

Optimization of a Malachite Green assay for detection of ATP hydrolysis by solubilized membrane proteins

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Enzymatic Assays and Analysis: Notes & Tips

Abstract:

We studied the activity of the fluorescently labeled membrane transporter MalGFK₂, which transports maltose at the expense of ATP hydrolysis. We used a commercially available Malachite Green assay (Sensolyte[®] MG Phosphate Assay Kit, Anaspec) to quantify the liberated phosphate upon ATP hydrolysis. However, strong variations in phosphate concentration were measured when using the supplier's handling protocol. We optimized the protocol, taking into account the effects mediated by glycerol, SDS and fluorescence label in the sample. As a result we obtained highly reproducible phosphate concentration values under conditions optimal for solubilized membrane proteins.

Key words: inorganic phosphate detection, malachite green, integral membrane proteins, enzymatic ATP hydrolysis, ABC transporter, fluorescent reporter groups

Advanced fluorescence based methods are widely used to obtain information about structure, function and dynamics of biological macromolecules. When endogenous fluorophores are not present, the biomolecule of interest can be covalently labeled with organic fluorescent dyes [1-4]. A prerequisite to use fluorescently labeled biomolecules in these studies is that the label does not interfere with the biological function of the molecule. Colorimetric assays are often utilized to test the activity of the biomolecule, such as colorimetric phosphate assays for enzymatic ATP hydrolysis [e.g. 5,6], and possible interference of the attached fluorescence reporter group with the color reaction has to be considered. Moreover, in most cases the available protocols are not optimally suited for solubilized proteins. Glycerol and SDS, used for stabilizing the solubilized protein and to quench enzymatic activity before adding the color reagent, respectively, are known to affect the color reaction [5, 7].

In this paper we describe a variation to a Malachite Green assay that introduces advantages for investigating solubilized membrane proteins, using the maltose/maltodextrin ABC-transporter MalGFK₂ as an example. The maltose transporter is a multimeric complex that consists of two transmembrane domains (MalG and MalK), and a cytoplasmic nucleotide binding domain dimer (MalK₂) that binds and hydrolyzes ATP and powers the transport. To study the dynamics of MalGFK₂ and its interaction with the maltose binding protein MalE [8], we labeled the periplasmic side of MalF (in position 177) and MalE in position 13 with the fluorescent dye fluorescein. To achieve covalent binding we used the reactive thiol group of cysteine residues introduced at the desired amino acid position by site-directed mutagenesis [9] and the iodoacetamido-derivative of fluorescein (IAF) [10,11]. Briefly, 400µM of IAF was reacted with 10µM of the respective cysteine variant of MalGFK₂ or MalE in 20%glycerol, 50mM Tris-HCl (pH8.0), 0.01% dodecyl maltoside (DDM), 40µM DTT at room temperature for 1 h. A labeling stoichiometry of about 1:1 was obtained.

Since the MalK subunits power the transport, functional activity of the ABC-transporter is determined by the ability of MalK to hydrolyze ATP under transport conditions, i.e. in the presence of maltose and MalE [9,12]. Non-radioactive colorimetric methods are common to determine the concentration of liberated inorganic phosphate upon ATP hydrolysis, such as the classical molybdate method [5] or the complex formation of phosphomolybdic heteropolyacid with the basic dye malachite green [6]. A disadvantage of the molybdate method is that the reagent containing ascorbic acid must be prepared freshly at the time of use and more than one pipetting step is required for the color reaction [5]. A known drawback of this method is the presence of SDS and glycerol in the sample [5]. SDS only from

certain companies and purity can be used, as other SDS products lead to blue color of the solution interfering with the phosphate-based color development [5]. Glycerol was shown to decrease the phosphate-based absorbance by 67% when using 20% glycerol [5]. The advantage of the malachite green method is that only one pipetting step is required and the coloring reagent stock can be stored at room temperature [6]. The stable malachite green (MG) reagent is also commercially available from Anaspec (Sensolyte[®] MG Phosphate Assay Kit). According to the suppliers' protocol, 20 μ l MG reagent were added to 80 μ l of the test sample and well mixed for 5-10 minutes at room temperature. Color will develop in 10-40 minutes and absorbance at 600-660nm can be measured to calculate phosphate concentration. When using this reagent to test the activity of the solubilized transporter sample, large variations in phosphate concentration (with a coefficient of variation of about 65%) were measured under conditions optimal for the solubilized membrane protein (0.01% DDM, 20% glycerol, 50mM Tris pH 8, SDS to quench ATP hydrolysis).

