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A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems

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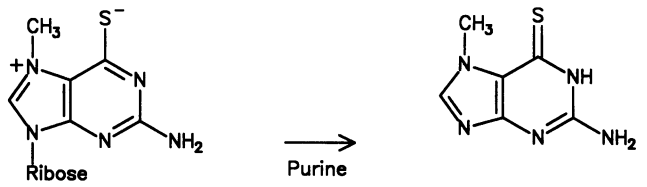
ABSTRACT **A spectrophotometric method for the measurement of inorganic phosphate (P_i) has been developed by using 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine-nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1). This substrate gives an absorbance increase at 360 nm on phosphorolysis at pH 6.5–8.5, and at pH 7.6 the change in extinction coefficient is 11,000 $M^{-1} \cdot cm^{-1}$. The Michaelis–Menten constants of the two substrates with the enzyme are 70 μM for the nucleoside and 26 μM for P_i; the k_{cat} is 40 s^{-1} (25°C). The assay was shown to quantitate P_i in solution at concentrations at least down to 2 μM. It can be used to measure the kinetics of P_i release from phosphatases, such as GTPases and ATPases, by coupling the two enzymic reactions. The utility of this assay was shown by three test systems: glycerol kinase plus D-glyceraldehyde acting as an ATPase and actin-activated myosin ATPase, and myosin subfragment 1, hydrolyzing a single turnover of ATP, releasing P_i with a rate constant the same as the steady-state ATPase activity.**

A spectrophotometric assay for inorganic phosphate (P_i) has been developed to probe the kinetics of P_i release from biological systems, such as GTPases and ATPases. Because of the importance of P_i in biological systems and in medical analysis, a variety of assays have been developed for this ion. Some are based on the colored phosphomolybdate complex (for example, see ref. 1) and others have used enzymes, particularly phosphorylases with fluorescent substrates (e.g., see refs. 2 and 3). However, none seemed particularly suitable for our purposes.

To be able both to quantitate P_i in solutions and to follow the kinetics of P_i release from enzymic reactions, the assay needs to be simple and rapid and the preferred method of detection is an increase in absorbance at a wavelength away from the absorbance regions of biological molecules, particularly proteins. Absorbance increases can have significant advantages over fluorescence changes mainly because the absorbance change is directly related to the change in $[P_i]$. This leads to simplicity in interpreting results (for example, there are no inner filter effects) and to instrument independence of results.

The assay is based on the difference in absorbance between 2-amino-6-mercapto-7-methylpurine ribonucleoside (methylthioguanosine, a guanosine analogue; MESG) and the purine base product of its reaction with P_i catalyzed by purine-nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) (Scheme I).



Scheme I

EXPERIMENTAL PROCEDURES

Glycerol kinase from *Escherichia coli* as well as bacterial and calf spleen purine-nucleoside phosphorylase were from Sigma. The bacterial protein was approximately one-third phosphorylase by gel electrophoresis and for some experiments was purified further on Q-Sepharose. Actin and chymotryptic subfragment 1 were prepared from rabbit skeletal muscle as described (4, 5).

MESG was synthesized by modification of the method of Broom and Milne (6). 2-Amino-6-chloro-purine ribonucleoside (2 g; Sigma) was dissolved in dry dimethylformamide (10 ml). Methyl iodide (4 ml) was added and the mixture was stirred for 14 h. Excess methyl iodide was removed *in vacuo*. Thiourea (2 g) was added and the mixture was stirred for 30 min. Methanolic ammonia was added until the solution became neutral (tested with pH indicator paper). The mixture was poured into stirred acetone to give a yellow precipitate. The solid was filtered and dried *in vacuo*. To ensure that the compound was pure with respect to other UV-absorbing compounds, it was dissolved in dimethylformamide and purified on a column of silica gel (2.5 × 15 cm), eluted with ethyl acetate/1-propanol/water (5:2:1; vol/vol). The compound was dried to a yellow solid by rotary evaporation and stored desiccated at −20°C. ^1H NMR analysis was as expected (6).

