by extensive dialysis. On the other hand, using nucleotide-free uncoating ATPase, we have obtained dissociation constants for ATP and ADP at least an order of magnitude stronger than these earlier reported values, which is consistent with our observation that the uncoating ATPase retains its bound ADP following enzyme purification and extensive dialysis. Since we find that ADP is bound to uncoating ATPase as strongly as ATP, our data may also explain why the enzyme binds to ATP–agarose so much more tightly than other cellular ATP-binding proteins; even if ATP is hydrolyzed on the column, the enzyme will still remain tightly bound.

This study has focused on the nucleotide-binding properties of the uncoating ATPase alone. In the present of protein substrates the affinities of ADP and ATP for the uncoating ATPase may be quite different. One suggestion that this may be the case comes from our previous observation (Greene & Eisenberg, 1990) that a 1:4 ratio of ADP to ATP caused about 50% inhibition in the extent of clathrin release form coated vesicles by uncoating ATPase, and a 1:1 ratio caused more than 80% inhibition. Moreover, preliminary studies also indicate that, during steady-state ATP hydrolysis in the presence of clathrin or synthetic peptides, the percentages of bound ADP on the uncoating ATPase increased by 2–4-fold compared to that in the absence of the protein substrates. We are currently investigating whether protein substrates indeed increase the ratio of ADP to ATP on the uncoating ATPase.

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