In the following we describe the modifications introduced in the protocol. Fig. 1A shows the absorption spectrum of the fluorescein labeled maltose transporter in the MalF subunit (MalF(T177C-AF)) with the absorbance band of fluorescein at around 500nm. The absorbance band of the malachite green phosphate complex is relative broad with an absorbance maximum at around 650nm (Fig. 1B). Addition of SDS to the reagent mixture yields a relatively narrow absorbance band with a peak centered at 620nm (Fig. 1B), directly within the recommended wavelength window for color detection between 600-660nm. Thus, a measurement within this wavelength region suffers from a large background absorbance and consequently yields a higher variability in the determined phosphate concentrations or was found to make phosphate analysis impossible [7]. This SDS complex absorbance band, however, is sufficiently blue shifted compared to the malachite green phosphate complex absorbance (Fig. 1B). Thus, using a detection wavelength of 700 nm, the background absorbance due to SDS is negligible and phosphate concentration can be determined with high precision (SEM<1%).

When 20% glycerol is present in the reaction mixture, we found a delay in the kinetics of color development and maximum absorbance is reached after 200 min (Fig. 1C). Using a 5-fold dilution to 4% glycerol, color development is complete after 60 min (Fig. 1C). Since a correlation between ammonium molybdate concentration and color development kinetics was documented [13], we added additional ammonium molybdate (2%) to the reaction mixture containing 4% glycerol to speed up color

development (Fig. 1C). The presence of additional ammonium molybdate accelerated the kinetics sufficiently (Fig. 1C) and reproducible absorbance values can be measured 20 min after addition of the coloring reagent. The accuracy of the determined phosphate concentrations is demonstrated by the calibration curve obtained in the presence of SDS and glycerol (Fig. 1B inset and D). These calibration curves are highly reproducible, with a coefficient of variation of about 4% (Fig. 1D), even when the measurements were several weeks apart (Fig. 1D inset).

Fig. 2A shows control measurements of ABC transporter activity. Since the functional activity of the ABC transporter is determined by the ability of the MalK subunits to hydrolyze ATP under transport conditions, i.e. in the presence of maltose and MalE, the basal activity of MalK (w/o maltose and MalE) serves as a background value. Possible artifacts leading to false positive results in the maltose transporter activity assay are hydrolysis of organic phosphate in acidic environment as present in the MG reagent [6], ATP hydrolysis in the presence of MalE or its fluorescently labeled variants, and color development in the presence of the liberated fluorescent dye fluorescein under acidic conditions. This was tested and the respective control measurements yield absorbance values hardly above the blank measurement, except for hydrolysis of ATP under acid conditions and ATP hydrolysis in the presence of wild type MalE with slightly elevated values (Fig. 2A). These values are, however, well below the values of basal transporter ATPase activity with A_{700} -values of about 0.2OD. As shown in Fig. 2B the basal ATP hydrolysis activity of the transporter is minor compared to the value under transport conditions, i.e. upon addition of MalE and maltose, in agreement with published data [9]. An increase in ATP hydrolysis activity under transport condition was also observed for the fluorescently labeled variants MalF(T177C-AF)GK₂(C40S) and MalE(G13C-AF). The corresponding kinetics of the enzymatic reaction are shown in Fig. 2C and D. For both fluorescently labeled proteins we observed a 2-3 fold increase in activity over the basal value, indicating that fluorescence labeling does not interfere with ABC transporter function.

