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Research paper

Methyl-β-cyclodextrin directly binds methylene blue and blocks both its cell staining and glucose uptake stimulatory effects

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Abstract

GLUT1, the most ubiquitously expressed member of the GLUT family of glucose transporters, can be acutely activated by a variety of cell stresses. Methylene blue activates glucose transport activity of GLUT1 in L929 fibroblast cells presumably by a redox cycling of MB, which generates an oxidative stress. Data shown here reveal that methyl-β-cyclodextrin (MCD) blocks both the staining of cells and activation of glucose uptake by directly binding to MB. MCD binding to MB was qualitatively demonstrated by a significantly slower dialysis rate of MB in the presence of MCD. Analysis of the complete spectra of aqueous MB solutions and MB plus MCD solutions by a factor analysis program called SIVVU indicated that these equilibria can be modeled by three species: MB monomer, MB dimer, and MCD–MB inclusion complex. The molar extinction coefficients for each species from 500 to 700 nm were determined. The equilibrium association constant (K_a) for MB dimer formation was measured at $5846 \pm 30\ M^{-1}$ and the K_a for formation of the MCD–MB complex was $310 \pm 10\ M^{-1}$. MCD also dramatically enhances the destaining rate of MB-stained cells. The loss of MB from the cell is tightly correlated with the loss of activated glucose uptake. This suggests that the MB activation of glucose uptake is likely not caused by its redox cycling, but more likely the result of a specific interaction between MB and a protein directly involved in the activation of GLUT1.
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Keywords: Methylene blue; Methyl-β-cyclodextrin; Glucose uptake; GLUT1; L929 fibroblast cells; Cell stress; SIVVU

1. Introduction

Glucose is a critically important energy source for a wide variety of cells, and the ability of cells to respond to changes in cell stress by moderating intracellular glucose concentrations is very important. A family of structurally related glycoproteins, designated as GLUT, is responsible for mediating passive glucose transport in mammalian cells. GLUT1, the most widely expressed member of this family, has traditionally thought to be only responsible for basal level glucose uptake. However, there is increasing evidence that GLUT1 can be acutely activated. Recent work has shown that C-peptide can activate glucose transport via GLUT1 in erythrocytes establishing a potential link between diabetes and GLUT1 activity [1]. GLUT1 is also activated by cell stress. Short-term exposure to cell stressors, such as hyperosmolarity or azide [2–4], nitric oxide [5] or glucose deprivation [6], activates GLUT1 within minutes. Methylene blue (MB), a cationic redox dye, with a history of medical use for the treatment of methemoglobinemia [7], septic shock [8], and endotoxemia [9] also acutely activates GLUT1 in L929 fibroblast cells [10]. There are two proposed mechanisms for this activation. First, MB may directly bind to and alter the activity of a protein within the activation pathway. Or second, MB may have an indirect effect by altering the intracellular concentration of redox cofactors. Methylene blue is known to undergo redox cycling, being reduced to leucomethylene blue by cofactors such as NADPH, and reoxidized by molecular oxygen or cellular proteins. This redox cycling generates oxidative stress in cells, potentially activating glucose uptake [11–13].

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MB-activated glucose transport can be completely blocked by methyl-β-cyclodextrin (MCD) [10], a form of β-cyclodextrin composed of seven methylated D-glucopyranose residues linked in a cyclic structure by α1–4 glycoside bonds. The cyclodextrins (CD) have a toroidal structure consisting of a hydrophilic exterior and a relatively hydrophobic interior, which makes them ideal to form inclusion complexes with guest molecules. Binding specificity is determined by chemical modifications of the CDs and the size of the interior space. As such, CDs have a number of applications, including uses for drug and gene delivery [14–16], and in the construction of thin layers, monolayer films or multilayered assemblies that contain selective binding sites [17,18]. MCD itself is known to bind cholesterol in its hydrophobic core, and therefore, has found wide use as a tool to alter the cholesterol content (deplete or deposit) of cell membranes [19,20].

