Adenosine diphosphate and adenosine monophosphate: enzymatic spectrophotometric determination

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Overview

The ATP/ADP ratio is an important measure of the cellular energy state. The enzymatic assay of ATP was described in a previous Methods of Nutritional Biochemistry.[1] In this enzymatic assay of ADP, phosphoenolpyruvate is used to phosphorylate ADP in the pyruvate kinase reaction. The resulting pyruvate is then reduced with NADH in the lactate dehydrogenase reaction. The stoichiometric consumption of NADH is followed spectrophotometrically at 340 nm. AMP can be subsequently assayed in the same reaction mixture by addition of myokinase. The nucleotides must first be extracted from tissue/cells by treatment with perchloric or trichloroacetic acid and centrifugation to remove the precipitated protein. The deproteinized supernatant must then be neutralized before the enzymatic analysis.

Reagents

Use distilled/deionized water for all solutions.

0.1 M Triethanolamine buffer, pH 7.6. Add 1.49 g triethanolamine (certified grade, Fisher Scientific, Medford, MA, USA, catalog No. T407) to 50 mL water. Adjust pH to 7.6 with 1N HCl. Make up volume to 100 mL. Store refrigerated. The solution is stable for at least a month. (The buffer and salt solutions are themselves stable for long periods but may become contaminated with bacterial or fungal growth after about one month.)

20 mM Triethanolamine buffer, pH 8.2. Add 0.3 g triethanolamine to 80 mL water. Adjust pH to 8.2 with 1N HCl. Make up volume to 100 mL. Store refrigerated. The solution is stable for at least a month.

1M $MgCl_2$. Add 5.08 g $MgCl_2$ (hexahydrate, Fisher) to 25 mL total volume with water. Store refrigerated. The solution is stable for at least a month.

1M KCl. Add 0.74 g KCl (Fisher) to 10 mL total volume with water. Store refrigerated. The solution is stable for at least a month.

Modified from Jaworek, D., Gruber, W., and Bergmeyer, H.U. (1974). Adenosine-5'-diphosphate and adenosine-5'-monophosphate. In *Methods of Enzymatic Analysis*, 2d ed. (H.U. Bergmeyer, ed.), pp. 2127–2131, Academic, New York.

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Pyruvate kinase buffer (0.1 M triethanolamine, pH 7.6, 10 mM $MgCl_2$, 40 mM KCl). Add 0.5 mL of 1 M $MgCl_2$ and 2 mL of 1 M KCl to 48 mL triethanolamine buffer, pH 7.6. Store refrigerated. The solution is stable for at least a month.

NADH, 2 mg/mL. Dissolve 4 mg NADH (Grade I, Boehringer Mannheim, Indianapolis, IN, USA) in 2 mL of 20 mM triethanolamine buffer, pH 8.2. (NADH is unstable at acidic pH.) Store refrigerated. The solution is stable for one week.

NADH, 0.4 mg/mL. Add 0.5 mL of 2 mg/mL NADH to 2 mL 20 mM triethanolamine buffer, pH 8.2. Make fresh daily.

5 mM Phosphoenolpyruvate. Dissolve 12 mg phosphoenolpyruvate (tricyclohexylammonium salt, Boehringer Mannheim) in 5 mL water. Use of the tricyclohexylammonium salt gives a neutral solution. Store frozen. The solution is stable for at least a year.

Lactate dehydrogenase, 1 mg/mL. Add 50 μL of 10 mg/mL suspension (Boehringer Mannheim) to 0.45 mL water. The enzyme from rabbit muscle or hog muscle should be used because of its greater activity and lesser contamination by potentially interfering enzymes. (Note that enzyme solutions should be mixed gently. Vigorous shaking may denature and inactivate them.) The solution may be kept refrigerated for several weeks.

Pyruvate kinase, 2.5 mg/mL. Add 0.1 mL of 10 mg/mL suspension (Boehringer Mannheim) to 0.3 mL water. The solution may be kept refrigerated for several weeks.

10 mM ADP. Add 25 mg ADP (monopotassium salt, Boehringer Mannheim) to 5 mL water. Neutralize (pH 6.5–7.5) with about 25 μL 1N NaOH. Store frozen. The solution is stable for several months. To calibrate the solution, measure the absorbance at 259 nm of a 500-fold dilution (e.g., 0.2 mL diluted to 100 mL); a quartz cuvette must be used. The concentration (in mM) is then $A_{259} \times 500/15.4$. The 1 mM ADP solution recommended to be used to check the enzymatic assay can be prepared by suitable dilution of this stock solution.

