Journal of Inorganic Biochemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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

Oxidative stress protection by manganese complexes of tail-tied aza-scorpiand ligands

Jorge González-García [a,b], Àlvar Martínez-Camarena [a], Begoña Verdejo [a], M. Paz Clares [a], Concepción Soriano [a], Enrique García-España [a,*], Hermás R. Jiménez [c], Antonio Doménech-Carbó [d], Roberto Tejero [e], Enrique Calvo [f], Laura Briansó-Llort [f], Carolina Serena [f], Sandra Trefler [f], Antonio Garcia-España [f]

[a] Instituto de Ciencia Molecular, Universidad de Valencia, C/Catedrático José Beltrán 2, 46980 Paterna, Valencia, Spain
[b] Department of Chemistry, Imperial College London, London SW7 2AZ, United Kingdom
[c] Departamento de Química Inorgánica, Facultad de Química, Universidad de Valencia, C/Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
[d] Departamento de Química Analítica, Facultad de Química, Universidad de Valencia, C/Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
[e] Departamento de Química Física, Facultad de Química, Universidad de Valencia, C/Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
[f] Unitat de Recerca, Hospital Joan XXIII, Institut d'Investigació Sanitaria Pere Virgili (IISPV), Universitat Rovira i Virgili, Tarragona, Spain

ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form 25 February 2016
Accepted 12 April 2016
Available online xxxx

Keywords:
Mn^{2+} complexes
Double polyazamacrocyclic scorpiands
Antioxidant activity
Bacteria
Anti-inflammatory activity

ABSTRACT

The Mn^{2+} coordination chemistry of double scorpiand ligands in which two polyazacyclophane macrocycles have been connected by pyridine, phenanthroline and bipyridine spacers has been studied by potentiometry, paramagnetic NMR and electrochemistry. All ligands show high stability with Mn^{2+} and the complexes were formed in a wide pH range. DFT calculations support the structures and coordination geometries derived from the study. A remarkable antioxidant activity was evidenced for these systems by the McCord-Fridovich assay and in *Escherichia coli* sodAsodB deficient bacterial cells. The three systems were tested as anti-inflammatory drugs in human macrophages measuring the accumulation of cytokines upon lipopolysaccharide (LPS) pro-inflammatory effect. All complexes showed anti-inflammatory effect, being $[Mn_2\mathbf{L1}]^{4+}$ the most efficient one.

1. Introduction

Oxidative stress is defined as an imbalance between generation and clearance of reactive oxygen species (ROS) by a biological system through endogenous free radical scavengers [1] and antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase or peroxiredoxin. Overproduction of ROS has been shown to oxidize various cellular components including DNA, RNA, proteins, enzymes and lipids, causing various forms of damage to cells and tissues. In humans, oxidative stress has been involved in a variety of degenerative processes, diseases and syndromes, including cardiovascular diseases [2], chronic inflammation [3], diabetes [4], neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [5–8], cancer [9–11] and a variety of other age-related pathologies [12].

To counterbalance these toxic species, eukaryotic cells display a number of antioxidant metalloenzymes. Among them, superoxide dismutases (SODs) play a pivotal role in the conversion of superoxide radical into oxygen and hydrogen peroxide; hydrogen peroxide is then transformed into water and dioxygen by the concerted action of catalase enzymes.

Purified and recombinant SOD enzymes have demonstrated therapeutic efficacy in animal models of some of these disease states including myocardial, cerebral ischemia-reperfusion injury, neurodegeneration, inflammation and cancer [13–15]. However, the use of purified SOD enzymes has several limitations like: i) absence of oral activity, ii) immunogenicity, iii) short half-lifes, iv) inability to gain access to intracellular space of cells and v) manufacturing costs [16]. Therefore, synthetic SOD mimetic compounds of low molecular weight could compensate the limitations of purified enzymes due to their lack of antigenicity, tissue penetrance, higher stability in solution, longer half-life and lower production expenses [15,17–20].

