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Molecular characterization of myoglobin from *Sciurus vulgaris meridionalis*: Primary structure, kinetics and spectroscopic studies

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ABSTRACT

Myoglobins (Mbs) are heme-proteins involved in dioxygen storage necessary for metabolic respiration. Mbs are intensely investigated as archetype to investigate structure/function relationship in globular proteins.
In this work, the myoglobin from *Sciurus vulgaris meridionalis* has been for the first time isolated and purified with a high yield and homogeneity. The primary structure characterization has been performed by applying a strategy based on high resolution tandem mass spectrometry. Proximal (position 93, α-helix F8) and distal (position 64, α-helix E7) histidinyl residues as well as most of the amino acid residues (i.e., Leu29, Lys45, Thr67, Val68) involved in the autoxidation mechanism are conserved in the squirrel Mb. The structural and dynamical properties of the squirrel Mb have been also deeply investigated by CD, NMR. Furthermore, molecular dynamics studies of Mbs from different species have been performed. In addition, the functional properties of squirrel Mb have been characterized by determining its autoxidation kinetic and thermal stability in comparison with crested porcupine and reindeer Mbs. Interestingly, a higher autoxidation rate was revealed for squirrel Mb with respect to reindeer and crested porcupine Mbs. Even considering the very similar structural fold, molecular dynamics data show a higher conformational mobility of squirrel Mb with respect to reindeer and crested porcupine.

1. Introduction

Myoglobin (Mb) is a water-soluble hemeprotein consisting of ~150 amino acid residues [1], which are involved as dioxygen storage structure necessary for the aerobic metabolism [2]. Mb is closely related to hemoglobin that is responsive for dioxygen carrying from the lungs to the tissues and displays quaternary structure due to interaction of four myoglobin-like subunits [3,4]. Mb and hemoglobin belong to vertebrate globin family [5], composed by four hemoprotein groups including also intracellular neuroglobin and cytoglobin; the first, expressed in nerve cells, is essential to protect neurons from hypoxic-ischemic injury, while the second is expressed in many different tissues and its function is not yet fully clarified [4,6]. The principal characteristic of this family is the presence of heme group containing a central iron atom (responsible of dioxygen metastable bond) embedded in a typical polypeptide chain fold, known as "globin fold", obtained by eight alpha-helices [7].

Mb is the first protein whose three-dimensional structure has been revealed by X-ray crystallography [1]. Since its discovery [8] it represents an excellent model for structural and functional studies of proteins. Despite its well-known role in O_2 storage several novel insights have been recently reported, such as its regulatory function in nitric oxide (NO) bioavailability [9] or low peroxidase activity in the presence of H_2O_2 in cardiac myocytes [10,11]. Moreover, in biotechnological sciences, immobilised Mb has been used for the construction of hydrogen peroxide and nitrite biosensors [12,13]. In food chemistry, vertebrate muscle Mb, due to its high concentration, is mainly responsible of meat red colour [14,15] due to the equilibrium between the purplish-red Mb form (DeoxyMb) and the cherry-red form (OxyMb). However, during meat storage, these two reduced Mb forms readily become oxidized to the brownish-red MetMb [16].

The Mb molecular evolution started ~600 Myr ago (Cambrian period) [17] and by structural studies it has been demonstrated that the apomyoglobin structure acquires the most suitable conformations to satisfy the reversible spin-crossover upon O_2 binding to porphyrin [18,19]. In this framework, though many aspects of the structure-function relationships of Mbs have been clarified [18,20–24], some important questions remain to be answered especially about Mb molecular evolution, which could be further investigated implementing the availability of their sequences and functional information from organisms still not investigated.

The European red squirrel (*Sciurus vulgaris* L.) is the only arboreal squirrel present in Italy and Europe. In Italy, there are three subspecies of red squirrel: *Sciurus vulgaris fuscoater* in the North, while in the Central and South there are two endemic subspecies, *Sciurus vulgaris italicus*

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and *Sciurus vulgaris meridionalis*, respectively [25]. *S. vulgaris meridionalis* is endemic in the southern Apennines and is the major Italian subspecies [26]. Today, the destruction of habitats and the introduction by man of the grey squirrel from North America are causing the gradual extinction of this rodent so that European research projects are ongoing for the protection and study of these animals [27]. Furthermore, although phylogenetic analyses using mitochondrial DNA (mtDNA) or microsatellites [28,29] and habitat studies are available [30–32], few studies on proteins isolated from *S. vulgaris meridionalis* have been reported.

In this study, we report the purification and characterization of myoglobin from *S. vulgaris meridionalis* (squirrel Mb), including the determination of its primary structure by using a strategy based on high-resolution mass spectrometry. In order to describe the structural characteristics of the squirrel Mb we first performed an accurate computational modeling study and then we validated the predicted 3D model through experimental data obtained by Nuclear Magnetic Resonance and Circular Dichroism techniques. In addition, the functional properties, in terms of autoxidation kinetic and thermal stability, were evaluated and compared with the data related to the crested porcupine (*Hystrix cristata* L.) and reindeer (*Rangifer tarandus* L.) Mbs [33]. Finally, to have a complete understanding of the structure-function relationships we investigated the dynamic personalities and molecular motions of the three myoglobins (squirrel, reindeer and crested porcupine) by performing a series of molecular dynamics simulation studies. Overall, our structural and dynamic characterization indicate that the squirrel Mb, while having a tri-dimensional structure very similar to that observed for the myoglobin from reindeer, has a completely different dynamical behaviour. In particular, the molecular dynamics simulations data show that the region located in the proximity of the distal pathway is more flexible in the squirrel Mb than either reindeer or crested porcupine Mbs.