In addition to inhibiting the activation of GLUT1 by MB, MCD also blocks cell staining by MB [10]. Staining of cells appears to require that MB be reduced at the surface of cells to the colorless, less polar leucomethylene blue, which then enters the cell. Treatment of cells with both MB and potassium ferricyanide, an agent that does not enter cells and maintains MB in its oxidized form, completely blocks cell staining [10,13]. The lack of any staining under these conditions also suggests that MB does not simply bind to the surface of cells. Two possible mechanisms could explain why MCD inhibits MB cell staining: MCD may interfere with the enzymatic reduction of MB at the cell surface [11–13], or it may physically bind to MB and block staining. The latter possibility is supported by a number of studies investigating the interactions between some CD derivatives and MB [21–26]. However, none of these studies have investigated interactions between the cationic MB and the more hydrophobic CD derivative, MCD. Therefore, the specific purposes of this study are: (1) to provide evidence for the formation of an MCD–MB complex which would explain the inhibition of cell staining, (2) to determine if MCD, in addition to inhibiting MB staining, can also accelerate the destaining of pre-stained cells, and (3) to correlate the level of MB staining to the magnitude of glucose uptake during the destaining process in order to gain a better understanding of how MB activates glucose uptake. If MB activates glucose uptake directly there should be a tight correlation between staining level and glucose uptake. On the other hand, if the MB effect is indirect, there should be a lag time as the concentrations of reduced cofactors recover, such that the loss of MB staining should be faster than the loss of activated glucose transport.

2. Materials and methods

2.1. Dialysis experiments

Three 4.0 mL aqueous solutions containing either 50 μM MB, 50 μM MB + 100 mM MCD, or 50 μM MB + 700 mM D-glucose were prepared and placed into dialysis tubing with 12,000–14,000 molecular weight cutoff. Each was placed in 300 mL of distilled water and dialyzed for 2 h with constant stirring. The absorbance of each solution was measured at 664 nm pre- and post-dialysis and absorbances were adjusted for volume changes. The percent optical density lost was calculated.

2.2. Measurement of equilibrium constants

Methylene blue has two absorbance peaks in the visible spectrum, one around 660 nm, largely attributed to the monomer form of MB, and a second, a shoulder, around 610 nm, largely attributed to the dimer form [22,27–29]. To measure the MB monomer–dimer equilibrium, MB solutions ranging from 0.5 to 60 μM were prepared. To analyze the MB–MCD equilibrium a set of solutions containing 20 μM MB with concentrations of MCD ranging from 0.01 to 50 mM were prepared. The absorbance spectra from 500 to 700 nm for both solution sets were obtained using a Cary50 UV–vis spectrophotometer and complete spectra were analyzed using an equilibrium-restricted factor analysis program called SIVVU. The free energy and equilibrium constant for each equilibria were determined as well as the molar extinction coefficients for the monomer, the dimer and the MB–MCD complex at each wavelength. Models with and without a chloride anion included in the dimer were tested. All spectra were obtained at equilibrium.

2.3. Cell growth and glucose uptake measurement

Approximately 1.2×10^5 L929 fibroblast cells were plated into each well of a 24-well culture-treated plate and incubated overnight at 37 °C in humidified room air supplemented with 5% CO_2 in low glucose (5.5 mM) DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The experiments investigating the destaining effects of MCD were initiated by staining the cells with medium containing 50 μM MB for 30 min. Cells were washed once to remove extracellular MB and then incubated in fresh DMEM medium (0.8 mL) with or without 10 mM MCD for the time indicated in the figure legends. Cells were then lysed in 0.5 mL lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 0.4% SDS), which exposed the cell contents to oxygen in order to oxidize any colorless leucomethylene blue in the cells back to the colored MB. Total MB content in the cells could then be determined by absorbance at 664 nm. In experiments directly comparing the level of MB staining to the magnitude of glucose uptake, glucose uptake measurements were performed before cell lysis.

Glucose uptake was measured using radiolabeled 2-deoxyglucose (2-DG) as previously described [6]. Briefly, the medium was replaced with 0.3 mL of glucose-free HEPES buffer (140 mM NaCl, 5 mM KCl, 20 mM HEPES/Na pH 7.4, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mM NaPyruvate, 1 mM mannitol) containing the same experimental agents and concentrations as in the incubation period, but supplemented with 1.0 mM [1,2-^{3}H]2-DG (0.3 μCi/mL) and 1.0 mM [1-^{14}C]mannitol (0.02 μCi/mL). After a 10-min incubation, cells were washed 3× with cold glucose-free HEPES. The

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cells were lysed, the absorbance at 664 nm was taken, and the [^{3}H]2-DG uptake with [^{14}C]mannitol as the extracellular marker was measured using scintillation spectrometry.

All 2-DG uptake data points on the tables and figures are at least triplicate measurements of a representative experiment. A two-tailed paired t-test was used to show statistical significance.

