the FEBS Journal

ADPase activity of recombinantly expressed thermotolerant ATPases may be caused by copurification of adenylate kinase of *Escherichia coli*

Baoyu Chen[1,*], Tatyana A. Sysoeva[2], Saikat Chowdhury[2], Liang Guo[3] and B. Tracy Nixon[2]

1 Integrative Biosciences Graduate Degree Program – Chemical Biology, The Pennsylvania State University, University Park, PA, USA
2 Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA, USA
3 BioCAT, Advanced Photon Source, Argonne National Lab and Illinois Institute of Technology, Chicago, IL, USA

Keywords
AAA+ ATPase; adenylate kinase; ADPase; σ54; thermophilic proteins

Correspondence
B. Tracy Nixon, 406 S, Frear Lab, Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA
Fax: +1 814 863 7024
Tel: +1 814 863 4904
E-mail: btn1@psu.edu

***Present address**
UT Southwestern Medical Center at Dallas, TX, USA

(Received 18 September 2008, revised 24 November 2008, accepted 2 December 2008)

doi:10.1111/j.1742-4658.2008.06825.x

Except for apyrases, ATPases generally target only the γ-phosphate of a nucleotide. Some non-apyrase ATPases from thermophilic microorganisms are reported to hydrolyze ADP as well as ATP, which has been described as a novel property of the ATPases from extreme thermophiles. Here, we describe an apparent ADP hydrolysis by highly purified preparations of the AAA+ ATPase NtrC1 from an extremely thermophilic bacterium, *Aquifex aeolicus*. This activity is actually a combination of the activities of the ATPase and contaminating adenylate kinase (AK) from *Escherichia coli*, which is present at 1/10 000 of the level of the ATPase. AK catalyzes conversion of two molecules of ADP into AMP and ATP, the latter being a substrate for the ATPase. We raise concern that the observed thermotolerance of *E. coli* AK and its copurification with thermostable proteins by commonly used methods may confound studies of enzymes that specifically catalyze hydrolysis of nucleoside diphosphates or triphosphates. For example, contamination with *E. coli* AK may be responsible for reported ADPase activities of the ATPase chaperonins from *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Methanococcus jannaschii* and *Thermoplasma acidophilum*; the ATP/ADP-dependent DNA ligases from *Aeropyrum pernix* K1 and *Staphylothermus marinus*; or the reported ATP-dependent activities of ADP-dependent phosphofructokinase of *P. furiosus*. Purification methods developed to separate NtrC1 ATPase from AK also revealed two distinct forms of the ATPase. One is tightly bound to ADP or GDP and able to bind to Q but not S ion exchange matrixes. The other is nucleotide-free and binds to both Q and S ion exchange matrixes.

ATPases associated with various cellular activities (AAA+ ATPases) form a large family of chaperone-like proteins that use ATP hydrolysis to remodel numerous macromolecular complexes [1]. The NtrC1 protein of *Aquifex aeolicus* is one such ATPase, belonging to the subfamily whose members are called bacterial enhancer binding proteins (EBPs). EBPs use ATP hydrolysis to activate transcription by the σ54-dependent form of RNA polymerase [2]. Although some AAA+ ATPases can operate by hydrolyzing other NTPs or even dNTP and ddNTPs [3,4], they specifically target the phosphodiester bond between β-phosphates and γ-phosphates of the nucleotides. They do not hydrolyze ADP, even though such hydrolysis releases free energy similar to that released by cleavage of the bond to the γ-phosphate. To our knowledge, such high specificity for the

Abbreviations
AAA+ ATPases, ATPases associated with various cellular activities; AK, adenylate kinase; Ap5A, diadenosine pentaphosphate; EBP, enhancer binding protein; Mg-ADP-BeF_x, ATP ground state analog composed of a complex of ADP and magnesium and beryllofluoride ions (*x* denotes uncertain stoichiometry of fluorine atoms); SAXS, small-angle solution X-ray scattering.

γ phosphate bond is also true for all members of the P-loop NTPase superfamily and most other nucleotide-binding proteins.

One well-known exception is apyrase (or NTPDase) of eukaryotic cells [5], which breaks both phosphodiester bonds of a nucleotide, hydrolyzing ATP and ADP to AMP and orthophosphate(s). Also, a novel ADPase activity of ATPases from thermophilic organisms, including four different chaperonins [6] and two DNA ligases [7,8], has been reported. It was hypothesized that using ADP as an energy source instead of ATP in thermophilic organisms may be beneficial because ATP is less stable at high temperatures. Furthermore, there are controversial observations that some ADP-dependent glucokinases and phosphofructokinases in thermophilic archeaons can also use ATP as a phosphoryl transfer donor [9–12].

Here we report an apparent ADPase activity in preparations of the recombinant ATPase domain of the AAA+ ATPase NtrC1 ($NtrC1^C$) from the extremely thermophilic bacterium *A. aeolicus* purified from *Escherichia coli*. Although conversion of ADP to AMP and P_i depends upon intact catalytic activity of the NtrC1 ATPase, we show that it also depends upon the action of 0.01% contamination by *E. coli* adenylate kinase (AK). Apparent catalysis of ADP hydrolysis by $NtrC1^C$ was in fact conversion of two ADP molecules to ATP and AMP by AK followed by hydrolysis of ATP to ADP and P_i by $NtrC1^C$.

Proteins that tolerate high temperatures, such as NtrC1, are popular subjects of structural studies. They are often purified by a similar strategy, which takes advantage of their thermostability. Our observation that AK of *E. coli* survives, and is indeed copurified, by such a method raises a concern about possible contamination of other protein preparations with AK. The presence of tiny amounts of this contaminant could confound studies of any nucleotide-hydrolyzing enzymes from thermophilic organisms. Chromatographic methods developed to remove the AK contamination revealed a heterogeneity in the ATPase preparation, yielding two subfractions. The resulting, more homogeneous preparation of an $NtrC1^C$ variant bearing a single amino acid substitution has led to diffracting crystals (to be described elsewhere).