Samples of MESG were analyzed by HPLC using a C_{18} reverse-phase column (Whatman Partisil 10 ODS; 25 × 0.4 cm). Elution was at 2 ml·min^{-1}, using 10 mM potassium phosphate (pH 5.5) containing 10% (vol/vol) methanol. This system could also be used to monitor the base product of the phosphorylase reaction. The elution times are 7.5 min for MESG and 15 min for the base.

RESULTS

The guanosine analog MESG has a strong UV absorbance peak at 330 nm at pH 7.6 (Fig. 1). In the presence of P_i and purine-nucleoside phosphorylase, the base 2-amino-6-mercapto-7-methylpurine forms, with the absorbance maximum shifted to 355 nm. Using the absorbance spectrum changes of MESG as a function of pH, it was shown that MESG has a pK_a of 6.5 (Fig. 2). The base product has a pK_a of 8.8 as determined by UV spectroscopy. This difference in ionization between base and nucleoside (Scheme II) is presumably due to the extra positive charge on the nucleoside. In the pH range 6.5–8.5, there is a UV absorbance difference between the phosphorylase substrate and product (Fig. 1).

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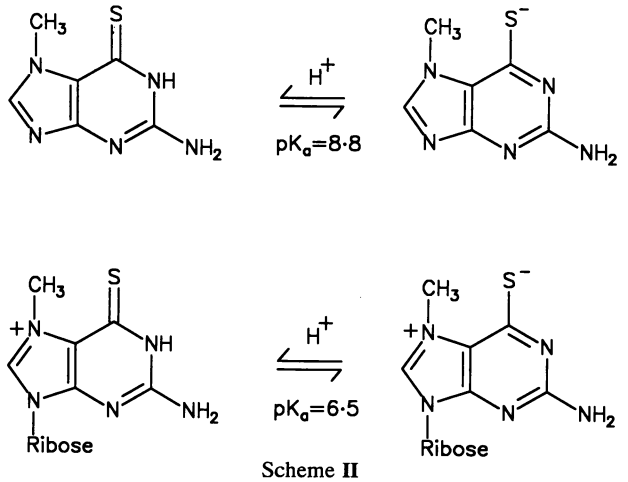
Abbreviation: MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside.



Scheme II

This difference provides a method to characterize MESG and P_i as substrates for the phosphorylase and also to quantitate P_i. The wavelength chosen to follow the phosphorylase reaction was 360 nm, a compromise between maximizing the change in absorbance during the reaction and minimizing the initial absorbance of the reaction mixture in the cuvette. At 360 nm and pH 7.6, the change in extinction coefficient is 11,000 $M^{-1}\cdot cm^{-1}$.

MESG is a good substrate for bacterial purine-nucleoside phosphorylase and the reaction follows Michaelis–Menten kinetics. At 25°C, the K_m is 70 μM for MESG and 26 μM for P_i; the k_{cat} is 40 s^{-1}. The K_m values compare well with other substrates tested (7, 8) and that for MESG is similar to 7-methylguanosine under the same conditions (data not shown). Calf spleen phosphorylase also uses MESG efficiently, but this system was not characterized fully.

The stability of MESG was studied within the pH range to be used both by following UV spectral changes and by HPLC. The spectral changes due to alkali-catalyzed breakdown were as described (9). By following the changes with time, MESG was shown to have a half-life of ≈4 h at pH 8.0 and 25°C; the half-life was ≈10-fold longer at pH 6.0. The type of base ring-opening mechanism suggested for 7-methylguanosine (10) may also apply for the thio analogue, although the breakdown products were not characterized.

The phosphorylase/MESG system was then used as an assay to determine the linearity of response for measuring a range of P_i concentrations (Fig. 3) and a range of rates of P_i

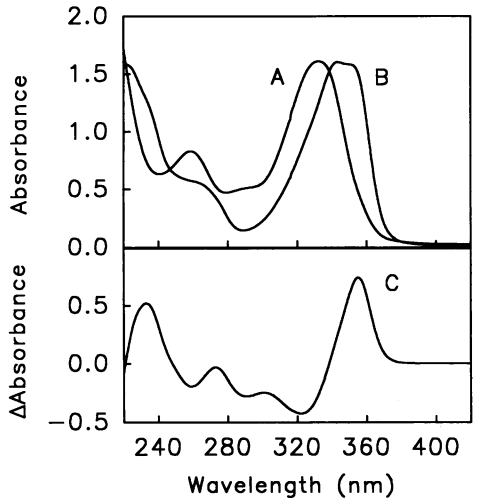


FIG. 1. UV spectra at pH 7.6. (*Upper*) MESG (50 μM) (trace A) and 2-amino-6-mercapto-7-methylpurine (50 μM) (trace B). (*Lower*) Difference spectrum (trace B − trace A).