3. Results and discussion

MCD completely blocks the MB staining of L929 fibroblast cells and thereby also blocks activation of glucose uptake [10]. It has been suggested that either MCD alters the reductase activity responsible for the reduction of MB to its more hydrophobic form, leucomethylene blue, or that there is a direct interaction between MCD and MB that blocks MB action. Direct interactions between MB and other cyclodextrins have been noted, but none of these studies have measured binding of MB to MCD. We employed dialysis in a simple experiment designed to detect direct MB–MCD binding. As seen in Table 1, 81.1% of MB diffused through the dialysis tubing, but in a solution containing MB plus MCD only 9.0% of the dye diffused out. The dialysis of MB in the presence of an equivalent amount of glucose was not altered (79.4% loss) suggesting a direct interaction of MB and the cyclized glucose derivative, MCD.

While binding constants between MB and other cyclodextrins have been measured [22–26], the binding constant to MCD has not. The 1:1 inclusion complex is illustrated by:

$$MB^+ + MCD \rightleftharpoons MB \cdot MCD^+ \quad (1)$$

However, this binding equilibrium is complicated by the formation of MB dimers in solution [22,27–29]

$$2MB^+ \rightleftharpoons MB_2^{+2} \quad (2)$$

This can be seen in a series of MB blue solutions as the ratio of the peak height to the shoulder height changes with the concentration of MB. Others have identified this equilibrium as written in reaction (2) and ascertained values for the equilibrium constant, but did so based on single wavelengths. We used a program called SIVVU to model the absorbance spectra of 11 aqueous MB solutions ranging in concentration from 0.5 to60 μM. When this program optimizes the free energy of reaction (2) and solves for the molar absorptivity values of the monomer and dimer forms of MB, the root-mean-square of the differences between the calculated and the observed absorbance values is 0.0166. The free energy for the dimerization is found to be −21.20 ± 0.02 kJ/mol, which corresponds to an equilibrium constant of 5846 ± 30 M^{-1} at 21 °C. This is in good agreement with previous results that report values ranging from 2800 M^{-1} [28] to 6940 M^{-1} [30]. The measurement reported in this study is based on the spectral data from 400 to 800 nm as opposed to analysis based on single wavelength measurements at the two peaks, 612 and 664 nm, resulting in a more precise measurement. However, because there is evidence in the literature that chloride anions may be associated with methylene blue aggregates [31], we also tested a model wherein dimerization proceeds with simultaneous inclusion of a single chloride anion.

$$2MB^+ + Cl^- \rightleftharpoons MB_2Cl^+ \quad (3)$$

This led to a better fit of our data with a root-mean-square of the differences between the calculated and the observed absorbance values of 0.0056, and ΔG value for reaction (3) of −44.4 ± 0.8 kJ/mol. This corresponds to a much greater equilibrium constant (7.7 ± 2.0 × 10^7 M^{-2}) for this three-way association reaction (see Table 2). This makes sense based on electrostatics, and we believe this represents a more accurate chemical model, but further studies are merited. The optimized molar absorptivity curves for the monomer and dimer are shown in Fig. 1.

Equilibria (1) and (3) were used together to model absorbance data from a series of eight solutions containing 20 μM MB and 0 to 2500 equivalents of MCD (see Table 2). The resulting molar absorptivity values of the MB–MCD inclusion complex were determined and are shown in Fig. 1. SIVVU analysis of these data yields a ΔG for reaction (1) of −14.02 ± 0.1 kJ/mol and a K_a = 310 ± 10 M^{-1}, with an RMS residual of 0.0045. This value fits nicely with data from Zhang et al. who utilized the fluorescence properties of MB to measure MB binding constants to a series of cyclodextrin derivatives [22]. Again, utilization of data from the entire spectra in contrast to using single fluorescent data points produced a more reliable measure of the K_a.

Whenever equilibrium constants are determined from spectroscopic data, it is important to verify that the constants are sensitive to changes in the composition of the solutions over the range of the measurements, and vice versa [31]. In both cases above, concentrations were sufficiently dilute to ensure weak binding ($K_a \cdot$[Host] < 12), a requirement if equilibrium concentrations are to be sensitive to K_a. Also, the reactions themselves were driven well past 20% completion (32% for reaction (1) and 99% for reaction (2)) to ensure that the K_a is sensitive to the variation in the composition of the solutions.

