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Determination of NTPase activities from measurement of true inorganic phosphate in the presence of labile phosphate compounds

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Abstract

One of the most common assays for NTPase activity entails the quantification of inorganic phosphate (P_i) as a colored phosphomolybdate complex at low pH. While this assay is very sensitive, it is not selective for P_i in the presence of labile organic phosphate compounds (OPCs). Since NTPase activity assays typically require a large excess of OPCs, such as nucleotides, selectivity for P_i in the presence of OPCs is often critical in evaluating enzyme activity. Here we present an improved method for the measurement of enzymatic nucleotide hydrolysis as P_i released, which achieves selectivity for P_i in the presence of OPCs while also avoiding the costs and hazards inherent in other methods for measuring nucleotide hydrolysis. We apply this method to the measurement of ATP hydrolysis by nitrogenase and GTP hydrolysis by elongation factor G (EF-G) in order to demonstrate the broad applicability of our method for the determination of nucleotide hydrolysis in the presence of interfering OPCs.

Keywords

nitrogenase; EF-G; ribosome; inorganic phosphate; nucleotide hydrolysis; phosphomolybdate

INTRODUCTION

Throughout biology, nucleoside triphosphate (NTP: ATP or GTP) hydrolysis is used to drive cellular processes such as chemical transformations, protein motion, and signaling [1–3]. A thorough mechanistic understanding of such ATP/GTP-driven processes requires a reliable means to quantify nucleotide hydrolysis under dynamic conditions in complex, multi-component media. Many assays have been developed to measure ATP/GTP hydrolysis, which focus on monitoring the amount of a) ADP/GDP formed [4,5] or b) inorganic phosphate (P_i) released [6,7] during the course of a reaction. In the case of the former, assays typically require separation of nucleoside diphosphate species formed from unused

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nucleoside triphosphate species by high-performance liquid chromatography (HPLC) [5]. In the case of the latter, P_i can be reacted with molybdic acid to yield a colored product, [6] or radioactive $^{32}P_i$ can be measured with a phosphorimager [4]. However, these assays often measure total phosphate content of the reaction mixture rather than true P_i released from enzymatic nucleotide hydrolysis, which can lead to substantial error in measurements of ATP/GTP hydrolysis activities [6,7]. Thus, there is an outstanding need to develop analytical methods for quantifying nucleotide hydrolysis that achieve the precision of chromatographic methods while maintaining the simplicity of colorimetric methods and are compatible with the multi-component composition of complex biochemical reactions.

Our interest in developing an improved assay for nucleotide hydrolysis is based on our work with nitrogenase [8,9], which catalyzes such a complex, ATP-driven biochemical reaction, namely the reduction of atmospheric N_2 into NH_3 [10]:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad \text{(Equation 1)}$$

Nitrogenase consists of two component proteins. The catalytic component of nitrogenase, MoFe-protein (MoFeP), is an $\alpha_2\beta_2$ heterotetramer that contains the site of N_2 binding and reduction, the so-called FeMo-cofactor [11]. The other component, Fe-protein (FeP), is a homodimeric ATPase that couples the hydrolysis of two ATP molecules to the transfer of an electron (e^-) to MoFeP. After each interprotein electron transfer (ET) event, oxidized ADP-bound FeP dissociates from MoFeP [12], followed by ADP/ATP exchange and re-reduction of FeP for the next cycle. Per Equation 1, this association/dissociation cycle must occur eight times for every N_2 reduced. It is important to note that both ATP hydrolysis by FeP and catalysis by MoFeP require the presence of both protein components: FeP by itself has no intrinsic ATPase activity [13] and MoFeP by itself cannot reduce N_2 [14]. ADP is a strong inhibitor of nitrogenase activity, not only because it binds more tightly to FeP than ATP, but also because ADP-bound FeP can compete with ATP-bound FeP for interacting productively with MoFeP [15]. Thus, standard nitrogenase activity assays require an ATP-regeneration system consisting of phosphocreatine (PC) and creatine kinase to continuously convert ADP to ATP and sustain enzymatic turnover [14]. Creatine kinase catalyzes the transfer of the phosphate group of PC to ADP to regenerate ATP and also produce creatine. The resulting mixture of multiple phosphate-containing species and their dynamic interconversions renders the quantification of ATP hydrolysis challenging, as discussed below.

