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Measuring the bioactivity and molecular conformation of typically globular proteins with phenothiazine-derived methylene blue in solid and in solution: A comparative study using photochemistry and computational chemistry☆

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ABSTRACT

Methylene blue is a phenothiazine agent, that possesses a diversity of biomedical and biological therapeutic purpose, and it has also become the lead compound for the exploitation of other pharmaceuticals such as chlorpromazine and the tricyclic antidepressants. However, the U.S. Food and Drug Administration has acquired cases of detrimental effects of methylene blue toxicities such as hemolytic anemia, methemoglobinemia and phototoxicity. In this work, the molecular recognition of methylene blue by two globular proteins, hemoglobin and lysozyme was characterized by employing fluorescence, circular dichroism (CD) along with molecular modeling at the molecular scale. The recognition of methylene blue with proteins appears fluorescence quenching via static type, this phenomenon does cohere with time-resolved fluorescence lifetime decay that nonfluorescent protein–drug conjugate formation has a strength of 10^4 M^{-1}, and the primary noncovalent bonds, that is hydrogen bonds, π-conjugated effects and hydrophobic interactions were operated and remained adduct stable. Meantime, the results of far-UV CD and synchronous fluorescence suggest that the α-helix of hemoglobin/lysozyme decreases from 78.2%/34.7% (free) to 58.7%/23.8% (complex), this elucidation agrees well with the elaborate description of three-dimensional fluorescence showing the polypeptide chain of proteins partially destabilized upon conjugation with methylene blue. Furthermore, both extrinsic fluorescent indicator and molecular modeling clearly exhibit methylene blue is situated within the cavity constituted by α_1, β_2 and α_2 subunits of hemoglobin, while it was located at the deep fissure on the lysozyme surface and Trp-62 and Trp-63 residues are nearby. With the aid of computational analyses and combining the wet experiments, it can evidently be found that the recognition ability of proteins for methylene blue is patterned upon the following sequence: lysozyme < hemoglobin < albumin. Basically, the distinction originates from different spatial structures of proteins and noncovalent interactions between proteins and methylene blue. In addition, biological relevance of the biorecognition of methylene blue with proteins was briefly discussed. We hope that this study could provide further standpoint so that one explore the biological activity of methylene blue and also phenothiazines.

1. Introduction

Methylene blue (structure shown in Fig. 1) is one of the basic dyes with the structure of heterocyclic aromatic chemical compound. It is an interesting compound, widely used in the different areas of industry, chemistry, biology and medicine [1]. For example, it is utilized for the manufacture of color pens, polygraphic paints, and coloring industrial products. The beneficial effects of methylene blue in the clinical fields including the management of septic shock, renal stones, encephalopathy, cyanide and nitrite poisoning, urinary tract infections, alleviate

Abbreviations: Ala, alanine; ANOVA, analysis of variance; ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; DNA, deoxyribonucleic acid; Ile, isoleucine; IRF, instrument response function; Leu, leucine; Lys, lysine; Phe, phenylalanine; R, correlation coefficient; S.D., standard deviation; Ser, serine; Tris, tris(hydroxymethyl)aminomethane; Trp, tryptophan; Tyr, tyrosine; UV/vis, ultraviolet–visible spectroscopy; Val, valine.

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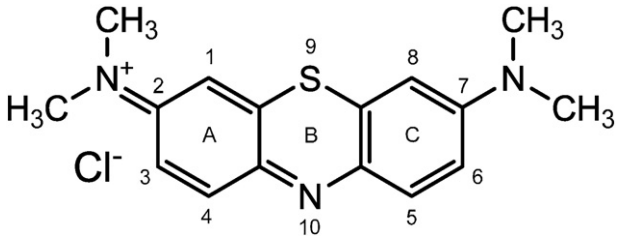

Fig. 1. Molecular structure of methylene blue.

the postoperative adhesions, treatment of cognitive disorders occurring in psychoses, slow down cognitive decline in Alzheimer's disease, etc. [1,2]. Methylene blue can also be used as an indicator to determine whether cells are alive or not. Nowadays it is a U.S. Food and Drug Administration grandfathered autoxidizable phenothiazine compound with potent antioxidant and metabolic enhancing properties at low doses [3].

However, in vivo clinical trials have confirmed that acute exposure to methylene blue will induce increased heart rate, vomiting, shock, Heinz body formation, cyanosis, jaundice and quadriplegia a tissue necrosis in humans [4]. In two very early studies, Nadler et al. [5] found that intravenous injection of high concentration of methylene blue in man and white male infant could yield pericardial pain, dyspnea, restlessness, apprehension, tremors, a sense of oppression, methemoglobinemia, hemolysis, reticulocytosis and slight anemia. Mahadevan et al. [6] impregnated methylene blue into women granulosa lutein cell and discovered that methylene blue repress progesterone production through granulosa lutein cell in a dose dependent mode, methylene blue may be taken up and handled by granulosa lutein cell, probably in lysosomes. This may somehow restrain progesterone synthesis or secretion or both by granulosa lutein cell without acute toxicity resulting in direct cell death, these data demonstrate now that methylene blue is a reproductive toxicant. Later, Lee and Wurster [7] clearly further clarified that methylene blue can cause cytotoxicity in SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cells in the concentration of micromolar level. In a more recent two-year study, finished in 2008 by the National Toxicology Program, it was verified that carcinogenic activity of methylene blue was measured in male F344/N rats and male B6C3F_1 mice, and equivocal carcinogenicity was also detected in female B6C3F_1 mice based on marginally raised incidences of malignant lymphoma [8]. Due to methylene blue having been universally employed in many realm (especially in medicine), in vitro and in vivo experiments have distinctly proved various dangerous symptoms evoked by methylene blue, thus it may symbolize a quite likely fatal risk for human beings. Therefore the medical use of methylene blue, e.g. phototreated plasma was no more permitted in some countries, such as Switzerland [9].

