Polyhedron 31 (2012) 530–538

Contents lists available at SciVerse ScienceDirect

Polyhedron

journal homepage: www.elsevier.com/locate/poly

Antioxidant activity of methimazole–copper(II) bioactive species and spectroscopic investigations on the mechanism of its interaction with Bovine Serum Albumin

Nora M. Urquiza [a], Luciana G. Naso [c], Silvia G. Manca [a], Luis Lezama [b], Teófilo Rojo [b], Patricia A.M. Williams [c], Evelina G. Ferrer [c,*]

[a] Cátedra de Química Analítica, Instituto de Química Analítica, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, 4000 San Miguel de Tucumán, Argentina
[b] Departamento de Química Inorgánica, Facultad de Ciencia y Tecnología, Universidad del País Vasco, Apdo 644, 48080 Bilbao, Spain
[c] Centro de Química Inorgánica (CEQUINOR/CONICET, UNLP), Facultad de Ciencias Exactas, UNLP, C. Correo 962, 1900 La Plata, Argentina

ARTICLE INFO

Article history:
Received 13 July 2011
Accepted 6 October 2011
Available online 17 October 2011

Keywords:
Methimazole
Methimazole–copper(II) complex
Graves' disease
EPR
FT-IR and UV–Vis spectroscopies
SOD activity

ABSTRACT

Free radical-mediated oxidative stress has been implicated in numerous autoimmune disorders including Graves' disease. Hyperthyroidism results in a marked increase in intracellular antioxidant enzymes including superoxide dismutase. The later activity is significantly increased in untreated Graves' patients while treatment with methimazole results in normalization of the free radical and antioxidant activity indices. In this context, and considering the findings mentioned above, the aim of the present study was to evaluate unknown biological activities of methimazole and its methimazole–copper(II) complex investigating their superoxide scavenger power. Under the applied experimental conditions, methimazole did not show superoxide dismutase (SOD) activity while the copper complexes exhibited a strong superoxide radical scavenging capacity. Bearing in mind that the capacity of drugs to bind and/or interact with albumin is essential for their pharmacokinetic and pharmacodynamic properties, a complete investigation of the binding ability of both compounds by using Fourier transform infrared (FT-IR), Raman and Fluorescence spectroscopies, and UV–Vis spectrophotometry was included. Besides, in order to probe the copper ligand environment the EPR spectra of such compounds were analyzed.

1. Introduction

Methimazole (1-methyl-3H-imidazole-2-thione) (Fig. 1) is one of the most widely used antithyroid drugs for treating Graves' hyperthyroidism. It is quickly absorbed from the gastrointestinal tract and persists in serum after drug ingestion (serum half-life is 6–8 h) [1]. The single daily dose of methimazole is 15–30 mg per day for hyperthyroidism, and "minor" side effects including cutaneous reactions (usually urticaria or macular rashes), arthralgia and gastrointestinal upset have been reported in approximately 5% of patients. Recently, our group began to investigate aspects related to unknown potential biological activities of methimazole and its copper(II) complexes demonstrating that both are capable to inhibit alkaline phosphatase activity [2]. In particular, our interest is to study the antioxidant activity of methimazole and methimazole–copper(II) bioactive species. Our interest in the determination of the superoxide dismutase (SOD)-like activity is based in the fact that during Graves' disease there is an increase in the formation of reactive oxygen species (ROS) and other free radicals producing oxidative stress [3]. Hyperthyroidism-induced dysfunction of the respiratory chain in mitochondria leads to ROS production and induces changes in the antioxidant protective system potential. ROS generate a drastic increment of the SOD activity in erythrocytes, which decreases significantly when patients are treated with methimazole [4].

It is well known that serum albumin is the most important transport protein in plasma and is also capable of binding and transporting metabolites, drugs, dyes and organic compounds. This protein often increases the apparent solubility of hydrophobic drugs in plasma and modulates the circulation, metabolism and effectiveness of many drugs both *in vivo* and *in vitro* [5,6]. It has been also demonstrated that protein–ligand interactions play an important role in a variety of biological processes. The investigation of compounds with respect to their binding to albumin becomes important because of the pharmacokinetic and pharmacodynamic role of such binding. These studies provide significant information on the structural features that determine the therapeutic efficacy of drugs, and hence constitute an important research field in chemistry, life sciences and medicine. Bovine Serum Albumin (BSA) (76%

* Corresponding author. Tel./fax: +54 0221 4259485.
E-mail address: evelina@quimica.unlp.edu.ar (E.G. Ferrer).

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doi:10.1016/j.poly.2011.10.008

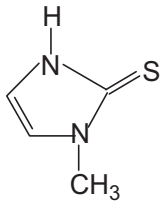

Fig. 1. Methimazol (1-methyl-3H-imidazole-2-thione).

similar to Human Serum Albumin, HSA) is a globular non-glycosylated single-chain protein of 582 amino acid residues cross-linked with 17 cystine residues (eight disulfide bonds and one free thiol). BSA has two tryptophans, Trp-134 and Trp-214, of which the first is more exposed to a hydrophilic environment, whereas the second is deeply buried in the hydrophobic loop. Aqueous BSA solutions are stable and homogeneous. There is evidence of conformational changes in BSA induced by its interaction with low molecular weight ligands. These changes appear to affect the secondary and tertiary structure of albumin. These molecular interactions are often monitored by spectroscopic techniques (FT-IR, Raman, UV–Vis). Fluorescence anisotropy has been found to be intrinsically high and sensitive to relevant environmental changes that increase the accessibility of quenchers to albumin's fluorophores, thus helping to clarify the mechanisms of albumin binding to compounds and providing evidences about the nature of the binding phenomenon [7,8].