While enzymatic ATP hydrolysis is routinely measured as the amount of ADP formed using reverse-phase HPLC to separate the nucleoside diphosphates from nucleoside triphosphates [16–18], when this strategy is applied to measure nitrogenase ATPase activity, the ATP-regeneration system must be omitted. Thus, the ATPase activity of nitrogenase is determined under conditions where inhibition by ADP becomes a concern [19]. Furthermore, baseline separation of ATP and ADP by HPLC takes at least several minutes to achieve for each data point, limiting the number of assays that can be performed in one day. Lastly, HPLC analysis generates a large volume of hazardous organic waste.

One way to measure ATP hydrolysis by nitrogenase in the presence of the ATP-regeneration system, which allows nitrogenase to be fully active as discussed above, is to colorimetrically determine the amount of creatine produced by enzymatic cleavage of PC in the regeneration of ATP [20]. However, this colorimetric method suffers from interference by excess PC and some nitrogenase substrates such as hydrazine [21]. To circumvent these interferences, individual activity assay solutions must be subjected to ion exchange chromatography [21], limiting the number of assays that can be performed in one day. Additionally, this technique is only useful in experiments utilizing the PC/kinase ATP-regeneration system, thus it is not broadly applicable to the study of NTPases.

Finally, nitrogenase ATPase activity can be evaluated as P_i released using a colorimetric method in which P_i is reacted with molybdic acid at low pH to form a colored complex. Nearly a century ago, Fiske and Subbarow [22] popularized this method of phosphate analysis, and their paper was recently listed as #37 of the top 100 most cited papers of all time [23]. This colorimetric method was initially developed to measure total phosphate content in blood and tissue, and it was desirable to work at low pH in order to catalyze the hydrolysis of organic phosphate compounds (OPCs) in the analyte such as nucleotides. However, the acidic conditions of this procedure are not conducive to the analysis of nucleotide hydrolysis in enzymatic assays including nitrogenase, since routine assays require the determination of true P_i in the presence of a large excess of OPCs. Additionally, it has been demonstrated that molybdate itself can accelerate the hydrolysis of OPCs [24].

Many attempts have been made to address the interference caused by OPCs in the colorimetric determination of P_i as a phosphomolybdate complex, including color development at substantially lower temperatures [25], in organic solvent [26], for shorter periods of time [27], and with increasing amounts of molybdic acid [28]. In some protocols, the phosphomolybdate complex is quickly extracted in alcohol before acidic conditions cause appreciable hydrolysis of labile OPCs in the analyte [29,30]. While these methods can improve the selectivity of the phosphomolybdate assay for P_i, they often decrease simplicity, while increasing time and cost. Here, we have adapted the colorimetric determination of P_i as a blue, phosphomolybdate complex for measuring ATPase activity by nitrogenase in a way that maximizes both precision and simplicity and is also more broadly applicable to the measurement of NTPase activity.

Inspired by additional work from Fiske and Subbarow, in which they achieved the first purification of PC by precipitating P_i as a Ca^{2+} salt to remove P_i from preparations of PC [31], we decided to separate true P_i from OPCs prior to reaction with molybdic acid in a similar way. This strategy has been used to measure acetyl phosphate content in enzyme preparations [32] and true P_i content in frog muscle [33], but it has never been applied to the study of enzymatic nucleotide hydrolysis activity.

We reasoned that this strategy had not been adapted for NTPase activity measurements because enzyme assays are typically stopped by addition of a strong acid, which will cause the hydrolysis of OPCs to begin even before the acidic color reagent is added. Furthermore, if the Ca^{2+} precipitation strategy is to be utilized, ethylenediaminetetraacetic acid (EDTA), since it chelates Ca^{2+}, cannot be used to quench enzyme activity, nor can sodium dodecyl

sulfate (SDS) [28], as it interferes with subsequent phosphomolybdate complex formation. However, substrate reduction by nitrogenase depends on electrostatic interactions between FeP and MoFeP to facilitate each ET cycle [9], and a large enough excess of salt ions fully inhibits activity [34]. As many other NTPase activities involve electrostatic interactions with protein partners [35,36], we reasoned that salt, rather than acid, can be used as a chemically mild quencher of NTPase activities. Here we have combined 1) mild enzyme activity quench with NaCl rather than acid, 2) precipitation of true P_i as a Ca^{2+} salt to separate it from all OPCs prior to analysis, and 3) the use of ascorbate to reduce the phosphomolybdate complex to a deep blue color with increased color stability and sensitivity to P_i in order to create a simple and precise way to measure enzymatic nucleotide hydrolysis that is fully compatible with the nitrogenase assay requirements (i.e., ATP-regeneration system). It is also applicable to the study of other NTPases, as demonstrated here by the quantification of the GTPase activity of elongation factor G (EF-G). This assay has the potential to replace the use of both HPLC and radioactive nucleotide analyses for the determination of NTPase activity.