In summary, our experiments show that two modifications in a commercially available malachite green assay overcome the drawbacks of the presence of SDS and glycerol in the sample to be tested. The detection wavelength of 700nm accounts for the interference with SDS-malachite green complex absorbance and the addition of higher concentrations of ammonium molybdate accelerate the delayed kinetics of color development in the presence of glycerol. Under these conditions highly reproducible

values for the concentration of phosphate can be obtained for membrane protein samples stabilized in glycerol and by using SDS to quench ATPase activity.

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Figure legends

Figure 1

A) Absorbance spectrum of MalF(T177C-AF)GK₂(C40S) in 50 mM Tris pH 8.0, 20% Glycerin, 0.01%DDM, 0.1mM PMSF, 20°C. Thr177 in MalF is exchanged to cysteine to allow fluorescence labeling with IAF. Cys40 of MalK is exchanged to serine to avoid unspecific labeling. The absorbance peak of fluorescein is indicated.

B) Absorbance spectrum of 0.3% SDS (- -) after 3 minutes incubation and absorbance spectra of phosphate standard solution (H₂PO₄-buffer, —) at different P_i concentrations (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50μM) after 20 minutes incubation in the presence of MG reagent. Inset: phosphate standard solution at different concentrations after 20 minutes incubation in the presence of MG reagent, additional 2% ammonium molybdate, 4% glycerol, 0.4% SDS, 0.002% DDM, 0.02mM PMSF, 10mM Tris pH 8.0.

C) Kinetics of color development measured at 700 nm with 6.25μM phosphate buffer in the presence of MG reagent and a) additional 2% ammonium molybdate, 4% glycerol, and b) 4% glycerol. Absorbance values were measured at 650nm for c) with a phosphate concentration of 12.5μM in 20% glycerol. The maximum absorbance of c) was normalized to the values obtained in a) and b).

D) Calibration curves (see text). Inset: Overlay of two calibration curves measured several weeks apart.

Figure 2

A) Control measurements of malachite green color development at 700nm and using additional 2% ammonium molybdate. The samples were incubated for 5 min at 37 °C prior to colorimetric reaction and analysis. Samples a)-c) are in the presence of 0.4% SDS, 10mM Tris pH 8.0, 4% glycerol, 0.002% DDM, 0.02mM PMSF. Samples d)-h) are in the presence of 1.92mM ATP, 9.6mM MgCl₂, 10μM maltose, 0.4% SDS, 10 mM Tris pH 8.0, 4% glycerol, 0.002% DDM, 0.02mM PMSF.

^{a)}Blank value, ^{b)}9.5μM fluorescein (IAF), ^{c)}1.92mM ATP and 9.6mM MgCl₂, ^{d)}0.416mg/ml MalE, ^{e)}0.416mg/ml MalE(G13C), ^{f)}0.416 mg/ml MalE(G13C-AF), ^{g)}0.013mg/ml MalFGK₂(C40S), ^{h)}0.013mg/ml MalF(T177C-AF)GK₂.

B) Basal and transport enzymatic activity of MalFGK₂(C40S), MalFGK₂(C40S) with MalE(G13C-AF) and MalF(T177C-AF)GK₂(C40S). Absorbance values of malachite green color development at 700 nm are given. ATPase reaction conditions in 60μl volume (1.92mM ATP, 9.62mM MgCl₂, 50mM Tris pH 8.0, 20% glycerol, 0.01% DDM, 0.1mM PMSF): Basal - 0.013mg/ml transporter; Transport - 0.013mg/ml transporter, 0.416mg/ml MalE, and 10μM maltose. ATPase activity was quenched by addition of 10μl 10% SDS. The colorimetric MG assay was performed with additional 2% ammonium molybdate after 5-fold sample dilution in H₂O resulting in a final concentration of 4% glycerol and 0.4% SDS. The samples were assayed in duplicate. SD is given.

C) Basal and transport enzymatic activity of MalF(T177C-AF)GK₂(C40S). The kinetics of the normalized P_i concentrations were fitted with $P_{i_{max}} - P_{i_{max}} * \exp(-k*t)$.

D) Basal and transport enzymatic activity of MalE(G13C-AF).