Myokinase, 0.5 mg/mL. Add 50 μL of 5 mg/mL suspension (Boehringer Mannheim) to 0.45 mL water. The solution may be kept refrigerated for a week.

10 mM AMP. Add 25 mg AMP (disodium salt, Boehringer Mannheim) to 5 mL water. Store frozen. The solution is stable for at least a year. The solution may be calibrated by absorbance at 259 nm, as described above for ADP.

Procedure

The enzymes, substrates (phosphoenolpyruvate and NADH), and samples should be kept on ice. Having the buffer at room temperature should prevent fogging of the cuvette. See discussion in Reference 1 for sample preparation and spectrophotometer settings.

1. In a 1-mL cuvette, add:

   0.5 mL pyruvate kinase buffer
   0.05 ml NADH, 2 mg/mL (use a 0.4-mg/mL solution for amounts of ADP <20 nmol)
   0.05 mL phosphoenolpyruvate, 5 mM (use a 0.5 mM solution for amounts of ADP <20 nmol)
   0.4 mL sample plus water (for example, 0.2 and 0.2 mL) (note that samples extracted with trichloroacetic acid may need to be weighed, and that no more than 0.25 mL should be used of samples extracted with perchloric acid; see Discussion)
   5 μL lactate dehydrogenase, 1 mg/mL

2. Mix by covering the top of the cuvette with Parafilm (Fisher) and inverting several times.

3. Place cuvette in the spectrophotometer, and take baseline measurement over 2 min to allow consumption of any endogenous pyruvate.
4. When stable readings have been obtained, add 5 μL pyruvate kinase (2.5 mg/mL) and mix. Avoid altering the position of the cuvette. This is most easily done by pipetting the enzyme solution onto a glass, or preferably plastic, stirring rod and mixing this into the cuvette. We use a plastic "plumper" (Hellma Cells, Jamaica, NY, USA) which has a shelf at the end that can be trimmed down as needed to fit the cuvette.
5. Follow the absorbance change to completion of the reaction (10–15 min). Extrapolate any drift back to the time of pyruvate kinase addition. A correction may also need to be made for absorbance change due to addition of enzyme in the blank reaction (see below).

The ADP concentration (in mM) in the sample is

$$\frac{\text{absorbance change}}{\text{extinction coefficient}} \times \frac{\text{total volume} = 1.01 \text{ mL}}{\text{sample volume}}$$

This value must be multiplied by any dilution during sample processing. The mM extinction coefficient is 6.2 or 5.9, depending on instrument configuration (see Discussion). If the absorbance change is greater than about 0.5 (or 0.1 when using 0.4 mg/mL NADH), the assay should be repeated with less sample. The assay can be checked by adding 50 μL of 1 mM ADP in place of sample; this should give an absorbance change of about 0.3. It is also advisable to run a blank reaction with no sample (or with neutralized perchloric or trichloroacetic acid dummy samples) to check for any contamination of the reagents, and to determine the dilution effect of enzyme addition (see below). If significant, the absorbance change in the blank must be subtracted from that of the samples.

6. If AMP is to be measured, add 5 μL myokinase (0.5 mg/mL) after the completion of the ADP/pyruvate kinase reaction. Follow the second absorbance change to completion of the myokinase reaction (10–20 min). Extrapolate any drift back to the time of myokinase addition.

Calculation of the concentration of AMP is the same as that given above for ADP, except that the extinction coefficient is doubled, since two equivalents of ADP are formed on phosphorylation of AMP by ATP in the myokinase reaction. Possible contamination of AMP in NADH, which used to be a problem with commercial preparations, should be checked by using 0.2 mL of 0.1 mM ATP in place of sample. The operation of the myokinase reaction should be checked by using AMP (and ATP) in place of sample (e.g., 50 μL of 0.5 mM AMP should give an absorbance change of about 0.3).