MATERIALS AND METHODS

Materials

Nucleotides were purchased from Roche Chemicals. All other chemicals were from Sigma-Aldrich unless otherwise noted.

Protein purification

Nitrogenase component proteins were purified from *Azotobacter vinelandii* as described previously [9]. The FeP and MoFeP had specific activities of 1600–2000 and 2200–2600 nmol C_2H_4 min^{-1} mg^{-1} protein, respectively. 70S ribosome was purified from *E. coli* MRE 600 cells as reported previously [37], except that cells were lysed at 16,000 psi with a microfluidizer. EF-G was purified from *E. coli* BL21-DE3 cells via the IMPACT method, as reported previously [38].

Nitrogenase activity assays

Nitrogenase activity assays were conducted anaerobically under an Ar atmosphere in an aqueous solution containing 0.2 μM MoFeP, 2.0 μM FeP, 50 mM Tris (pH 8.0), 60 mM NaCl, 5 mM Na_2ATP, 5 mM $MgCl_2$, 30 mM PC, 0.00125 mg/mL creatine kinase, 6.5 mM $Na_2S_2O_4$, and 0.072 atm C_2H_2 in a final volume of 1.16 mL. Sealed 14-mL vials containing all components except FeP, MoFeP, $Na_2S_2O_4$, and C_2H_2 were made anaerobic on a Schlenk line. After solutions were made anaerobic, a 1.0 M $Na_2S_2O_4$ solution in 1.0 M Tris base was prepared anaerobically and added to each vial to a final $Na_2S_2O_4$ concentration of 6.5 mM. Next, 1.0 mL 1 atm C_2H_2 was transferred to each vial. After MoFeP was added to each vial, vials were shaken at 30°C. The enzymatic reactions were initiated by the addition of FeP and terminated with 0.30 mL of 5 M NaCl after 10 minutes. Protein concentrations were determined via Fe chelation in 6.4 M guanidine hydrochloride by 2,2-bipyridine using an extinction coefficient of 8650 $M^{-1}cm^{-1}$ at 522 nm [9].

Quantification of C_2H_2 and CH_4 formed by nitrogenase

The amount of e^- transferred to product by nitrogenase was determined by quantifying the amount C_2H_4 evolved with gas chromatography (GC); CH_4 was also measured by GC in reactions containing CN^-. Briefly, 50 μL aliquots of headspace from quenched reaction vials were injected into an SRI 8610C GC at 150°C containing an alumina column (Alltech) and a flame induction detector. C_2H_4 (Airgas) was used to generate the standard curve before sample analysis each day. For assay solutions that contained NaCN, aliquots of a 100 mM NaCN stock solution were added to vials before degassing and salt concentrations were adjusted accordingly.

Quantification of P_i formation during nitrogenase turnover

ATP hydrolysis was monitored through the quantification of released P_i during the enzymatic reaction. After product formation was measured by GC, 500 μL of each assay solution was removed and added to 800 μL of a solution containing 200 mM $CaCl_2$ and 50 mM Tris (pH 8.0). Solutions were mixed and centrifuged for 5 min at 13,000 g. Because the force of pipetting could rupture the soft, white pellet, an aliquot of 1.2 mL of the supernatant fraction was removed, leaving 0.1 mL remaining in the tube with the precipitate, and 1.0 mL deionized (DI) H_2O was added to the tube. Solutions were centrifuged for an additional 5 min at 13,000 g. An aliquot of 1.0 mL of the supernatant fraction was removed, leaving 0.1 mL remaining in the tube with the precipitate, and 1.0 mL DI H_2O was again added to the tube. This process was repeated for a total of four centrifugation steps. The white pellet was then resuspended in a 1.0 mL solution of 0.2 M HCl, and 100 μL of the resulting suspension was added to 10.0 mL DI H_2O. Then, 1.0 mL of the color reagent was added. The method of Strickland and Parsons [39] was used for the preparation of the color reagent, which contained 4.9 mM ammonium molybdate, 61 mM ascorbate, and 0.42 mM potassium antimonyl tartrate in 1.2 M sulfuric acid. Absorbance of the blue solutions was measured at 885 nm after 1 h using an Agilent 8453 UV-visible spectrophotometer.