Molecular recognition in biopolymer–ligand complexes is responsible for the selective binding of a low molecular weight chemical to a specific target. In biological systems, the biomacromolecule–ligand interactions undertake a particular object, i.e. signal transduction, enzyme cooperativity, physiological response, substance transportation and other processes such as carrying oxygen by hemoglobin [10,11]. These are generally noncovalent in nature and the noncovalent interaction of molecular recognition is essential for nearly all biological processes. The interactions between ligands and biological macromolecules are thereby of vital significance for the drug design and the invention of new medicines to profit human health [12,13]. Under these circumstances, the study of methylene blue conjugation to globular proteins represents a very significant device to obtain pharmacological and toxicological information. A wide range of experimental and to a lesser extent theoretical techniques have been developed for inspecting the bioactivity and molecular conformations of protein–ligand interaction, including calorimetry, chromatography, circular dichroism, crystallology, electrophoresis, equilibrium dialysis, fluorescence, Fourier transform infrared spectroscopy, light scattering, nuclear magnetic resonance, rheology, small-angle neutron scattering, surface enhanced Raman scattering, surface tension, turbidity, ultracentrifugation and ultrafiltration [10–25]. Among them, fluorescence is the only appropriate tool to determine the most comprehensively qualitative and quantitative parameters on the protein–ligand reaction according to the notions of Hovius et al. [26] and MacManus-Spencer et al. [27,28]. Very recently, Ortiz et al. [29], Masetti et al. [30], Vardevanyan et al. [31] and Xu et al. [32] tested the binding of methylene blue to single stranded DNA by using different biophysical techniques, and found that methylene blue bound to DNA occurs essentially with the 2′-deoxyguanosine 5′-phosphate nucleotide, a highly electrostatic interaction contribution to the binding process.

The aim of the present contribution was to examine the biomolecular recognition of the complex formed between phenothiazine-derived methylene blue and the globular proteins hemoglobin and lysozyme by means of steady-state and time-resolved fluorescence, circular dichroism (CD), synchronous fluorescence and three-dimensional fluorescence spectra. Binding location of methylene blue was probed with 8-anilino-1-naphthalenesulfonic acid (ANS) displacement experiments and affirmed by computer-aided molecular modeling utilizing the available X-ray crystal structure of the two proteins. We have also compared the biorecognition of methylene blue by albumin, since this macromolecule is capable of binding to a diverse range of bioactive ligands, including methylene blue. These original outcomes will likely provide a better understanding of the molecular recognition occurring between charged biomacromolecules and the controversial compound methylene blue in vivo and thus give insight into processes that take place in several biological systems.

2. Experimental

2.1. Materials

Hemoglobin human (H7379, lyophilized powder), lysozyme (L4919, BioUltra, lyophilized powder, ≥98%), methylene blue (M9140) and 8-anilino-1-naphthalenesulfonic acid (A1028, ≥97%) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Deionized water was generated by a Milli-Q Ultrapure Water Purification Systems from Millipore (Billerica, MA) and all the experiments were performed in Tris (0.2 M)–HCl (0.1 M) buffer of pH = 7.4, with an ionic strength 0.1 in the presence of NaCl, except where specified, the pH was checked with an Orion Star A211 pH Benchtop Meter (Thermo Scientific, Waltham, MA). Dilutions of the hemoglobin (30 μM) and lysozyme (16 μM) stock solution in Tris–HCl buffer were prepared immediately before use and the concentrations of proteins were measured by the method of Lowry et al. [33]. All other reagents utilized were of analytical grade and received from Sigma-Aldrich. To remove any undissolved matter, all samples were filtered through a 0.22 μm Millex-GV Filter (Millipore, Billerica, MA).

2.2. Fluorescence Emission

Steady-state fluorescence measurements were carried out with a 1.0 cm path length quartz cell using an F-7000 spectrofluorometer (Hitachi, Japan) equipped with a thermostatic bath. The excitation and emission slits were set at 5.0 nm each, intrinsic fluorescence was acquired by exciting the continuously stirred protein solution at 295 nm to favor tryptophan (Trp) excitation, and the emission spectra were gathered in the wavelength range of 300–450 nm at a scanning speed of 240 nm min^{-1}. The reference sample consisting of the Tris–HCl buffer of methylene blue in corresponding concentrations was subtracted from all fluorescence measurements.