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Manganese binding to antioxidant peptides involved in extreme radiation resistance in *Deinococcus radiodurans*

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ABSTRACT

A decapeptide, DEHGTAVMLK (DP1), and its random scrambled version, THMVLAKGED (DP2), have been studied for their interactions with manganese. The amino acid composition of the peptides was selected to include the majority of the most prevalent amino acids present in a *Deinococcus radiodurans* bacterium cell-free extract that contains components capable of conferring extreme resistance to ionizing radiation. The extract appears to be rich in Mn(II) complexes which seem to be responsible for protecting proteins from Reactive Oxygen Species damage. We focused our attention on the interaction of the decapeptides with Mn(II) ion with the aim of obtaining information on the possible complexes formed, by using NMR, EPR, and ESI-MS techniques.

1. Introduction

The development of radioprotective agents is very important in order to try to prevent or reduce radiation-induced injury and mortality derived from clinical (radiotherapy, exposure to radionuclides in nuclear medicine) or non-clinical (accidental exposure to high radiation, nuclear disasters) settings. For this purpose it is important to study not only the molecular and cellular basis of radiation toxicity, but also the molecular and chemical mechanisms of protection that are exerted, for example, in radioresistant organisms [1–4]. *Deinococcus radiodurans* (DR) is one of the most remarkable bacterium that exhibits extreme resistance to radiation, being able to survive ionizing radiation exposure 20 times higher than *E. coli* and 3000 times more than the lethal human dose [5–8], approximately 5 Gray (Gy). In particular, DR has been shown to be resistant to γ-radiation doses higher than 10,000 Gy, or even to lethal effects of Ultraviolet Radiation (UVR) higher than 1000 J/m^2 [9–11], with no significant loss of viability. The importance of manganese for resistance to ionizing radiation has been reported [12] together with that of small peptides derived from a variety of DR proteins [13,14]. It is known that DR accumulates high levels of manganese and small peptides. Additionally, a hydrolyzed ultrafiltrate extract obtained from DR homogenate was found to provide remarkable protection to enzymatic activity from ionizing radiation insult. The extract appears to be rich in Mn(II) complexes which seem to be responsible for specifically protecting proteins from ROS (Reactive Oxygen Species) induced damages [13]. Further, DR has been found to lack unique, highly efficient DNA repair enzyme machinery, thus DR's resistance to ionizing radiation is attributed to efficient ROS scavenging activity. This process appears to be mediated by the accumulation of small Mn(II) complexes that can protect essential enzymes from oxidative damage [8,15,16]. It is known that the free Mn(II) ion on its own is poorly reactive against the superoxide O_2^- anion; nevertheless, it can act as a SOD (Superoxide Dismutase) mimic when coordinated to various ligands [17], though toxic when present in high concentration [18–20]. Besides, peptides containing histidine and oxygen donor amino acids can be interesting ligands for manganese ions [21,22].

In this paper, a decapeptide DEHGTAVMLK (DP1) and an its random scrambled version THMVLAKGED (DP2) have been chosen to study the Mn(II) interaction with DR metabolites.

The amino acid composition of the decapeptides derived by including the most prevalent amino acids found in the cell-free extract obtained from DR. The two peptides were tested both in the D- or L-form. Here we report a spectroscopic study through NMR, EPR and ESI-MS techniques on the Mn(II) and DP1 and DP2 peptides in order to get information on the molecular and structural behavior of these Mn(II)-antioxidant species in solution.

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2. Materials

The two decapeptides, DEHGTAVMLK and THMVLAKGED, were synthesized by Elim Biopharmaceuticals (Hayward, CA, USA). $MnCl_2$, D_2O and other solvents were purchased from Sigma Aldrich, and used without any further purification.

2.1. EPR spectroscopy

Electron paramagnetic resonance (EPR) spectra were recorded using a Bruker ELEXSYS E500 CW-EPR spectrometer equipped with an NMR teslameter (ER 036TM) and frequency counter (E 41 FC) at X-band frequency, at 77 K and room temperature. The peptide concentration was 1 mM and metal to ligand molar ratio 1:1.2. The solution for EPR was prepared with ethylene glycol (5–30%) as a cryoprotectant. The EPR parameters were obtained by using the Bruker WinEPR SimFonia program and Doublet new (EPR OF S = 1/2) program by Dr. Andrew Ozarowski, National High Field Magnetic Laboratory, University of Florida.

2.2. Electrospray ionization-mass spectrometry (ESI-MS)

Electrospray ionization mass spectrometry (ESI-MS) data were collected using a Bruker MicrO-TOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an Apollo II electrospray ionization source with an ion funnel. Spectra were recorded in the positive and negative ion modes in the range 100 to 1500 *m/z*. The instrumental parameters were as follows: scan range *m/z* 400–1600, dry gas–nitrogen, temperature 170 °C, ion energy 5 eV. The capillary voltage was optimized to the highest S/N ratio and it was 4500 V. The small changes in the voltage (±500 V) did not significantly affect the optimized spectra. Samples (with metal to ligand 1:1.2 stoichiometry, and [ligand] = 10^{-5} M) were prepared by using a MeOH/H_2O mixture (50/50 v/v) as a solvent, at pH 7.4 and 9.0. The instrument was calibrated using the Tunemix mixture (BrukerDaltonik, Germany) in the quadratic regression mode. The overall charge of the analyzed ions was calculated on the base of the distance between isotopic peaks. Data were processed by application of the Compass DataAnalysis 4.0 (BrukerDaltonik, Germany) program.