Quantification of GTPase activity of EF-G through the measurement of released P_i

Steady-state GTP hydrolysis by EF-G was measured in reaction mixtures containing activated 70S ribosome (0.2 μM), 5 mM Na_2GTP, 20 mM Hepes (pH 7.6), 6 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol and varying concentrations of EF-G (0–9 μM) in a total volume of 200 μL. Activity assays were conducted for 30–90 minutes in 1.7-mL Eppendorf tubes at room temperature and quenched by adding 80 μL of 5 M NaCl. Appropriate controls were carried out to ensure the rate of product formation was linear over the entire course of each assay and that the NaCl quench was as effective as routine quenching of EF-G activity with SDS. GTP hydrolysis was measured as P_i released as described above for nitrogenase but with the following modifications: 180 μL quenched sample was mixed with 120 μL of 400 mM $CaCl_2$ in 100 mM Tris (pH 8.0) followed by 80 μL of 100 mM K_2CO_3 in 100 mM Tris (pH 8.0). K_2CO_3 was added to precipitate $CaCO_3$ and increase the size of the white pellet. All trials were conducted at least in triplicate, and the error bars represent ± 1 standard deviation. Data were fit to the Michaelis-Menten equation for enzyme kinetics using Prism.

Quantification of GTPase activity of EF-G through the measurement of $^{32}P_i$ formed

Steady-state GTP hydrolysis by EF-G was measured in reactions containing, in a final volume of 50 μL, activated 70S ribosome (0.2 μM), 2 mM Na_2GTP (trace ^{32}P-γ-GTP, specific activity 12.5 Ci/mmol), 20 mM Hepes (pH 7.6), 6 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, and varying concentrations of EF-G (0–9 μM). Assays were conducted in 1.7-mL Eppendorf tubes at room temperature. 3-μL aliquots were withdrawn at different time points and quenched with 1 μL of 5% SDS. 1-μL aliquots of the quenched solutions were spotted on cellulose thin-layer chromatography (TLC) plates and developed in 0.5 M KH_2PO_4 (pH 3.5). The amount of $^{32}P_i$ formed was quantified using a phosphorimager. The initial velocities were plotted versus [EF-G] and fit to the Michaelis-Menten equation for enzyme kinetics using Prism. All trials were conducted at least in triplicate, and the error bars represent ± 1 standard deviation. [Note: we used ^{32}P-γ-GTP, which is hydrolyzed to $^{32}P_i$ and non-radioactive GDP. We monitor the formation of $^{32}P_i$ by TLC].

RESULTS AND DISCUSSION

Development of the colorimetric assay for true P_i released

In addition to the conversion of N_2 into NH_3 (Equation 1), nitrogenase is also capable of reducing other substrates by multiples of 2 e^-, such as C_2H_2 to C_2H_4 [40]. The ATP requirement of nitrogenase is independent of the substrate being reduced [41]. In some cases, however, site-directed mutants and small-molecule inhibitors have been shown to cause uncoupling of ATP hydrolysis from productive ET, significantly elevating the number of ATP molecules that must be hydrolyzed for the transfer of 2-e^- (ATP/2e^-) above the theoretical value of 4 [42–44]. While ET by nitrogenase is routinely measured by GC, to monitor the 2-e^- reduction of C_2H_2 to C_2H_4, ATP hydrolysis by nitrogenase has historically been much more difficult to measure due to the complex, dynamic nature of the nitrogenase activity assay components.