Results

Highly pure $NtrC1^C$ preparation catalyzes hydrolysis of ADP

$NtrC1^C$ purified by heat denaturation and anion exchange chromatography was highly pure (> 99%) as judged from SDS/PAGE (Fig. 1A) and gel filtration (not shown). However, addition of 5 mM ATP to the protein produced 8–10 mM free P_i (data not shown), suggesting further hydrolysis of ADP. This was confirmed by ion exchange chromatography permitting quantification of fluxes in the concentrations of ATP, ADP and AMP, beginning with an initial concentration of 10 mM ATP (Fig. 1B).

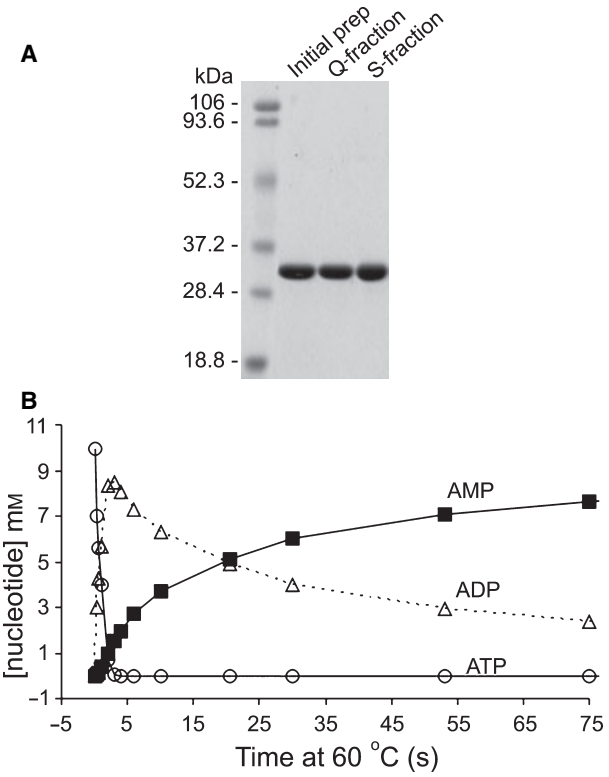

Fig. 1. Apparent ADP hydrolysis by highly pure $NtrC1^C$. (A) SDS/PAGE of purified $NtrC1^C$ (initial preparation) and subsequent Q-fraction and S-fraction. Ten micrograms of each protein was loaded. (B) Products of ATP hydrolysis by 2 mg·mL^{-1} $NtrC1^C$ Q-fraction at 60 °C, as quantified by anion exchange chromatography.

The apparent ADPase activity displays high thermal stability, requires an ATPase-competent $NtrC1^C$ protein, and is associated with structural changes in $NtrC1^C$

To determine how the apparent ADPase activity is associated with the NtrC1 ATPase, we first examined the rate of ADP turnover by $NtrC1^C$ preparations that had been pre-equilibrated to different temperatures. The optimal temperature for ADP hydrolysis was 60 °C, which is somewhat lower than the 82 °C optimum seen for ATP hydrolysis by $NtrC1^C$

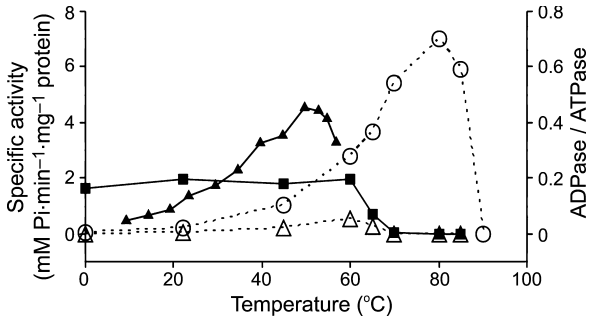

Fig. 2. Thermostability of ATP and apparent ADP hydrolysis. The initial rate of P_i release was measured upon addition of 5 mM ATP (open circles) or ADP (open triangles) to the NtrC1^C Q-fraction (2 mg·mL^{-1}) incubated with 5 mM $MgCl_2$ at the desired temperatures. The ratio of ADPase activity to ATPase activity is shown as filled rectangles. Data for AK of *E. coli* (filled triangles) were taken from [14] and plotted using arbitrary units to show its optimal temperature for activity.

(Fig. 2). The ratio of ADP turnover to ATP turnover remained constant and close to 20% over a wide range of temperatures, from 0 °C to about 60 °C. At higher temperatures, ADP turnover started to decrease, and it ceased above 70 °C. After thermal inactivation by incubation at 80 °C for 30 min, the ADPase activity was completely recovered as soon as it could be measured upon cooling to 60 °C (not shown). Studies of several NtrC1^C single amino acid substitution variants showed that both ADPase and ATPase activities require the same active site residues (Table 1).

Using small-angle solution X-ray scattering (SAXS) and size exclusion chromatography, we previously established that a large conformational change in NtrC1^C is stabilized upon binding of ADP-BeF$_x$, a ground state analog of ATP. This conformational change allows NtrC1^C to interact with σ54 [13]. Here, we used the same methods to determine whether the apparent hydrolysis of ADP is associated with structural changes in NtrC1 ATPase. Substitution of the conserved glutamate of the Walker B motif (Glu239) by alanine abolished ATP hydrolysis, but the altered protein still underwent a conformational change similar to the wild-type when ATP was added, and it could then bind to σ54. Likewise, this substitution abolished hydrolysis of ADP, but addition of ADP caused a conformational change similar to that seen upon the addition of ATP and it promoted binding to σ54 (Fig. 3A,B).