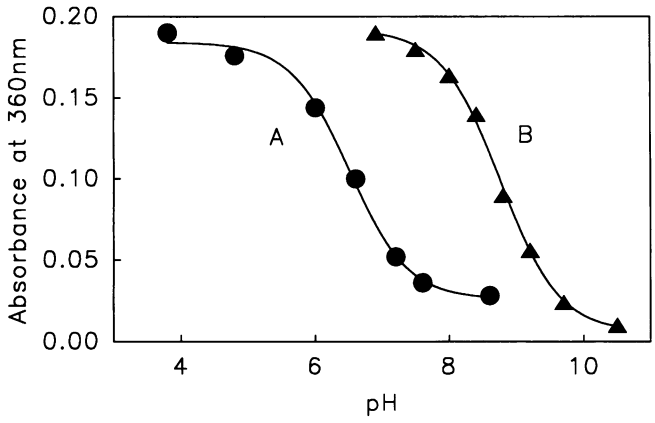


FIG. 2. Change in absorbance at 360 nm as a function of pH for MESG (curve A) and 2-amino-6-mercapto-7-methylpurine (curve B). The curves are theoretical best fits for pK_a values of 6.5 (curve A) and 8.8 (curve B).

production. For the former, the conditions (e.g., the amount of enzyme) were chosen such that the reaction was complete during monitoring the absorbance over ≈5 min. The slope of this graph in Fig. 3 can be used to determine the unknown concentrations of P_i (μM) added to the assay solution

$$[P_i] = 91 \times \Delta A \cdot cm^{-1} \times \text{dilution factor.}$$

These experiments and similar ones at different [MESG] confirm that the phosphorylase reaction is essentially irreversible with this substrate, as described for the 7-methylguanosine analog (11). An equilibrium constant >130 was estimated from measuring the extent of reaction after mixing approximately equimolar P_i and MESG. These experiments also indicate that the reaction is sensitive down to concentrations of 2 μM P_i and can be used to quantify this amount of P_i.

To determine the suitability of this assay system to measure the rate of P_i production, a simple test system was used in which ATP is hydrolyzed to ADP and P_i catalyzed by D-glyceraldehyde and glycerol kinase from *E. coli* (12). In this single protein system, the glyceraldehyde is probably phosphorylated on the aldehyde oxygen, and the resulting product breaks down rapidly to glyceraldehyde and P_i. The measured initial rate of P_i release (data not shown) was linear with

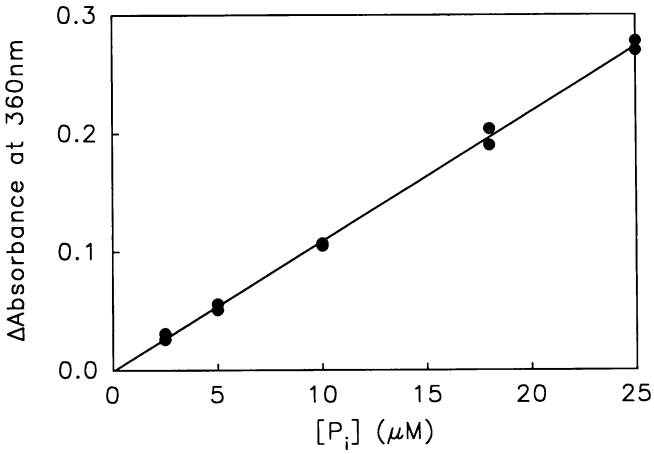


FIG. 3. Change in absorbance at 360 nm due to the phosphorylase-catalyzed reaction of MESG as a function of P_i concentration. Assay conditions (in 2.5 ml) were 50 mM Tris·HCl buffer, pH 7.6/50 μM MESG/phosphorylase (0.2 unit·ml^{-1}). Aliquots (5–20 μl) of KH_2PO_4 solutions were added to the required concentration and the absorbance at 360 nm was monitored for ≈5 min.