Colorimetric determination of P_i released initially seemed to us the simplest, quickest, and least expensive method to quantify ATP hydrolysis by nitrogenase. Figure 1, however, highlights the difficulty in P_i quantification that occurs when P_i is in a complex mixture containing OPCs. Standard solutions of 0–20 μM P_i made in DI H_2O turned blue when mixed with the color reagent containing ammonium molybdate, ascorbate, and potassium antimonyl tartrate in sulfuric acid (Figure 1A) [39]. However, when these standard solutions were instead made up in the nitrogenase activity assay matrix, which includes 30 mM PC and 5 mM MgATP, both of which are OPCs, all solutions turned dark blue (Figure 1B), demonstrating that OPCs are susceptible to acid-catalyzed hydrolysis at low pH. This process releases P_i and causes a dramatic increase in the effective concentration of P_i available to complex with molybdic acid. When we instead added an excess of Ca^{2+} to the P_i standard solutions containing OPCs (prior to mixing P_i with the acidic color reagent), a white precipitate formed (Figure 1C) wherein the true P_i is sequestered while OPC-associated P_i remains in the supernatant fraction. After removing the supernatant fraction and resuspending the precipitate in weak acid (~0.2 M HCl), the color development of these P_i standards (Figure 1D) matched that of the P_i standards made in DI H_2O (Figure 1A).

Next, we measured the rate of the blue phosphomolybdate complex formation. The absorbance of a P_i standard solution, when made up in a complex matrix containing an excess of OPCs, continues to increase with time due to continuous release of labile P_i from OPCs (Figure 2A). In contrast, when true P_i is separated from OPCs by precipitation with Ca^{2+} ahead of the addition of the acidic color reagent, the blue color stabilizes after about 1 h (Figure 2A), since no OPCs remain in solution to release additional P_i. Figure 2B shows that standard curves of P_i diluted to a final concentration of 0–20 μM P_i made in DI H_2O (pictured in Figure 1A) and those made in the nitrogenase activity assay matrix, which includes a large excess of OPCs, (pictured in Figure 1D) overlay well when P_i is first precipitated as a Ca^{2+} salt. These experiments firmly establish that the Ca^{2+} precipitation procedure is effective at separating labile OPCs from true P_i. Furthermore, quantitative recovery of P_i is achieved by this procedure, since there is no change in the absorbance of P_i standards made in DI H_2O whether or not the Ca^{2+} precipitation step is used. P_i was quantitatively recovered whenever the white precipitate was visible. This initially required a minimum of 1 μmol P_i and an excess of Ca^{2+}. However, additional salts (i.e. K_2CO_3) could be added to co-precipitate insoluble Ca^{2+} salts, thereby increasing the size of the white pellet. Using this technique, as little as 100 nmol P_i was routinely pelleted and quantitatively recovered.

Application of method for measuring true P_i to quantify ATPase activity

To assess the ability of our method to quantify ATP hydrolysis during nitrogenase turnover, we measured the ATP/$2e^-$ ratio for wild-type (wt) nitrogenase under standard assay conditions and compared our measurements to published results. The ATP/$2e^-$ ratio for wt nitrogenase should approach 4 when the activity assay is performed at 30°C and $Na_2S_2O_4$ is used as the external reducing agent [45], as demonstrated by Eq. 1. Experimentally, ratios of 4–5 are typically measured, but with standard deviations that are often not reported or can be greater than the mean [43–47]. First, we quenched our nitrogenase activity assays with 5 M NaCl rather than concentrated acid, base, chelator, or detergent. We quantified the amount of e^- productively transferred to substrate by wt nitrogenase by quantifying the amount of C_2H_4 formed from C_2H_2 using GC. Then, we added $CaCl_2$ to the assay solutions to capture released P_i in a white precipitate. We centrifuged the assay solutions, removed the supernatant fraction, resuspended the white pellet, and added an aliquot of each activity assay solution to the acidic color reagent. After measuring the absorbance of these solutions at 885 nm, our method yielded an ATP/$2e^-$ value of 4.8 ± 0.3 with a percent relative standard deviation (%RSD) of 5.3% (Table 1). Thus, our method is able to reproduce the accepted ATP/$2e^-$ literature value in the presence of the regeneration system, which contains a large excess of OPCs, (30 mM PC, 5 mM MgATP, 0.125 mg/mL creatine kinase) with a substantial increase in precision. This will greatly aid in the analysis of slight differences in the activities of site-directed nitrogenase mutants.