Table 1. ATPase and ADPase activities of NtrC1^C variants with single amino acid substitutions. The location of each substitution shows the structural role of the residue in the function of the ATPase [2,21]. '+' and '–' represent the presence or absence of specified activities, respectively. For a given ATP-hydrolyzing mutant protein, the rate of ADP turnover was typically 10–20% of ATP hydrolysis.

Substitution	Location	ATPase	ADPase
Wild-type		+	+
T217A	GAFTGA loop	+	+
N280A	Sensor 1	+	+
K173A	Walker A	+	+
E239A	Walker B	–	–
R299A	Arg-finger	–	–
R357A	Sensor 2	–	–

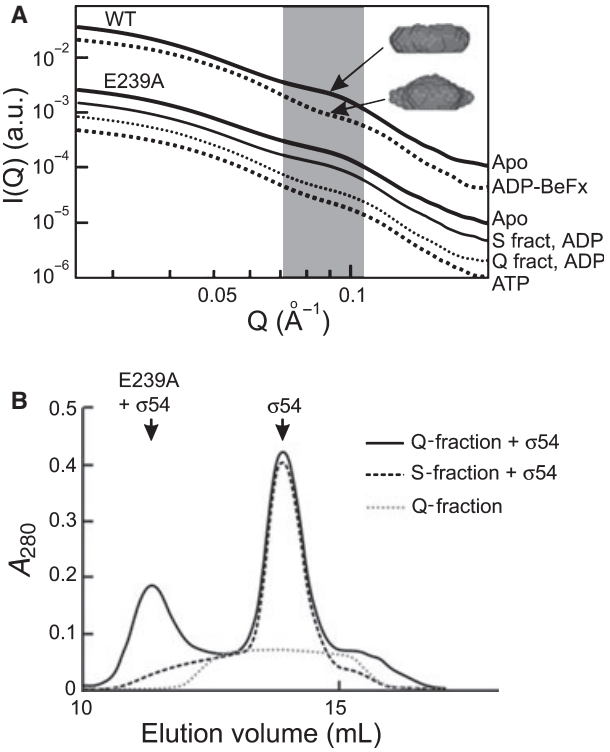

Fig. 3. Structural and functional effects of turning over ADP. (A) Small-angle solution scattering from 10 mg·mL^{-1} NtrC1^C wild-type (WT) and the E239A variant in the presence of 5 mM specified nucleotides or analogs (Q-fraction and S-fractions are specifically noted for E239A; otherwise, similar results were seen for the initial preparation, Q-fraction and S-fraction). The shaded area contains signatures of relevant conformational changes, with the 'bending-up' and 'bending-down' trajectories (arrows) suggesting either a flattened, non-σ54-binding, or a pore-region extruded, σ54-binding conformation, respectively (shapes illustrated as space-filled models) [13]. (B) Gel filtration chromatography profiles of NtrC1$^C_{E239A}$ in the presence of 2 mM ADP, monitoring complexation of the ATPase with σ54.

Cation exchange chromatography separates the NtrC1^C preparation into two fractions, one of which lost the apparent ADPase activity and the other of which was enriched for ADPase activity

Despite the fact that the above results were consistent with NtrC1 ATPase being able to hydrolyze ADP, the ADPase activity could not be visualized on native gels by enzymatic staining (Fig. 4A). This suggested the presence of another factor in the apparent ADP hydrolysis reaction. As the protein was purified by anion exchange chromatography, we tried cation exchange for further purification. The protein fractionated into two parts (Fig. 4B). Both the S-fraction (bound to the SP HP column) and Q-fraction (in the flow-though) had similar ATPase activities, and MS showed similar molecular masses for the respective proteins (S-fraction, 30 537.5 ± 6 Da; Q-fraction, 30 537.0 ± 6 Da). However, the S-fraction lost apparent ADPase activity and the Q-fraction had an elevated apparent ADPase activity. [Note that this cation exchange chromatography was performed at room temperature (22 °C); when it was performed at 4 °C, the resulting S-fraction did not lose the apparent ADPase activity (not shown).] Chromatography of the E239A variant also yielded a Q-fraction and an S-fraction. Only the Q-fraction showed conformational change and binding to σ54 when presented with ADP. These results suggest that at room temperature, a separate factor needed for apparent ADP hydrolysis activity does not bind to the S-column, but that the column does nonetheless bind to a subfraction of NtrC1 ATPase.

The Q-fraction has tightly bound nucleotides, but this does not cause the apparent ADPase activity

We searched for differences between the Q-fraction and S-fraction that could shed light on the source of the apparent ADPase activity. No differences were observed by staining SDS/PAGE (Fig. 1A) or 2D electrophoresis gels with Coomassie Blue, or by gel filtration chromatography and *in vitro* transcription assay (not shown). A major difference was that the Q-fraction but not the S-fraction of NtrC1^C retained nucleotides (ADP > GDP > >; AMP > GMP, data not shown) that were released when heated in the presence of 8 M urea at 70 °C (Fig. 5). Under native conditions, dialysis of the Q-fraction against four changes of buffer containing 5 mM EDTA for 4 days at 22 °C failed to release these 'tightly bound' nucleotides (not shown). However, they could be released by repeated dilution and spin-concentration of the Q-fraction. As the ATPase functions as a ring-shaped heptamer that