Discussion

ATP is the major energy currency of the cell, and the ATP level and the ATP/ADP ratio are measures of the cellular energy state. (Assay of ATP was described in a previous Methods of Nutritional Biochemistry.[1]) Since ATP may be rapidly dephosphorylated in excised tissues at room temperature, or even in broken cells due to ATPases and phosphatases, samples for analysis of ATP and ADP must be quickly frozen and/or deproteinized with perchloric or trichloroacetic acid, as discussed previously.[1]

The choice of appropriate amounts of sample, NADH, and phosphoenolpyruvate deserves some comment. The residual potassium perchlorate in neutralized perchloric acid extracts appears to inhibit pyruvate kinase. Up to 25% of the reaction mixture volume can be sample, but a higher percentage will slow the assay reaction excessively. For samples deproteinized with trichloroacetic acid and subsequently neutralized by ether extraction, some ether remains dissolved in the water layer. This residual ether can cause a 5–20% error in sample pipetting with automatic pipets, which tend to drip because of lowered surface tension and volatil-

ization of the ether. We therefore routinely measure sample volumes in this case by weighing what is added to the assay cuvette.

The amount of NADH and PEP to be used depends on the amount of ADP (and AMP) in the sample. The 2-mg/mL NADH solution gives about 140 μM in the cuvette, or an aborbance of about 0.8. The 0.4-mg/mL NADH solution gives about 25 μM in the cuvette, or an absorbance of about 0.15. There should be at least a 20% excess of NADH. The sample may need to be diluted to avoid exhausting the cosubstrates. If there is a question as to whether sufficient NADH remains unconsumed at the end of the reaction, observe the absorbance change on adding ADP (e.g., 5 μL of 10 mM). Note that any endogenous pyruvate also consumes NADH. Pyruvate is unstable at neutral pH and is largely lost in neutralized samples that have been frozen and thawed, but is likely to be present in fresh samples. Pyruvate can also be present as a contaminant in the phosphoenolpyruvate. This is a reason for using a low concentration of phosphoenolpyruvate when assaying low amounts of ADP. One may check for the presence of pyruvate by following the absorbance change on adding lactate dehydrogenase, rather than including that enzyme in the initial reaction mixture. To know how much NADH is initially available, we usually run one preliminary reaction with excess ADP.

The addition of 5 μL of enzyme to the 1-mL reaction volume means a 0.5% dilution of the initial absorbance. This absorbance change can be significant at low levels of ADP and/or AMP. It is also a reason for using less NADH at low ADP, since the NADH contributes much of the background absorbance. The absorbance change in the ADP (AMP) assay of a sample should be corrected by subtracting the change observed on adding pyruvate kinase (myokinase) to a blank reaction lacking sample, with the same NADH. Alternatively, especially if the sample itself contributes significant absorbance, one may subtract the absorbance change observed on adding a second portion of pyruvate kinase (myokinase) after the ADP (AMP) assay has finished.

Pyruvate kinase does phosphorylate other nucleoside diphosphates, though at somewhat lower rates.[2] Hence, this assay actually measures total nucleoside diphosphate. This is generally satisfactory, since the cellular nucleoside diphosphate pool is predominantly ADP. Myokinase is specific for AMP.

Any spectrophotometer set at 340 nm can be used for this NADH-linked assay. As explained previously,[1] for sensitivity down to 0.2 nmol we use a Hewlett Packard (Palo Alto, CA, USA) diode array spectrophotometer (model 8450A) set to measure $A_{335\text{-}345}$ minus $A_{390\text{-}400}$. In this optical configuration, the millimolar extinction coefficient is reduced slightly to 5.9, from the value of 6.2 at 340 nm. Sensitivity might be improved somewhat by using a fluorometer to measure NADH fluorescence at 460 nm; however, an ADP standard must be added subsequently to calibrate the fluorescence change. ADP can be measured by bioluminescent assay with firefly luciferase, once the ADP is converted to ATP by incubation with pyruvate kinase and phosphoenolpyruvate.[3] ADP and AMP can also be separated from other nucleotides by anion exchange or reverse-phase HPLC and quantitated by their UV absorbance.[4,5] Sensitivity of the HPLC methods can be increased to the picomole range by prior chemical conversion of the adenine nucleotides to strongly fluorescent 1,N^6-etheno derivatives.[6,7] HPLC methods are more time-consuming, require careful calibration with standards, and the instrumentation is relatively expensive; however, they have the advantage that ATP, ADP, AMP, and other nucleotides can be quantitated at the same time.

There is a final caveat regarding the use of any of these analytical methods to determine ADP. They all measure the total cellular ADP. In some tissues, notably muscle, much of the ADP is protein bound. Calculations of the free (unbound) ADP concentration and the free cytosolic ATP/ADP ratio have been made, using the creatine kinase equilibrium and assayed concentrations of creatine phosphate, creatine, and ATP.[8,9] Similar

considerations may apply to AMP, and the free AMP concentration has been calculated using the myokinase and creatine kinase equilibria.[8,10]

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