As an additional test, we also measured the ATP/$2e^-$ ratio for wt nitrogenase under conditions known to cause uncoupling of ATP hydrolysis from ET. CN^- is known to inhibit nitrogenase activity by uncoupling the hydrolysis of ATP from productive ET to substrate. The ATP/$2e^-$ increases with increasing [CN^-] and plateaus at about 18 when [CN^-] ≥ 80 μM [42]. We chose to measure the ATP/$2e^-$ for wt nitrogenase proteins in the presence of 5 mM

NaCN at pH 8.0, so the effective [CN^-] would be much larger than 80 μM. Though CN^- is a reversible inhibitor of nitrogenase activity, HCN is a substrate of nitrogenase. Thus, in order to measure the amount of e^- transferred to product, we measured both the C_2H_4 formed from 2-e^- reduction of C_2H_2 and the CH_4 formed from 6-e^- reduction of HCN to CH_4 and NH_3. As expected, the ratio of ATP/2e^- under these conditions, 16 ± 1.1, as measured by our NaCl/Ca^{2+}/P_i assay, lies within the range of previously reported values (Table 1) [42].

Application of method for measuring P_i to quantify GTPase activity

As an independent validation of our NaCl/Ca^{2+}/P_i assay, we measured ribosome-dependent GTP hydrolysis by EF-G. EF-G is a ribosome factor that participates in protein synthesis. Translocation of mRNA and tRNA along the ribosome is coupled to the binding and hydrolysis of GTP by EF-G [48]. In order to interrogate how the hydrolysis of GTP is coupled to the efficient synthesis of proteins, it is crucial to precisely measure GTP hydrolysis by wt EF-G and site-directed mutants. An established method for measuring GTP hydrolysis by EF-G requires the use of radiolabeled GTP [49]. Each activity assay is initially spiked with ^{32}P-γ-GTP. After the enzyme activity is quenched, ^{32}P-γ-GTP and $^{32}P_i$ are separated by TLC and the ratio of radioactivity counts is used to extrapolate the extent of hydrolysis. While this assay offers a substantially higher sensitivity than colorimetric measurements, radiolabeled nucleotides are expensive, hazardous and have a short shelf life.

To establish that our method for measuring ATPase activity was also applicable to GTPase activity measurements, we determined the steady-state Michaelis-Menten parameters for ribosome-dependent GTP hydrolysis by EF-G using two techniques. First, we measured GTP hydrolysis using the established ^{32}P-γ-GTP method, and we determined the k_{cat} for ribosome-dependent GTP hydrolysis to be 5.0 ± 0.3 s^{-1} and the K_m to be 8.7 ± 0.8 μM (Table 2). We subsequently measured 70S ribosome-dependent GTP hydrolysis by EF-G under the same reaction conditions using our colorimetric method. Like nitrogenase assays, these EF-G activity assays were quenched with 5 M NaCl rather than acid or detergent. Using this method, we determined the k_{cat} for ribosome-dependent GTP hydrolysis to be 3.1 ± 0.7 s^{-1} and the K_m to be 9.4 ± 3.5 μM. It is important to note that though these k_{cat} values lie within two standard deviations of one another, both fall well within the range of previously reported k_{cat} values for ribosome-dependent GTP hydrolysis by EF-G [36,49,50]. Additionally, while the K_m values determined by these techniques are slightly higher than what has been reported previously, which can be explained by the inherent preparation-to-preparation variance in ribosome purity among laboratories [36,51], they are within one standard deviation of one another. Figure 3 shows the Michaelis-Menten curves obtained in this work. Because the standard ^{32}P-γ-GTP assay and our new NaCl/Ca^{2+}/P_i method yielded overall similar steady-state kinetic parameters, while achieving a decrease in cost, hazard, and time, our method is well suited to assess changes in the GTP hydrolysis activity of EF-G, for example, due to mutagenesis.

CONCLUSIONS

We have presented here an improved assay for nucleotide hydrolysis through the determination of true P_i in the presence of labile OPCs that is applicable to ATPases as well

as GTPases. Compared to established methods for measuring nucleotide hydrolysis, this method has minimal requirements for specialized instrumentation, requiring only a single-beam UV-vis spectrophotometer, utilizes non-hazardous and inexpensive reagents, and is at least as precise as standard available techniques. The major limitation of this assay is the amount of P_i released that is required for reliable quantification. Currently, our method requires a minimum of 100 nmol P_i for precise measurements. Further exploration with insoluble Ca^{2+} salts could lead to strategies to lower this minimum requirement.