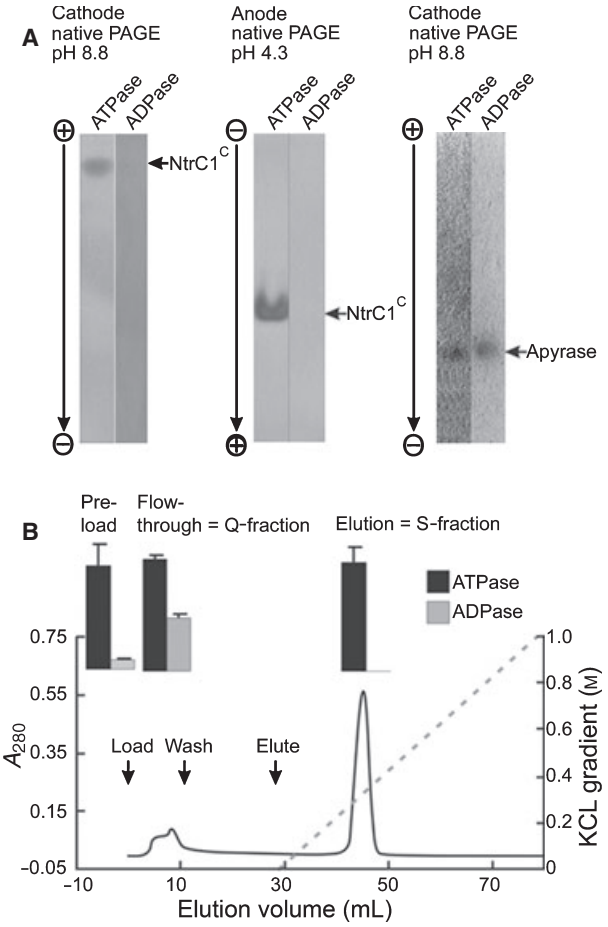

Fig. 4. Apparent ADPase activity is separable from NtrC1^C ATPase activity. (A) Native gels showing *in situ* enzymatic staining for ATP or ADP hydrolysis activity of the Q-fraction of NtrC1^C. Arrows indicate positions of the NtrC1^C and apyrase proteins located by Coomassie Blue staining (not shown). Similar enzymatic staining for an apyrase (Sigma) is shown in parallel as a positive control for this method in detecting P_i released from ATP or ADP hydrolysis. Both regular cathode native PAGE for acidic proteins and anode native PAGE for basic proteins were performed to ensure that the unidentified ADPase-stimulating factor migrated into the gel. Electrode directions are shown by vertical arrows, with ⊕ representing the anode and ⊖ the cathode. (B) Further purification of NtrC1^C with a 5 mL SP HP cation exchange column at 22 °C. The flow-through is the Q-fraction and the elution is the S-fraction. The relative rate of ATP or ADP turnover is shown as bars aligned to corresponding fractions of the chromatography profile.

is unstable below a concentration of a few mg·mL^{-1} [13], this manipulation presumably cycled the protein through disassembled and assembled states, releasing the nucleotides. Release of the bound nucleotides from the Q-fraction did not affect the ATPase or apparent ADPase activity, or enable the Q-fraction to bind to the HP SP column (not shown). The S-fraction of protein remained free of 'tightly bound' nucleotide after it

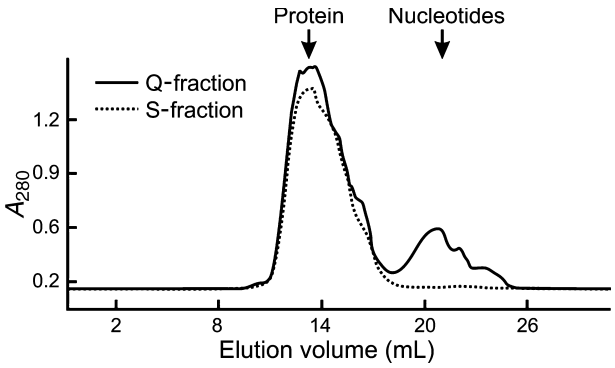

Fig. 5. The Q-fraction of $NtrC1^{C}$ contained tightly bound nucleotides. After denaturation in 8 M urea at 70 °C, 50 mg of the $NtrC1^{C}$ Q-fraction or S-fraction were applied to a 24 mL Superdex 200 column with 8 M urea included in the elution buffer.

was incubated at various incubation temperatures and for various times with numerous combinations of nucleotides in the presence or absence of Mg^{2+}.

Contamination of $NtrC1^{C}$ ATPase with AK causes the apparent ADP hydrolysis

A different form of the NtrC1 ATPase domain that has a C-terminal His6 tag, $NtrC1^{Cshort\text{-}his6}$, behaved similarly to $NtrC1^{C}$ in purification and functional assays (not shown) – however, it could be further purified by nickel affinity chromatography, due to the His6 tag. The eluate from the nickel resin retained 98% of the applied $NtrC1^{Cshort\text{-}his6}$, and it was free of apparent ADP hydrolysis activity. Fractionation of the flow-through by gel filtration showed that the apparent ADPase activity coeluted with the remaining $NtrC1^{Cshort\text{-}his6}$ (not shown). Our previous work established that NtrC1 ATPase oligomerizes from a mixture of monomers and dimers into a heptamer ring in the presence of ADP-BeF_x, resulting in a dramatic shift of its elution peak in gel filtration. To test whether the ADPase-causing factor still coeluted with the $NtrC1^{Cshort\text{-}his6}$ when the ATPase oligomerized, we fractionated the flow-through by gel filtration in the presence of ADP-BeF_x (Fig. 6A). The fraction of oligomerized ATPase lost the apparent ADP hydrolysis activity. Examination of all the gel filtration fractions identified an 'ADPase-stimulating' peak that itself could not hydrolyze ATP or ADP, but when added to several ADPase-free ATPase preparations caused the latter to appear to hydrolyze ADP (not shown). The tested ATPases included the S-fraction of $NtrC1^{C}$ ATPase, two other EBPs (PspF and NtrC), the more distantly related ClpX ATPase, and the transcription terminator Rho. Hence, the apparent ADP hydrolysis was clearly stimulated by a factor that was copurified in the $NtrC1^{Cshort\text{-}his6}$ ATPase Q-fraction. The Q-fractions of purified ATPase-deficient $NtrC1^{C}$ variants listed in Table 1 also contained such a factor. When tested separately, these Q-fractions did not stimulate ADP turnover; however, apparent hydrolysis was observed when these Q-fractions were mixed with the S-fraction of the wild-type $NtrC1^{C}$ (itself competent to hydrolyze ATP but devoid of ADP hydrolysis activity).