MCD effectively blocks the staining of cells with MB [10]. We were curious to learn if MCD would also enhance the rate of destaining. L929 fibroblast cells were stained, washed, and incubated in fresh medium with or without 10 mM MCD. After 10, 20, 30, or 60 minutes the medium was removed, cells were lysed and absorbance at 664 nm was measured. The results are shown in Figure 2. MCD clearly enhances the destaining rate. After 10 minutes in the presence of MCD, only 11.6% of MB remains in the cell and after 60 minutes

Table 1
Loss of MB dialysis in the presence and absence of MCD

Solutions dialyzed	% MB loss
50 μM MB	81.1
50 μM MB + 100 mM MCD	9.0
50 μM MB + 700 mM D-glucose	79.4

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Table 2
Equilibrium-restricted factor analysis of spectrometric data

	Dimerization of MB	Dimerization of MB with chloride	Complexation of MB with MCD
K_a (M^{-1})	5846 ± 30	7.7 ± 2.0 × 10^7	310 ± 10
ΔG_{294} (kJ/mol)	−21.20 ± 0.02	−44.4 ± 0.8	−14.02 ± 0.1
RMS residual	0.0166	0.0056	0.0045
Final R^2 (%)	99.91	99.99	99.994
Restricted data reconstruction (2 chemical factors) (%)	99.551	98.41	99.30
Unrestricted data reconstruction (2 mathematical factors) (%)	99.558	99.558	99.36
Total curves	11	11	8
Fixed molar extinction curves	0	0	0
Activity coefficients model	None	None	None
Concentration range	0.50–60 μM MB	0.50–60 μM MB	20 μM MB, 0–50 mM MCD
Reaction coordinate range (%)	0–32	0–32	0–99
Overall contribution of MB in absorbance data (%)	86.85	91.57	42.78
Solvent	Water	Water	Water

only 2.9% remains. In contrast, after 30 minutes of destaining without MCD, 52.0% of the stain remains and 30.6% is still present in the cell after an hour. It is not clear if the enhanced destaining rate is entirely due to simple trapping of MB as it exits the cell, or if MCD actually enhances the efflux rate of MB.

Methylene blue activates glucose transport in L929 fibroblast cells [10], but it is not clear if MB interacts directly with a protein required in the activation mechanism, or if MB acts indirectly by a redox cycling process that drains the cell of reduced cofactors, stimulating glucose uptake [10–13]. This second possibility predicts that there would be a lag between loss of MB from the cell and the recovery of the concentrations of reduced cofactors. In order to gain insight into whether MB acts directly or indirectly, the correlation between the level of staining and the magnitude of glucose uptake was investigated. In two control experiments, L929 cells were initially stained for 30 min in the presence and absence of 10 mM MCD after which the 2-DG uptake and concentration of MB were measured immediately. The experimental cells were stained for 30 min without MCD present and then destained in the presence and absence of 10 mM MCD for 15 min. The results are shown in Table 3. It is clear the MCD prevents both the staining and activation of glucose transport as previously reported [10]. Cells treated with MB in the

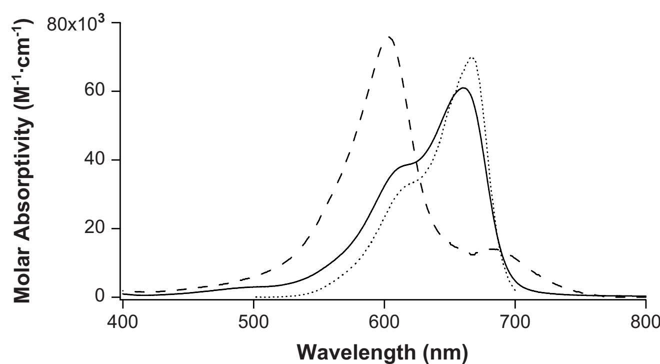

Fig. 1. Molar absorptivity curves for methylene blue monomer (solid), dimer monochloride (dashed), and methylcyclodextrin–methylene blue complex (dotted) as determined via equilibrium-restricted factor analysis. The magnitude of the absorptivity of the dimer per molar of MB would be half of what is shown.