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Abbreviations

P_i	inorganic phosphate
OPC	organic phosphate compound
EF-G	elongation factor G
HPLC	high-performance liquid chromatography
MoFeP	MoFe-protein
FeP	Fe-protein
e^-	electron
ET	electron transfer
PC	phosphocreatine
EDTA	ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulfate
GC	gas chromatography
DI	deionized
TLC	thin-layer chromatography
wt	wild-type
%RSD	percent relative standard deviation

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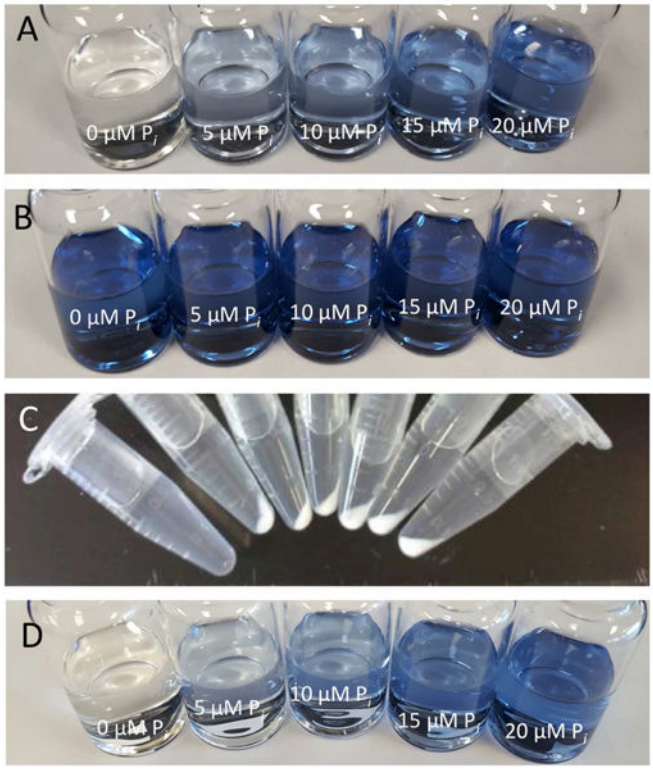

Figure 1.

A. Blue phosphomolybdate complex formed from reaction of the molybdic acid color reagent with 0–4 mM P_i standard solutions in DI H_2O diluted to a final concentration of 0–20 μM P_i, with increasing concentration from left to right. **B.** Blue phosphomolybdate complex formed from reaction of the molybdic acid color reagent with 0–4 mM P_i standard solutions in a mock nitrogenase activity assay solution containing 50 mM Tris (pH 8.0), 30 mM phosphocreatine, and 5 mM MgATP diluted to a final concentration of 0–20 μM P_i, with increasing concentration from left to right. **C.** Solutions containing 0–4 mM P_i after addition of excess $CaCl_2$ and centrifugation. **D.** Blue phosphomolybdate complex formed from reaction of the molybdic acid color reagent with the 0–4 mM P_i standard solutions shown in C after the supernatant fraction is removed and precipitate is resuspended in weak acid. These solutions have been diluted to a final concentration of 0–20 μM P_i, with increasing concentration from left to right.

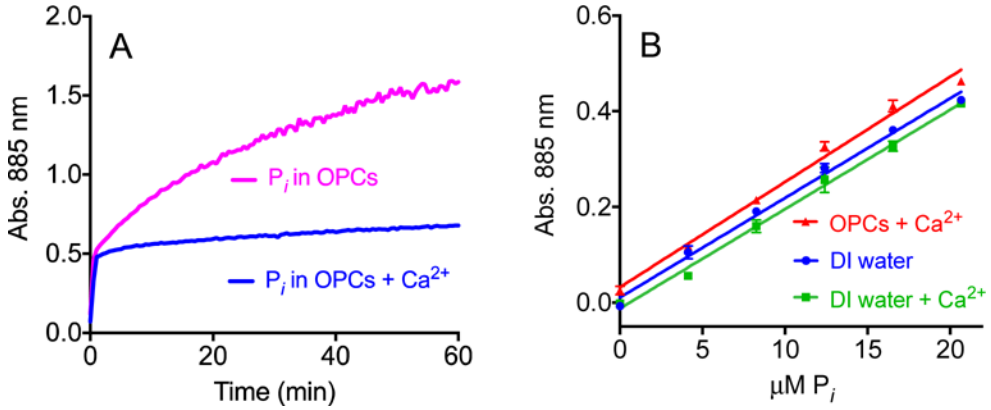

Figure 2.