The present work provides a deeper insight into the formation of complexes between bioactive substances (methimazole and methimazole–copper(II) species) and BSA using Fourier transform infrared (FT-IR), Raman and Fluorescence spectroscopies, UV–Vis spectrophotometry, and also EPR measurements to probe the copper ligand environment. It is important to remark that the structural and conformational changes of the protein and the alterations in the metal complex surroundings upon binding to the coordination complex have not been adequately addressed by previous studies.

2. Materials and methods

Bovine Serum Albumin BSA (A-6003, essentially fatty acid-free) was obtained from Sigma Chemical Company (St. Louis, MO) and used as supplied. Copper(II) nitrate trihydrate was obtained from Merck. Methimazole, Nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide/reduced (NADH) and all the other analytical grade chemicals used were purchased from Sigma. Methimazol–copper(II) complex ([Cu(MeimzH)$_2$(H$_2$O)$_2$](NO$_3$)$_2$·H$_2$O═Cu(II)–MeimzH) was prepared and purified according to published procedures [2].

2.1. SOD assays

The SOD mimetic activity was determined by a non-enzymatic method. In this method, the system (PMS)/(NADH) produces the superoxide anion radical. The system contains 0.5 mL of sample, 0.5 mL of 1.404 mM NADH, 0.5 mL of 300 μM NBT, in 0.05 M phosphate buffer (pH 7.5). After incubation at 25 °C for 15 min, the reaction starts by the addition of 0.5 mL of 120 μM PMS [9]. The solutions of methimazol–copper(II) complex were prepared in hot dimethylsulfoxide (DMSO) before adding the phosphate buffer to obtain the desired final concentrations. The final DMSO to buffer concentration ratio never exceeded 5:100. Then, the reaction mixture was incubated for 5 min at 25 °C. For comparative purposes free ligand and free copper(II) were also tested under the same experimental conditions. The results were determined by reading the absorbance at 560 nm. The amount of complex that produced a 50% inhibition of NBT reduction was obtained from a plot of percentage of inhibition *versus* complex concentration.

2.2. BSA interactions

BSA was dissolved in Tris–HCl (0.1 M, pH 7.4) buffer to attain a final concentration of 4% w/v (~0.6 mM). Methimazole and copper(II) salts solutions were added dropwise to the 2% w/v BSA solution (~0.3 mM) with constant stirring to ensure the formation of an homogeneous solution and to obtain the desired concentration of 0.08–0.5 mM. Whereas Cu(II)–MeimzH was not soluble in water and only scarcely soluble in phosphate buffer, an adequate solubility was reached under these experimental conditions allowing measurements. For each sample and concentration, three independent replicates were performed. These solutions were used for fluorescence measurements, which were carried out on a Perkin–Elmer LS-50B luminescence spectrometer (Beaconsfield, England) equipped with a pulsed xenon lamp (half peak height <10 μs, 60 Hz), an R928 photomultiplier tube and a computer working with FL Winlab software. Both excitation and emission slits were set at 5 nm throughout this study. BSA 2% w/v was titrated by successive additions of Methimazol and copper(II) salts solution from 0.08 to 0.5 mM and the fluorescence intensity was measured (excitation at 280 nm and emission at 348 nm) at 298 K. The 0.5 mM solutions were also assayed by UV–Vis electronic spectroscopy. Electronic absorption spectra were recorded with a Hewlett-Packard 8453 diode-array spectrophotometer, using 1 cm quartz cells in the 200–800 nm range. Buffer solutions were placed in the reference compartment. Three independent replicates of BSA solution and the corresponding complexes were measured. FT-IR spectra of the freeze-dried powdered samples were measured with a Bruker IFS 66 FT-IR-spectrophotometer from 4000 to 400 cm^{-1} in the form of pressed KBr pellets. For data processing, spectra of the buffer were collected under the same conditions. Then, the absorbance of the buffer was subtracted from the spectra of the sample to get the FT-IR spectra of modified proteins. Raman spectra were collected on a Bruker IFS 66 FT-IR-Raman spectrophotometer provided with the NIR Raman attachment equipped with an Nd:YAG laser at 1064 nm. Frequency calibration of the instrument was performed using the sulfur line at 217 cm^{-1}. Spectra were recorded at room temperature with a laser power of 500 mW, and a spectral resolution of 6 cm^{-1}. Each spectrum was obtained after collecting and averaging 1000 scans in order to obtain spectra with high signal-to-noise ratio. FT-Raman spectra were plotted as intensity (arbitrary units) against Raman shift in wavenumber units (cm^{-1}). All spectra were vector normalized in the whole range (4000–500 cm^{-1}). In order to improve signal-to-noise ratio in FT-IR and Raman spectra, Tris–HCl buffer, BSA solutions and methimazole and copper(II) salts solutions were lyophilized. As with other proteins, particularly globulins, FT-Raman spectra of protein solutions and freeze-dried powders are almost identical, therefore, freeze drying does not affect protein conformation as determined by FT-Raman spectroscopy [10]. This was taken into account during the procedure. The plotting, processing, normalizations, manipulations, and evaluation of spectra (FT-IR and Raman) were carried out through OPUS software (Bruker Optics, Germany). Band intensities were calculated after a linear baseline correction performed with an integration method developed within OPUS software. The intensity values obtained for the tyrosine doublet were calculated taking into account the local baseline of each peak (830 and 850 cm^{-1}). Bands of the major vibrational motions of the side chains or the peptide backbone were assigned by comparison to Raman data reported in the literature [2,10]. All analyses were performed in three independent experiments, and the results were