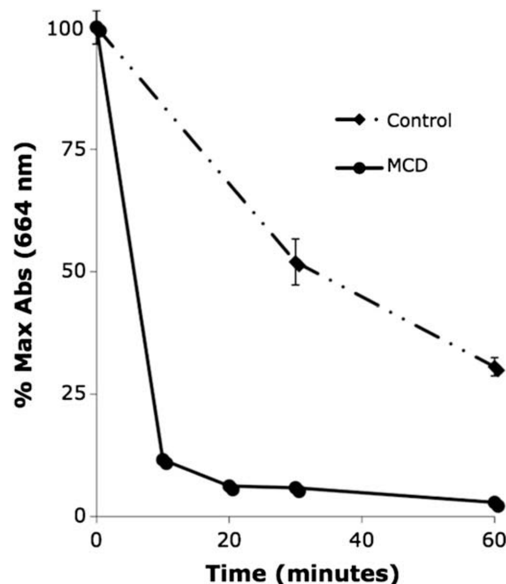

Fig. 2. Rates of destaining in the presence and absence of MCD. L929 fibroblast cells were stained for 30 min using medium containing 50 μM MB. The medium was removed and fresh medium without (Control) or with 10 mM MCD (MCD) was added. The destaining medium was removed at the indicated times, cells were lysed, and absorbances at 664 nm were obtained. Data are normalized to a percent of maximum absorbance and are displayed as means ± standard errors. All absorbances from MCD-treated cells are statistically lower than their corresponding control at $P < 0.01$.

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Table 3
Effects of MCD on staining and destaining by MB and 2-DG uptake

Staining conditions	Destaining	OD (664 nm)	2-DG uptake (nmol/10 min per well)
50 μM MB + 10 mM MCD	None	0.011 ± 0.001	1.31 ± 0.10
50−μM MB	None	0.578 ± 0.018	5.75 ± 0.22
50−μM MB	15 min	0.394 ± 0.014	4.15 ± 0.16
50−μM MB	15 min + 10 mM MCD	0.058 ± 0.007	1.37 ± 0.03

presence of MCD incorporated less than 2% of the MB that was taken up by the cell when MCD was not present (OD of 0.011 compared to 0.578) and 2-DG uptake was 1.31 ± 0.10 nmol/10 min per well, which was not different from unstained cells (data not shown). The 15-min incubation with MCD removed a significantly greater amount of the dye (0.058 OD remaining compared to 0.394) and it reduced 2-DG uptake more effectively (1.37 ± 0.03 compared to 4.15 ± 0.16 nmol/10 min per well). The parallel losses of MB staining and activation of 2-DG uptake are illustrated in Fig. 3 as a percent of the maximum response. The data indicate that when 67.0% of the MB remains in the cell, the stimulation of 2-DG is 64.0% of the maximum and when only 8.3% of the MB remains, 2-DG uptake is 1.4% of the maximum response. There appears to be no lag between the loss of MB and the loss of activated glucose uptake. These data are consistent with MB activating glucose uptake by directly binding to a protein, rather than acting indirectly through the loss of reducing cofactors.

In conclusion, this study enhances our understanding of the MB monomer–dimer equilibrium and reports for the first time the value of the equilibrium constant for the 1:1 inclusion complex between MB and MCD. MCD can block both MB staining and activation of glucose uptake in L929 fibroblast cells and significantly enhances the destaining of MB-stained cells. While the exact mechanism of how MB activates

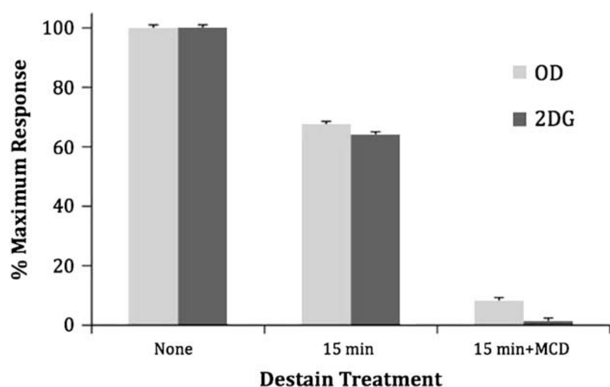

Fig. 3. Comparison of the concentration of MB and the magnitude of 2-DG uptake. OD and 2-DG uptake data sets from Table 2 were each normalized to their maximum response. Maximum response was determined by subtracting data obtained when cells were treated with MB + MCD from the values obtained when cells were treated with MB with no destaining. Results from experiments in triplicate are displayed as means ± standard error for direct comparison.

glucose uptake in L929 cells requires further investigation, the tight correlation between the level of MB staining and the activation of glucose uptake reported here, suggests that MB acts directly rather than indirectly via redox cycling as previously proposed [10–13].

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