2.3. NMR spectroscopy

Nuclear Magnetic Resonance (NMR) measurements were performed on a Bruker Ascend™ 400 MHz spectrometer equipped with a 5 mm automated tuning and matching broad band probe (BBFO) with z-gradients, as previously described [23–28]. NMR experiments were carried out using 5 mM concentration of the ligands in 90/10 (v/v) H_2O/D_2O at 298 K in 5 mm NMR tubes. 2D ^1H–^{13}C HSQC spectra (Heteronuclear Single Quantum Coherence) were acquired using a phase-sensitive sequence employing Echo-Antiecho-TPPI gradient selection with a heteronuclear coupling constant J_{XH} = 145 Hz, and shaped pulses for all 180° pulses on f2 channel with decoupling during acquisition. Sensitivity improvement and gradients in back-inept were also used. Relaxation delays of 2 s and 90° pulses of about 10 μs were employed in all the experiments. Solvent suppression in 1D ^1H and 2D ^1H–^1H TOCSY (Total Correlation Spectroscopy) experiments was carried out by using excitation sculpting with gradients. The spin-lock mixing time of TOCSY experiments was obtained with MLEV17. ^1H–^1H TOCSY spectra were performed using mixing times of 60 ms. A combination of 1D, 2D TOCSY, HSQC experiments was used to assign the signals of both free and metal-bound ligands at different pH values. All NMR data were processed with TopSpin (Bruker Instruments) software and analyzed by Sparky 3.11 and MestRe Nova 6.0.2 (Mestrelab Research S.L.) programs.

2.4. Model calculations

The models of DP1 and DP2 peptides were obtained from HyperChem(™) 8.0.7 molecular modeling software [29]. Mn(II) coordination with the peptides was modeled according to the spectroscopic results, as previously described [21,22], using the molecular mechanics geometry optimizations obtained by an AMBER force field implemented in HyperChem. Molecular graphics of the most likely coordination sphere for Mn(II) species were generated by using the UCSF Chimera package.

3. Results and discussion

Manganese complexes with DEHGTAVMLK (DP1) and THMVLAKGED (DP2) have been investigated by means of a combination of spectroscopic and spectrometric techniques. The titration of the peptides with Mn(II) ion has been followed by the selective paramagnetic line-broadening and signals disappearance in the 1D ^1H, 2D ^1H–^{13}C HSQC and ^1H–^1H TOCSY NMR spectra at physiological pH value (pH 7.4) (with and without phosphate buffer) by increasing Mn(II) to ligand molar ratios. Depending on the metal-nucleus distance, the electron-nucleus coupling effects may prevent the observation of proton signals of the residues close to the metal site due to the severe induction of broadening in the NMR signals [30]. Mn(II), in particular, has one electron per d orbital and displays the longest electronic relaxation times among the 3d metal ions, so the proton NMR spectra for Mn(II) systems are expected to have very broad signals [31]. To overcome this phenomenon, substoichiometric amounts of metal were added to the peptide solutions to monitor the relaxation effect of any specific nucleus, even in closer proximity to the paramagnetic center [19,21,22,32]. The line-broadening effects, in this way, have been considered to localize and characterize the metal binding sites along the sequence, whereas ESI-MS and EPR techniques provided additional information on the species formed, the peptide-metal ion stoichiometry and geometry of the metal complexes. A complete ^1H and ^{13}C resonance assignment of DP1 and DP2 peptides was made by a combination of 1D and 2D ^1H–^{13}C HSQC ,^1H–^1H TOCSY and ^1H–^1H ROESY experiments (Table 1S).

3.1. Manganese binding to DP1 and DP2.

Sequential addition of substoichiometric amounts of Mn(II) ion to the peptide solution at pH 7.4 yields a selective metal-induced line broadening. As shown on the top in Fig. 1a for the 1D ^1H traces (see also Fig. S1 for more clarity) in the aromatic region of the spectra, the protons experiencing a gradual but specific decrease in intensity are those of the imidazole ring Hδ2 and Hε1 of His-3 residue. The interaction of Mn(II) with His-3 is further proven by the disappearance of its aliphatic β protons (Fig. 1b). Moreover a severe paramagnetic effect on the Asp-1 (β protons) and Glu-2 (γ and β protons) side chain resonances strongly supports the involvement in manganese binding of both of these residues together with the His residue. Additional proofs to support this finding are given by the heteronuclear NMR experiments. The complete disappearance or severe reduction in intensity of the carbon-proton correlation in the 2D ^1H–^{13}C HSQC NMR spectra (Fig. 1a–b) of free peptide DP1 and Mn(II):DP1 system at 0.02:1 M ratio, clearly indicate that the first three residues in the peptide sequence, DEH, are involved in the coordination to Mn(II) ion. A quantitative analysis of cross-peak intensity in the HSQC spectra as a function of the residue number is presented in Fig. 2A. The nuclei closest to the Mn(II) center (Asp-1, Glu-2 and His-3) show the largest paramagnetic relaxation effect, whereas those located further are relatively unaffected. While the Asp-1 cross-peaks completely disappeared upon addition of 0.02 eq of Mn(II) to DP1, the decrease in intensity of the Glu-2 (with γ > β) and His-3 (with ε1 = δ2 = β > α) cross-peaks, which is strictly dependent on the metal-nucleus distance [30], provides evidence of

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