Further fractionation of the 'ADPase-stimulating' fraction from the above by MonoQ chromatography and analysis by MS (MALDI and, separately, LC/MS) identified AK as a contaminant that could cause the apparent ADP hydrolysis activity when coupled with the ATPase activity of NtrC1 (Fig. 6B; MALDI and LC/MS identified masses matching tryptic fragments of AK of *E. coli* that covered 72 or 25.4% of the entire polypeptide, respectively). Other identified contaminants include YjgF and the ω-subunit of RNA polymerase. The presence of AK was confirmed by the ability of the fraction to catalyze the reaction 2ADP ⇔ ATP + AMP (Fig. 6C). This reaction and the apparent hydrolysis of ADP by the Q-fraction of $NtrC1^{C}$ were both strongly inhibited by the specific AK inhibitor diadenosine pentaphosphate (Ap5A) (Fig. 6D). Addition of purified recombinant AK to ADPase-free NtrC1 ATPase caused similar apparent ADPase activity (not shown). The total yield of AK from 30 g of *E. coli* cell paste was 50 μg, 10 000 times less than the yield of NtrC1 ATPase. The presence of trace quantities of AK thus caused the apparent ADP hydrolysis, by generating ATP to be used by the ATPase.

Discussion

It is widely known that proteins cannot be purified from biological samples to 100% purity, even though many published studies describe their samples as 'purified to homogeneity'. For at least three reasons, potential contamination can easily be overlooked in the purification of recombinant proteins of thermophilic organisms that are expressed in *E. coli*. First, the purification involves heating at 60–80 °C. Most *E. coli* proteins irreversibly denature and aggregate at such temperatures. The identities of the *E. coli* proteins that do survive the heat treatment are not known, and they are thus largely overlooked. Second, the activity assays for thermophilic proteins are usually performed at relatively high temperatures, again presumed to inactivate most *E. coli* proteins. Third, these thermophilic proteins are usually expressed at high levels, so preparations of them contain such low levels of impu-