In this respect, we have recently reported that mononuclear manganese complexes of "aza-scorpiand" ligands [21] with high SOD-like activity [22,23] show anti-inflammatory activity in macrophages and in a murine model of sepsis [24]. On the other hand, we had previously reported the synthesis, Zn^{2+} and Cu^{2+} complexation and some aspects of the anion coordination chemistry of polytopic receptors constituted by two scorpiand ligands linked through pyridine (**L1**) and phenanthroline (**L2**) aromatic spacers [25–28]. In this paper we have prepared a new compound of the series in which the scorpiand ligands are connected by a bipyridine spacer (**L3**); we have studied the coordination chemistry of the three receptors towards Mn^{2+} and we have tested the

* Corresponding author.
E-mail addresses: enrique.garcia-es@uv.es (E. García-España), hermas.jimenez@uv.es (H.R. Jiménez), antonio.domenech@uv.es (A. Doménech-Carbó), roberto.tejero@uv.es (R. Tejero), agarciae.hj23.ics@gencat.cat (A. Garcia-España).

http://dx.doi.org/10.1016/j.jinorgbio.2016.04.020
0162-0134/

antioxidant properties of their mono- and binuclear complexes by the McCord-Fridovich method and their antioxidant activity in *Escherichia coli* cultures. Finally we have checked the anti-inflammatory activity of theses complexes in human macrophages.

2. Materials and methods

2.1. Reagents

L1 and **L2** have been obtained by a procedure described previously [25]. All the compounds were isolated as their hydrochloride salts and gave satisfactory elemental microanalysis. All reagents were obtained from commercial sources.

2.2. Synthesis of ***L3***

6-(2′-Aminoethyl)-3,6,9-triaza-1(2.6)-pyridinacyclodecaphane (443.2 mg, 1.78 mmol) and 2.2′-bipyridine-4.4′-dicarbaldehyde (198 mg, 0.93 mmol) were dissolved in 40 mL of anhydrous ethanol and the mixture was stirred for 2 h at room temperature. $NaBH_4$ (550 mg, 14.5 mmol) was then added and the resulting solution stirred for 2 h at room temperature. The ethanol was removed under reduced pressure. The resulting residue was treated with H_2O (10 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was removed at reduced pressure, and the resulting residue was dissolved in ethanol and precipitated as the hydrochloride salt of **L3** in 75% yield. mp: >345 °C. ^1H NMR (300 MHz, D_2O): δ_H (ppm) = 8.88 (d, J = 5.5 Hz, 2H), 8.55 (s, 2H), 7.94 (d, J = 5.5 Hz, 2H), 7.92 (t, J = 8 Hz, 2H), 7.42 (d, J = 8 Hz, 4H), 4.60 (s, 4H), 4.59 (s, 8H), 3.47 (t, J = 8 Hz, 4H), 3.26 (t, J = 5.3 Hz, 8H), 3.13 (t, J = 8 Hz, 4H), 2.92 (t, J = 5.3 Hz, 8H). ^{13}C NMR (75.43 MHz, D_2O): δ_C (ppm) = 149.4, 148.9, 147.7, 146.1, 139.8, 127.0, 124.3, 122.2, 50.8, 50.5, 50.0, 49.5, 45.9, 43.9. Calc for $C_{38}H_{54}N_{12}\cdot 8HCl\cdot 9H_2O$: C 40.3%, H 7.1%, N 14.8%. Found: C 40.4%, H 7.3%, N 14.9%. *MS* (*FAB*) *m/z* 680 $[MH]^+$.

2.3. EMF measurements

The potentiometric titrations were carried out at 298.1 ± 0.1 K using NaCl 0.15 M as supporting electrolyte. The experimental procedure (burette, potentiometer, cell, stirrer, microcomputer etc.) has been fully described elsewhere [29]. The acquisition of the emf data was performed with the computer program PASAT [30]. The reference electrode was an Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen ion concentration probe by titration of previously standardised amounts of HCl with CO_2-free NaOH solutions and the equivalent point determined by the Gran's method [31,32], which gives the standard potential, E°, and the ionic product of water (pKw = 13.73(1)). The computer program HYPERQUAD was used to calculate the protonation and stability constants [33]. The HYSS [34] program was used to obtain the distribution diagrams. The pH range investigated was 2.5–11.0. The concentration of Mn^{2+} and of the ligands ranged from 0.5 to 5 mM with Mn^{2+}:L molar ratios varying from 3:1 to 1:2. The titration curves for each system (at least two titrations, ca. 150 experimental points) were treated either as a single set or as separated curves without significant variations in the values of the stability constants.