2. Material and methods

2.1. Chemicals and proteolytic enzymes

Endoproteinases (trypsin, chymotrypsin, pepsin and Asp-N) were purchased from Sigma-Aldrich (Milan, Italy). Solvents for RP-HPLC were supplied by Carlo Erba (Milan, Italy). Bicinchoninic acid (BCA) kit was purchased from Pierce (Rockford, IL, USA). Materials for chromatography were described elsewhere [33,34]. For apoMb separation by reversed-phase HPLC (RP-HPLC), the C-4 (0.46 × 25 cm; 5-μm particle size) was obtained from Phenomenex (Castel Maggiore, BO, Italy). The following solvents were used: solvent A, 0.1% TFA (Carlo Erba) and solvent B, acetonitrile containing 0.1% TFA. All other reagents and chemicals were of analytical grade.

2.2. Extraction and purification procedure

Meat samples used for this study were taken from six carcasses of animals dead naturally. Mb was isolated from squirrel striated muscle applying a previously reported protocol for highly water-soluble proteins [33,34] with slight modifications. Briefly, following partial removal of the fat and connective tissues, meat samples (about 50 g) were homogenized in milli-Q water (1:2; w:v) using a Waring blender (Waring Products, Torrington, CT, USA), at 4 °C. The homogenate was centrifuged at 10,000 *g* for 40 min at 4 °C by using a centrifuge Avant-J (Beckman Coulter, Cassina de Pecchi Milan, Italy), filtered through Miracloth paper (Calbiochem, San Diego, CA, USA) and dialysed in milli-Q water. Mb was then gel-filtered on Sephacryl S-100 HR (GE Healthcare, Milan, Italy), previously equilibrated in 10 mM Tris·Cl, pH 8.0 (buffer A) containing 0.2 M NaCl. The Mb-containing fractions (~17 kDa) were concentrated using an ultrafiltration cell (Amicon Inc., Beverly, USA; membrane molecular mass cut-off: 10,000) and then dialyzed against 2 L of buffer A (three changes). The dialyzed protein solution was centrifuged at 15,800 *g* using a centrifuge Avant-J (Beckman Coulter) for 30 min, at 4 °C. The supernatant was subjected to anion exchange chromatography on Source™ 15Q FPLC column (GE Healthcare), using the AKTA purifier FPLC (GE Healthcare), equilibrated with buffer A and eluted with a gradient made up by buffer A increasing NaCl concentration (from 0 to 50 mM), in 60 min, at a flow rate of 1.0 mL/min. During purification procedure, the absorbance was measured at 280 and 409 nm to monitoring heme-proteins, and the protein homogeneity checked by SDS-PAGE (15% polyacrylamide gel).

2.3. Analytical procedures for myoglobin characterization

2.3.1. Visible spectra

Vis-spectra were measured in the 500–700 nm range in 50 mM Na-phosphate and 50 μM EDTA, pH 7.2 (buffer B) on a Cary-1E UV–Vis spectrophotometer (Varian-Agilent Technologies, Santa Clara, CA, USA), at 25 °C. Ferrous and ferric Mb derivatives were prepared, as previously described [35].

2.3.2. Autoxidation rate measurements

The autoxidation of OxyMb to MetMb was monitored by recording the changes of the absorption spectrum in the 500–700 nm range and estimating the absorbance decrease of OxyMb α-peak (at 582 nm for crested porcupine Mb or 581 nm for squirrel and reindeer Mb) using a Synergy HT Multi-Mode Microplate Reader (BioTek, Bad Friedrichshall, Germany). For the characterisation of the autoxidation process, spectra were collected every 10 min for 6 h. Ferrous and ferric Mb derivatives were prepared as previously described [35]. All experiments were carried out in buffer B, at 37 °C, and performed in triplicate with freshly prepared OxyMb.

2.3.3. Preparation of apomyoglobin

The separation of apoMb from the heme group was performed by RP-HPLC on a C-4 column, as previously reported [36,37].

2.3.4. Enzymatic digestion apoMb

Digestion with pepsin was performed in 0.2 M KCl, pH 1.3 by two additions of the enzyme with a final enzyme-to-substrate ratio of 1:100 (w:w). Subsequently, the hydrolysis was carried out at 30 °C. The digestion was monitored by collecting aliquots each hour for MALDI-ToF analysis with a total incubation time of 6 h (data not shown). Digestions with trypsin, chymotrypsin and endoproteinase Asp-N were performed as previously reported [33,36].

2.4. Mass spectrometry analysis of intact protein

The relative molecular mass (Mr) of myoglobin was determined on a Q-TOF Micro mass spectrometer (Waters, Manchester, UK) as previously described [33,38]. The sample in acetonitrile: 0.1% formic acid in water (50:50; v:v) at a concentration of 10 pmol/μL was infused into the system at a flow rate of 5 μL/min. The acquisition and deconvolution of data were performed by using the Mass Lynx 4.1 software (Waters). The capillary source voltage and the cone voltage were set at 3000 and 43 V, respectively. The source temperature was kept at 80 °C and nitrogen was used as drying gas (flow rate about 50 L/h).

2.5. High resolution nanoLC–Tandem Mass Spectrometry

Mass spectrometry analyses on myoglobin tryptic, chymotryptic, Asp-N and pepsin digests (50 fmol) were performed on a Q Exactive Orbitrap mass spectrometer equipped with an EASY-Spray nano-electrospray ion source (Thermo Fisher Scientific, Bremen, Germany) and coupled to a Thermo Scientific Dionex UltiMate 3000RSLC nano system (Thermo Fisher Scientific). Solvent composition was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Peptides were loaded on a trapping PepMap™100 μCartridge