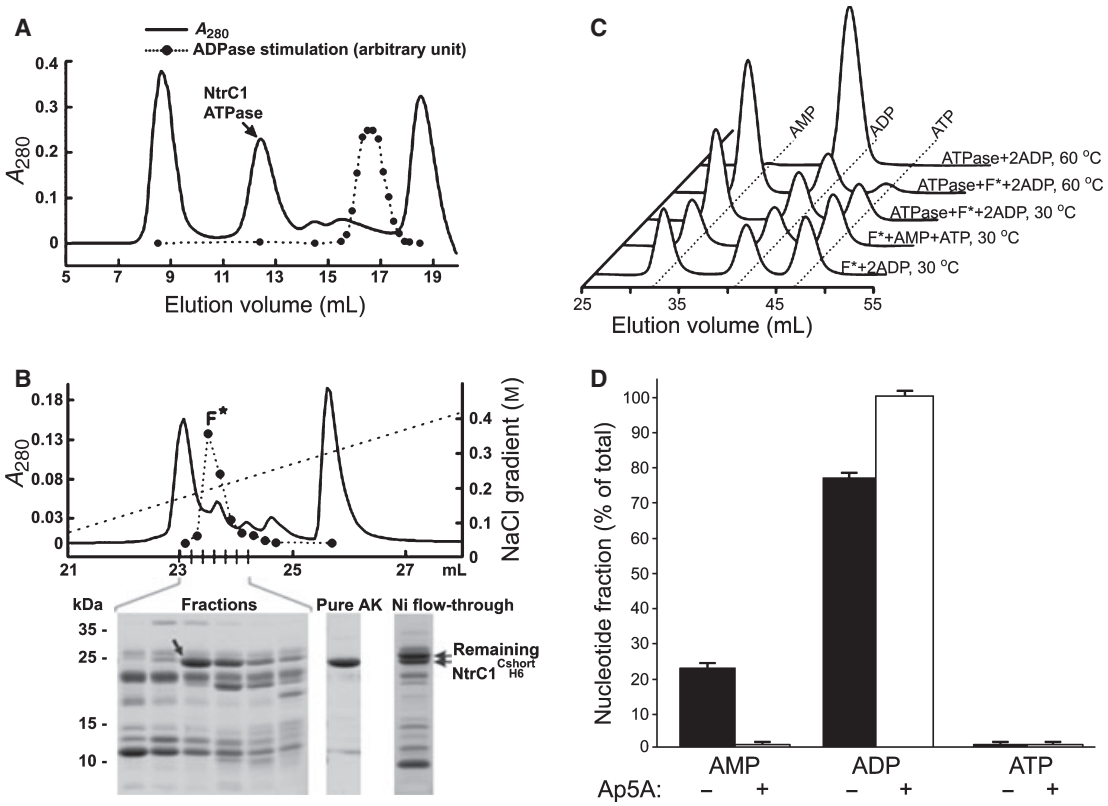

Fig. 6. Identification of AK contamination. (A) Gel filtration profile (solid line) of the flow-through from a nickel column of NtrC1$^{Cshort\text{-}his6}$ in the presence of 1 mM ADP-BeF_x. Each 200 μL fraction was diluted 100-fold before being mixed 1 : 1 with 1.5 mg·mL^{-1} ADPase-free NtrC1$^{Cshort\text{-}his6}$ to measure apparent ADP hydrolysis. The metal fluoride ATP analog stabilized assembly of the residual NtrC1^{C} ATPase into its ring form (eluting at 12.8 mL; arrow) and clearly separated it from material that stimulated apparent ADP hydrolysis (dashed line, peak at ~ 16.8 mL). (B) Further fractionation of the pooled 'ADPase-stimulating' fractions in (A) (16–17.5 mL) by MonoQ chromatography. Stimulation of apparent ADPase activity was measured as in (A), with the peak fraction denoted as F*. SDS/PAGE analysis of the first six fractions shows that the stimulating activity coincides with enrichment of *E. coli* AK (arrow; purified recombinant AK is shown as a reference). The flow-through from the nickel column shows overlap between residual NtrC1$^{Cshort\text{-}his6}$ and AK, plus all other impurities. (C) Interconversion of ADP and ATP/AMP by fraction F*. Solutions containing $MgCl_2$ and ADPase-free NtrC1$^{Cshort\text{-}his6}$ or fraction F* were equilibrated at the given temperatures and mixed with the indicated nucleotides (5 mM ATP or AMP, 10 mM ADP). After 5 min of incubation, 100 μL of each reaction was applied to a 5 mL Q HP column. Bound nucleotides were eluted with a 120 mL gradient of 0–1 M KCl, but only the 83–333 mM range is shown. Labels and dotted lines indicate elution condition for standards of AMP, ADP and ATP. (D) Ap5A blocks conversion of ADP to ATP and AMP. The above reaction with 10 mM ATP was repeated in the absence or presence of 10 mM Ap5A. Data for a single time point show that the inhibitor does not block ATP hydrolysis, but does prevent production of AMP.

rities that the latter go unnoticed. Finally, even within a 'pure' population of protein molecules, differences in ligand occupancies or conformational states can generate diversity. Here we report an example where these issues turn out to have important, confounding impacts on studies of an AAA+ ATPase.

We see that a common method for purifying thermophilic proteins (by ion exchange and size exclusion chromatography of cleared, heated extracts) yields a few hundredths of a per cent of residual *E. coli* proteins, one of which is AK. This enzyme is a strong catalyst, stimulating the reaction 2ADP ⇔ ATP + AMP with a maximum k_{cat} of 1400 s^{-1} at 50 °C (Fig. 2 and [14]). Given its high catalytic activity and K_m for ADP of 90 μM [15], nanomolar concentrations of AK are sufficient to generate ATP from ADP to fuel the NtrC1^{C} ATPase and cause the effect of apparent hydrolysis of ADP by NtrC1^{C}. Similar contamination by AK may be relevant to other studies of thermophilic proteins. ADPase activities were reported for thermophilic chaperonins (*Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Methanococcus jannaschii*, and *Thermoplasma acidophilum*) and a DNA ligase (*Aeropyrum pernix* K1 and *Staphylothermus marinus*). These proteins were purified in ways similar to that reported here [6–8]. Although the chaperonins exhibited an ADPase activity at 80 °C, at which the *E. coli* AK is inactive, it is possible that the chaperonin protected

AK from thermal inactivation just as it protected malate dehydrogenase from thermal unfolding for 60 min at 80 °C [6]. The observations of these ADPase activities were novel and unexpected, and were discussed in the context of possible metabolic differences between mesophilic and thermophilic organisms. It is important to establish that the reported ADPase activities are indeed intrinsic for the enzymes and were not caused by the interconversion of adenine nucleotides catalyzed by AK. AK is also able to produce ADP from ATP and AMP, the latter of which is often present (or slowly generated) at low levels in most ATP preparations. The reported ATP dependence of the ADP-dependent phosphofructokinase from *P. furiosus* may thus also be caused by contamination with AK [9]. Once ADP hydrolysis begins, fresh AMP would be produced to feed the coupled catalysis.

It is also clear from this study that prior preparations of AAA+ $NtrC1^{C}$ ATPase domain were not homogeneous. An uncharacterized conformational difference must exist that causes a 2 : 1 partitioning of Q-column binding material into forms that bind or fail to bind to an S-column. Also, mixed purine nucleotides are tightly bound to the non-S-binding fraction, but this does not explain the partitioning among the ion exchange resins, because the nucleotides can be removed by cycles of dilution and reconcentration without affecting the charge-based partitioning. It remains to be determined whether the heterogeneity revealed here has significance for how the NtrC1 AAA+ ATPase functions. We have noted no distinction between the SAXS signals for the Q-fraction and S-fraction of $NtrC1^{C}$ in the apo state or when provided with different nucleotides or nucleotide analogs [13] (B. Chen and B. T. Nixon, unpublished observations). This suggests that the tightly bound nucleotide diphosphates participate in (or at least do not interfere with) intersubunit communication that occurs in response to subsequently bound nucleotides or metal fluorides. We have been able to generate diffracting crystals of the S-fraction of the E239A substitution variant bound to Mg^{2+}-ATP (to be described elsewhere). Examples of nucleotides being tightly bound to AAA+ ATPases have been reported [16], as have sites of differential affinity for nucleotides [17–19], but how these are integrated into ATPase function is not yet clear [18–20].