2.4. Paramagnetic NMR spectroscopy

The paramagnetic NMR measurements were acquired on a Bruker Avance 400 spectrometer operating at 399.91 MHz. One-dimensional spectra were recorded in D_2O with presaturation of the H_2O signal during part of the relaxation delay. Relaxation delay times of 50–200 ms, 30–75 kHz spectral widths and acquisition times of 60–200 ms were used. NMR spectra were processed using exponential line-broadening weighting functions as apodization with values of 5–40 Hz. Chemical shifts were referenced to residual solvent protons of D_2O resonating at 4.76 ppm (298 K) relative to TMS. Sample concentration of the complexes used in the paramagnetic ^1H NMR experiments was 2.0–3.7 mM. The longitudinal relaxation times of the hyperfine shifted resonances were determined using the inversion recovery pulse sequence (d_1-180°-τ-90°-acq, where d_1 is the relaxation delay and acq the acquisition time), 15 values of τ were selected between 0.4 ms and 200 ms [35]; (d_1 + acq) values were at least five times higher than the longest expected T_1 ranging from 100 to 400 ms and the number of scans was 10,000. The T_1 values were calculated from the inversion-recovery equation. Transversal relaxation times were obtained measuring the line broadening of the isotropically shifted signals at half-height through the equation $T_2^{-1} = \pi \Delta\nu_{1/2}$.

2.5. DFT calculations

DFT calculations of the Mn^{2+} complexes with the studied ligands were performed with the density functional theory as a computational method using B3LYP/6–31 + G** and B3LYP/LANDL2DZ combinations. Specifically, the 6–31 + G** basis set has been used for the non-metallic atoms, while the LANDL2DZ has been used for the transition metals, in which it has been generated ab initio effective core potentials to replace the Coulomb, core-orthogonality and exchange effects of the chemically inert core electrons in manganese. The Gaussian 09 package was used for these DFT calculations and the Molden package for the modelization analysis [36].

2.6. In vitro McCord-Fridovich SOD activity assays

SOD-like activity was determined by using the nitroblue tetrazolium (NBT) reduction method [37–39]. The assays were carried out in a pH = 7.4 50 mM HEPES buffer at 298 K. The concentration of NBT was 50 mM and the superoxide was generated with the xanthine oxidase-xanthine system.

2.7. Escherichia coli growth assays

E. coli strains WT (AB1157) and SOD-deficient, *sodAsodB*-(PN134) were obtained from J. A. Imlay [40]. Bacterial growth assays were carried out as described previously [23,24,41]. Briefly, bacteria were resuspended in casamino acid medium (M9CA) at a final concentration of 0.04 O.D at 620 nm, and bacterial growth at 37 °C in 96 well plates was followed turbidimetrically at 620 nm using an automatic multiwell thermostated spectrophotometer with shaking. Stock solutions at 1 mM of Mn_2-**L1**, Mn_2-**L2** and Mn_2-**L3** in water were diluted as required with M9CA medium. Three independent experiments were performed in which all compounds were tested in triplicate.

2.8. Cell culture and treatments

THP-1 human monocyte cells (ATCC, Rockville, USA) were cultured at 37 °C in 95% humidified air, 5% CO_2 in RPMI-1640 medium containing 10% (*v*/v) fetal bovine serum (FBS), supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. THP-1 monocytes were seeded at 3 × 10^5 cells/well in 6-well-plates and differentiated to macrophages with 200 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) during 3 days. Prior starting the treatment, the macrophages were maintained in fresh medium without PMA for two days, washed with sterile phosphate-buffered saline (PBS) and incubated for 4 h in serum free medium [19–20,42]. The complexes were then added to the cells at 0, 1, 5, 10 and 25 μM and 4 h later, the cells were treated with 1 μg/mL of *E. coli*-derived LPS (lipopolysaccharide serotype 0111:B4, SIGMA) dissolved in PBS for 16 h. The cell culture media and the cells for protein determination were collected and frozen at −80 °C.

Please cite this article as: J. González-García, et al., Oxidative stress protection by manganese complexes of tail-tied aza-scorpiand ligands, J. Inorg. Biochem. (2016), http://dx.doi.org/10.1016/j.jinorgbio.2016.04.020