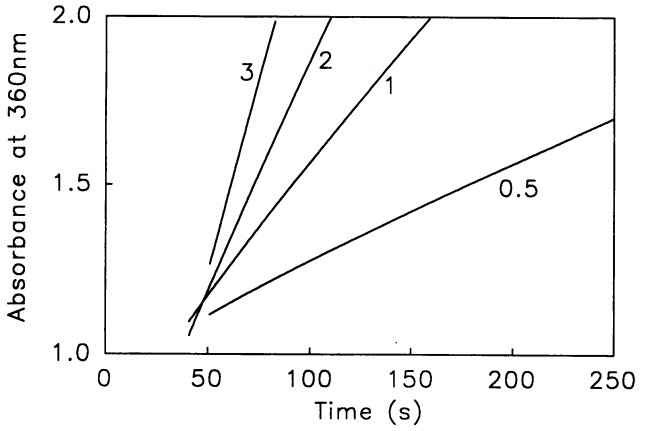


FIG. 4. Changes in absorbance at 360 nm due to the phosphorylase-catalyzed reaction of MESG with P_i released from the actomyosin subfragment 1 ATPase. The solutions at 25°C contained the following in 20 mm Tris·HCl/1 mM $MgCl_2$/5 mM dithiothreitol, pH 7.5: 0.4 mM ATP, 0.2 mM MESG, phosphorylase (12.5 units·ml^{-1}), 0.67 μM myosin subfragment 1, and actin at the micromolar concentration shown on the graph.

glycerol kinase concentration up to the limit imposed by manual mixing in the spectrophotometer cuvette (≈0.02 absorbance unit·s^{-1}).

The linearity of response was tested by using actin-activated ATPase activity of myosin subfragment 1 at different concentrations of actin (Fig. 4). This measured ATPase rate is proportional to actin concentration in the range of actin concentrations used. At the highest [actin], the k_{cat} is 3.4 s^{-1}, which is within the range expected for these conditions. The methodology was also tested by using a single turnover of ATP hydrolysis by myosin subfragment 1 in the absence of actin and the data were fitted to a single exponential (Fig. 5).

DISCUSSION

It has previously been shown that methylation of guanosine at N-7 produces a good substrate for purine-nucleoside phosphorylase in the direction of phosphorolysis (8). This system has been used to measure P_i (3) and to synthesize other nucleoside analogues from the bases, using 7-methylguanosine to cycle P_i back to ribose 1-phosphate, which condenses with the new base (11). 6-Thioguanosine nucleotides have been used to study GTPase mechanisms (13)

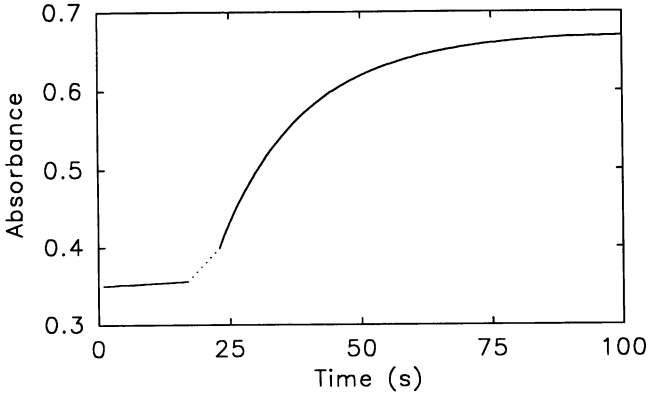


FIG. 5. Single turnover of ATP hydrolysis by myosin subfragment 1, as monitored by P_i release, using the MESG/phosphorylase system. The reaction at 30°C was monitored at 360 nm. The solution contained 20 mM Tris·HCl buffer (pH 7.5), 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM MESG, phosphorylase (12.5 units·ml^{-1}), 34 μM myosin subfragment 1. The reaction was initiated with 31 μM ATP and the period during addition is shown by a dotted line. The data fit a single exponential, with a rate constant of 0.06 s^{-1}.