A. Time course for the blue phosphomolybdate complex development monitored by absorbance at 885 nm of P_i standard solutions in a matrix containing OPCs with (blue) and without (pink) the Ca^{2+} precipitation step to sequester true P_i and separate it from OPCs. **B.** Standard curves in triplicate of P_i standards in DI H2O (blue; y = (0.021 ± 0.001) x + (0.011 ± 0.011)), in DI H_2O and treated with the Ca^{2+} precipitation step (green; y = (0.021 ± 0.001) x − (0.012 ± 0.001)), and in the nitrogenase activity assay matrix, which contains and excess of OPCs, but that has been treated with the Ca^{2+} precipitation step (red; y = (0.022 ± 0.001) x + (0.032 ± 0.013)).

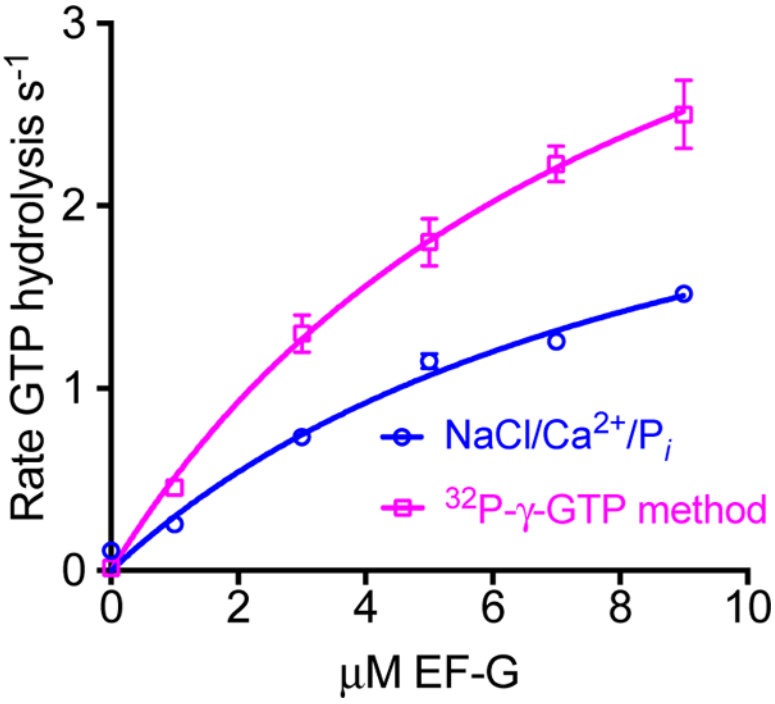

Figure 3.

Michaelis-Menten curves show the rate of 70S ribosome-dependent GTP hydrolysis by EF-G as a function of [EF-G]. Data obtained using our NaCl/Ca^{2+}/P_i method for measuring nucleotide hydrolysis is shown in blue and compared to data obtained using the standard radiolabeled nucleotide technique, shown in pink. Error bars represent ± 1 s.d.

Table 1

ATP/2e$^-$ values for wt nitrogenase under standard conditions and in the presence of 5 mM NaCN at pH 8.0

	ATP/2e$^-$ (in literature)	**ATP/2e$^-$ (this work)**
WT nitrogenase, standard conditions	4–5 (%RSDs >100% reported)	4.8 ± 0.3 (5.3% RSD)
WT nitrogenase with 5 mM NaCN at pH 8.0	17–18 (%RSDs not reported)	16 ± 1.1 (6.7% RSD)

Table 2

Steady-state Michaelis-Menten kinetic parameters for 70S ribosome-dependent GTP hydrolysis by EF-G

	K_m (μM)	k_{cat} (s^{-1})
^{32}P-γ-GTP method (this work)	8.7 ± 0.8	5.0 ± 0.3
NaCl/Ca^{2+}/P_i method (this work)	9.4 ± 3.5	3.1 ± 0.7