Experimental procedures

Protein preparation

Two NtrC1 ATPase constructs from *A. aeolicus* (GI #2983588) were used: $NtrC1^{C}$ (residues 121–387) [21] and $NtrC1^{Cshort\text{-}his6}$ (residues 137–387 plus a C-terminal His6 tag). Both proteins were overexpressed from pET21 vectors in Rosetta *E. coli* cells (Novagen). Typically, 15–20 g of frozen cell paste was resuspended in chilled buffer A [20 mM Tris, 5% (w/v) glycerol, pH 8.0] plus 500 mM KCl, 5 mM EDTA and EDTA-free complete protease inhibitor (Roche Diagnostics Corporation, Indianapolis, IN, USA), and disrupted by sonication as previously described [21]. Lysate was cleared by centrifugation at 100 000 ***g*** for 45 min at 4 °C, incubated at 70 °C for 30 min, and recleared by centrifugation as before. Supernatant was applied to a Sephacryl S-200 HR 26/60 column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) equilibrated with buffer B (20 mM Tris, 5 mM EDTA, pH 8), giving fractions containing NtrC1 ATPase that were applied to a 70 mL Q Sepharose Fast Flow column or 5 mL HiTrap Q HP column (GE Healthcare) and eluted with a salt gradient (0.05–1 M KCl added to buffer A, 5 °C). Additional purification of protein diluted to 50 mM final KCl concentration was achieved at 22 °C, using a 5 mL cation exchange HiTrap SP HP column (GE HealthCare), which split the protein into two portions: two-thirds bound to and eluted from the S-column with a similar salt gradient (named the S-fraction), and one-third failed to bind (named the Q-fraction). Also at 22 °C, the Q-fraction of $NtrC1^{Cshort\text{-}his6}$ was bound to and eluted from a 5 mL nickel affinity column (Sigma) using imidazole (500 mM), and the flow-through was concentrated by filter-centrifugation at 3000 ***g*** for three minute intervals (Amicon Ultra-15 10K; Millipore). The concentrated flow-through was supplemented with 1 mM Mg-ADP-BeF_x), and fractionated on a Superdex 200 10/30 size exclusion column (GE Healthcare) equilibrated with buffer A containing Mg-ADP-BeF_x (1 mM) to promote oligomerization of NtrC1. This caused it to elute at 12.5 mL, well ahead of fractions peaking at 16.7 mL, which enabled the S-fraction of $NtrC1^{Cshort\text{-}his6}$ (ADPase-free) to 'hydrolyze' ADP. The pooled active fractions were desalted into low-salt buffer (20 mM Tris, 5% glycerol, pH 8.0) and further fractionated on a MonoQ HR 5/5 column using a gradient of KCl.

σ54 with His6 tag from *Klebsiella pneumoniae* was purified as previously described [22]. SDS/PAGE, native PAGE [13,23], IEF and analytical gel filtration chromatography were used to determine the protein composition of various fractions.

Functional and structural assays

Nucleotide hydrolysis was measured by determining the concentration of released P_i using a heteropolyacid system with slight modifications [24]. NtrC1 ATPase was pre-equilibrated with 5 mM $MgCl_2$ in buffer A at the desired temperature (typically 60 °C) for 3 min before 5 mM ADP or ATP was added to start the reaction. At each time point, 5 μL of the reaction mixture was aliquoted into 270 μL of

0.88 M HNO_3 to quench the reaction. Finally, 225 μL of color-developing solution (44.4 mM bismuth nitrate, 0.6 M HNO_3, 31.1 mM ammonium molybdate, 0.11% ascorbic acid, freshly mixed from stock solutions) was added, and $A_{700\ nm}$ was measured after 3 min. Alternatively, free nucleotides were separated from protein by centrifugation at 10 000 ***g*** for 20 s through Nanosep 3K membranes (Pall Life Sciences Corp., New York, NY, USA). Recovered nucleotides were identified and quantified by anion exchange chromatography and UV spectroscopy, using known nucleotides as standards (Sigma-Aldrich Corp., St Louis, MO, USA) [13]. Nucleotides tightly bound to protein in the Q-fraction were released by either repeated dilution and concentration (Amicon Ultra-15 10K; Millipore) or incubation in buffer A supplemented with 8 M urea at 70 °C for 30 min followed by gel filtration on a Superdex 200 10/30 column equilibrated with the urea buffer. Enzymatic staining on native gels was performed by trapping the P_i released from ADP or ATP hydrolysis at 60 °C as previously described [25]. To track the activity of AK during its enrichment (and prior to its identification), the fractions were diluted and mixed with the S-fraction of $NtrC1^{Cshort\text{-}his6}$ (ADPase-free) to measure apparent ADP hydrolysis. The single-round *in vitro* transcription assay, SAXS and gel filtration experiment to measure the complexation of $NtrC1^{C}$ with σ54 were performed as previously described [13,26].

Acknowledgements

This work was funded by NIH grant GM069937 to B. T. Nixon. Use of the Advanced Photon Source was supported by the DOE, and the BioCAT is an NIH-supported Research Center. EIF and MS were performed by Hassan Koc and Emine Koc (Penn State) and by the Proteomics and Mass Spectrometry Facility of the Huck Institutes of the Life Sciences at Penn State. AK, ClpX ATPase and Rho were generous gifts from H. Yang (Chemistry, University of California, Berkeley, CA, USA), R. T. Sauer (Biology, Massachusetts Institute of Technology, MA, USA), P. Babitzke (Biochemistry and Molecular Biology, The Pennsylvania State University, PA, USA), respectively.