because of their absorbance properties at 300–360 nm that change on protonation with a pK_a of 8.2–8.6 (14). The combination of these properties was considered to make a substrate for purine nucleoside phosphorylase that might show an absorbance change around physiological pH on phosphorolysis. This nucleoside MESG has a pK_a at 6.5 and shows considerable changes in absorbance properties as a function of pH.

MESG is apparently well behaved as a substrate for the phosphorylase, fitting Michaelis–Menten kinetics with a K_m for P_i of 26 μM. The reaction is essentially irreversible in favor of phosphorolysis: the equilibrium constant is >130. This is very different from the situation with nonmethylated substrates such as guanosine or deoxyinosine, where nucleoside formation is favored and the equilibrium constant is ≈0.02 at pH 7.0 (7, 15, 16). Because the reaction greatly favors phosphorolysis, this MESG/phosphorylase system also acts as a "P_i mop," enabling concentrations of P_i to be reduced below micromolar in solutions being used for biological experiments. Another reaction catalyzed by nucleoside phosphorylase has been suggested for this purpose (17).

The MESG/phosphorylase assay is useful for measuring P_i concentrations in the micromolar range, which is often that of interest for biological solutions. P_i contamination is often present in solutions at micromolar concentrations, so that added P_i at less than this concentration may not give the true value. The assay is simple relative to other real-time assays of P_i as it requires addition of only one enzyme, an important consideration when the reaction is used to measure the kinetics of P_i release. In this case, conditions must be met such that the phosphorylase is not contributing to rate limitation—i.e., that the observed rate of absorbance change is the rate of P_i release. If two enzymes are required to measure the P_i, suitable conditions to measure P_i release rates are likely to be more difficult to find. Although assays based on phosphomolybdate complex formation generally have high sensitivity, they are not particularly suitable for measuring P_i release rates. The need to take aliquots at particular time points for the color reaction limits this approach and makes it unsuitable for rapid reaction kinetics, where a stopped-flow apparatus might be used. A continuous assay as described here allows the complete time course to be followed without further manipulation of samples.

A limitation of the MESG/phosphorylase assay is the thermal instability of the nucleoside MESG to base-catalyzed decomposition, a property shared with 7-methylguanosine (10). This arises because of the positive charge on the purine in turn caused by the methylation, but it is this feature that makes it a good substrate for the phosphorylase (11) and ensures the pK_a change on phosphorolysis. However, aqueous solutions of MESG are stable for >1 month stored at −20°C, and breakdown in the cuvette is minimal over the time course of most assays (a few minutes). A further limitation is imposed by the range of pH values over which there is a significant difference in absorbance between substrate and product (Fig. 2). The assay may not give correct results in the presence of other molecules that are also substrates for the phosphorylase. This includes some other nucleosides (7), arsenate (7), and vanadate (data not shown). In comparison, a wide range of species, including, for example, some buffers, interfere with nonenzymic assays such as those that involve phosphomolybdate complex formation.

The phosphorylase-catalyzed reaction can be coupled to phosphatases to measure the kinetics of phosphate release, and this has been established for a variety of systems to measure rates up to the limits imposed by manual mixing. The D-glyceraldehyde/glycerol kinase system is an active ATPase and this was used to test the linearity of rate response of the MESG/phosphorylase. Using the actomyosin ATPase system also shows linear response with increasing actin

concentration. A single turnover of ATP hydrolysis by myosin subfragment 1 was monitored by this system. The rate of P_i release is the same as the ATPase rate (0.06 s^{-1}) as shown (18) to confirm that P_i release is rate limiting in this reaction. The method has been used to investigate the GTPase-activating protein-activated GTP hydrolysis on $p21^{ras}$ (M.R.W. and J. L. Hunter, unpublished results). Stopped-flow techniques can be used to investigate rapid reaction kinetics for these systems.

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