References

1 Erzberger JP & Berger JM (2006) Evolutionary relationships and structural mechanisms of AAA+ proteins. *Ann Rev Biophys Biomol Struct* **35**, 93–114.
2 Rappas M, Bose D & Zhang X (2007) Bacterial enhancer-binding proteins: unlocking sigma54-dependent gene transcription. *Curr Opin Struct Biol* **17**, 110–116.
3 Berger B, Wilson DB, Wolf E, Tonchev T, Milla M & Kim PS (1995) Predicting coiled coils by use of pairwise residue correlations. *Proc Natl Acad Sci USA* **92**, 8259–8263.
4 Lee JH, Scholl D, Nixon BT & Hoover TR (1994) Constitutive ATP hydrolysis and transcription activation by a stable, truncated form of *Rhizobium meliloti* DCTD, a sigma 54-dependent transcriptional activator. *J Biol Chem* **269**, 20401–20409.
5 Komoszynski M & Wojtczak A (1996) Apyrases (ATP diphosphohydrolases, EC 3.6.1.5): function and relationship to ATPases. *Biochim Biophys Acta* **1310**, 233–241.
6 Hongo K, Hirai H, Uemura C, Ono S, Tsunemi J, Higurashi T, Mizobata T & Kawata Y (2006) A novel ATP/ADP hydrolysis activity of hyperthermostable group II chaperonin in the presence of cobalt or manganese ion. *FEBS Lett* **580**, 34–40.
7 Jeon SI & Ishikawa K (2003) A novel ADP-dependent DNA ligase from *Aeropyrum pernix* K1. *FEBS Lett* **55**, 69–73.
8 Seo MS, Kim YJ, Choi JJ, Lee MS, Kim JH, Lee JH & Kwon ST (2007) Cloning and expression of a DNA ligase from the hyperthermophilic archaeon *Staphylothermus marinus* and properties of the enzyme. *J Biotechnol* **128**, 519–530.
9 Kengen SW, Tuininga JE, de Bok FA, Stams AJ & de Vos WM (1995) Purification and characterization of a novel ADP-dependent glucokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **270**, 30453–30457.
10 Ronimus RS, Koning J & Morgan HW (1999) Purification and characterization of an ADP-dependent phosphofructokinase from *Thermococcus zilligii*. *Extremophiles* **3**, 121–129.
11 Verhees CH, Tuininga JE, Kengen SW, Stams AJ, van der Oost J & de Vos WM (2001) ADP-dependent phosphofructokinases in mesophilic and thermophilic methanogenic archaea. *J Bacteriol* **183**, 7145–7153.
12 Verhees CH, Koot DG, Ettema TJ, Dijkema C, de Vos WM & van der Oost J (2002) Biochemical adaptations of two sugar kinases from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochem J* **366**, 121–127.
13 Chen B, Doucleff M, Wemmer DE, De Carlo S, Huang HC, Nogales E, Hoover TR, Kondrashkina E, Guo L & Nixon BT (2007) ATP ground- and transition states of bacterial enhancer binding AAA+ ATPases support complex formation with their target protein, σ54. *Structure* **15**, 429–440.
14 Wolf-Watz M, Thai V, Henzler-Wildman K, Hadjipavlou G, Eisenmesser EZ & Kern D (2004) Linkage between dynamics and catalysis in a thermophilic–mesophilic enzyme pair. *Nat Struct Mol Biol* **11**, 945–949.
15 Monnot M, Gilles A-M, Girons IS, Michelson S, Barzu O & Fermandjian S (1987) Circular dichroism investigation of *Escherichia coli* adenylate kinase. *J Biol Chem* **262**, 2502–2506.
16 Zhang X, Shaw A, Bates PA, Newman RH, Gowen B, Orlova E, Gorman MA, Kondo H, Dokurno P, Lally J

et al. (2000) Structure of the AAA ATPase p97. *Mol Cell* **6**, 1473–1484.
17 DeLaBarre B & Brunger AT (2003) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nat Struct Biol* **10**, 856–863.
18 Hersch GL, Burton RE, Bolon DN, Baker TA & Sauer RT (2005) Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine. *Cell* **121**, 1017–1027.
19 Schumacher J, Joly N, Claeys-Bouuaert IL, Azia SA, Rappas M, Zhang X & Buck M (2008) Mechanisms of homotropic control to coordinate hydrolysis in a hexameric AAA+ ring ATPase. *J Mol Biol* **381**, 1–12.
20 Martin A, Baker TA & Sauer RT (2007) Distinct static and dynamic interactions control ATPase–peptidase communication in a AAA+ protease. *Mol Cell* **27**, 41–52.
21 Lee SY, DeLaTorre A, Yan D, Kustu S, Nixon BT & Wemmer DE (2003) Regulation of the transcriptional activator NtrC1: structural studies of the regulatory and AAA+ ATPase domains. *Genes Dev* **17**, 2552–2563.
22 Rappas M, Schumacher J, Beuron F, Niwa H, Bordes P, Wigneshweraraj SR, Keetch CA, Robinson CV, Buck M & Zhang X (2005) Structural insights into the activity of enhancer-binding proteins. *Science* **307**, 1972–1975.
23 Reisfeld RA, Lewis UJ & Williams DE (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195**, 281–283.
24 Chen B, Guo Q, Guo Z & Wang X (2003) An improved activity assay method for arginine kinase based on a ternary heteropolyacid system. *Tsinghua Sci Technol* **8**, 422–427.
25 Zlotnick GW & Gottlieb M (1986) A sensitive staining technique for the detection of phosphohydrolase activities after polyacrylamide gel electrophoresis. *Anal Biochem* **153**, 121–125.
26 Xu H, Gu B, Nixon BT & Hoover TR (2004) Purification and characterization of the AAA+ domain of *Sinorhizobium meliloti* DctD, a sigma54-dependent transcriptional activator. *J Bacteriol* **186**, 3499–3507.