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Enhanced heme accessibility in horse heart mini-myoglobin: Insights from molecular modelling and reactivity studies

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ABSTRACT

Mini-myoglobin (mini-HHMb) is a fragment of horse-heart myoglobin (HHMb) considered to be the prototype of the product encoded by the central exon of the HHMb gene. For this reason, mini-HHMb has been studied extensively showing that carbonylation and oxygenation properties of the ferrous form are similar to those of the full-length protein, while kinetics and thermodynamics of azide binding to the ferric form are significantly different from those of HHMb. To analyze the structure–function relationships in mini-HHMb and the role of conformational fluctuations in ligand accessibility, the molecular model of mini-HHMb has been built and refined by molecular dynamics simulations, and analyzed in parallel with that of full length HHMb. Moreover, imidazole binding parameters of ferric mini-HHMb and HHMb have been determined. Furthermore, structural data of ferric mini-HHMb and HHMb have been correlated with the imidazole and previously determined azide binding properties. Present results indicate that, despite the extensive trimming, the heme-α-helices E-F substructure is essentially unaltered in mini-HHMb with respect to HHMb. However, the heme-Fe atom displays an enhanced accessibility in mini-HHMb, which may affect both ligand association and dissociation kinetics.

1. Introduction

Mini-myoglobin (mini-HHMb) is a fragment of horse-heart myoglobin (HHMb) obtained by limited proteolysis and made up by residues 32–139 [1,2], which makes it closely similar to the central exon of the HHMb gene, which encodes residues 31–105. Mini-HHMb has been studied quite extensively, as it represents a general model to study the role played by the central exon in heme binding and globin folding [1–3]. The central exon of myoglobin (Mb) and hemoglobin genes encodes the domain that forms the hydrophobic pocket in which the heme is bound, while the *C*- and *N*-terminal fragments are thought to contribute additional heme stabilization and to modulate the metal-center reactivity [4–6]. However, the central exon of the leghemoglobin gene is splitted in two, as it is formed by four exons coding, for instance in soybean, for protein regions 1–32, 33–68, 69–103, and 104–144 [7].

Mini-HHMb functionally resembles the native full-length protein in the carbonylation and oxygenation properties as well as in the O_2 replacement reaction with CO [1], although the stability of the oxygenated derivative of mini-HHMb is drastically reduced [2]. From the structural viewpoint, circular dichroism studies and fluorescence properties of the complex of mini-HHMb with 1-

Abbreviations: 6cHS, six-coordinated high spin form; 6cLS, six-coordinated low spin form; HHMb, horse-heart myoglobin; HHMb(III), ferric full-length HHMb; Mb, myoglobin; mini-HHMb, horse heart mini-myoglobin; mini-HHMb(III), ferric mini-HHMb; MD, molecular dynamics.

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anilino-8-naphtalene sulphonate indicate that the constraints imposed by heme binding allowed the protein to acquire a conformation resembling that of the native protein. These data led to the hypothesis that mini-HHMb could be considered as a model for an ancestor oxygen-carrier hemoprotein [2]. On the other hand, kinetics and thermodynamics of azide binding to ferric mini-HHMb (mini-HHMb(III)) are significantly different from those of ferric full-length HHMb (HHMb(III)) [8], indicating that the removal of the *N*- and *C*-terminal portions affects mainly the structural and functional properties of the ferric form.

To date no structural data of mini-HHMb are available at atomic level. In fact, at mini-HHMb higher than 5×10^{-5} M, the heme-protein loses the heme and the globin precipitates, impairing the possibility of collecting direct structural data by both X-ray crystallography and NMR (G. De Sanctis, unpublished data). Therefore, to study in deeper detail the structure–function relationships of mini-HHMb and the role of conformational fluctuations in ligand accessibility to the heme-Fe atom, and hence the reactivity, in the present study a molecular model of mini-HHMb has been built and refined by molecular dynamics simulations, and analyzed in parallel with that of full length HHMb. Further, structural data have been correlated with azide [8] and imidazole (present study) binding properties of mini-HHMb(III) and HHMb(III).

2. Materials and methods

2.1. Mini-HHMb sample preparation

HHMb was purchased from Sigma (Sigma Chemical Co., St. Louis, MO,USA) and mini-HHMb was prepared as previously described [1]. The HHMb and mini-HHMb concentration was determined spectrophotometrically using $\varepsilon = 157$ cm^{-1} mM^{-1} at 409 nm for HHMb(III) [9], and $\varepsilon = 207$ cm^{-1} mM^{-1} at 423 nm for ferrous carbonylated mini-HHMb [1]. All other reagents were of analytical grade and used without further purification.

2.2. Molecular dynamics simulations

The starting mini-HHMb three-dimensional structure has been obtained by trimming the *N*- and *C*-terminal regions of HHMb (PDB ID: 1WLA [10]) to match the mini-HHMb primary structure. In detail residues 1–31 and 140–153 of HHMb were deleted to generate the new Leu32 and Arg139 *N*- and *C*-termini, respectively. The modeled mini-HHMb structure has been subsequently equilibrated in water by molecular dynamics (MD) simulations in explicit solvent using the CHARMM33 macromolecular mechanics package [11] and the CHARMM27 parameters and force field [12]. The three-site TIP3p model [13] was used for water molecules. In detail, hydrogen atoms were added to the modeled structure using the routine HBUILD of the CHARMM package. The structure has been placed in a truncated octahedron, constructed from a cubic volume of water molecules of dimension 77.758 Å × 77.758 Å × 77.758 Å, and water molecules overlapping with protein atoms (cut-off = 2.8 Å) have been removed. The solvated structure, containing 7140 water molecules, has been energy minimised by applying a harmonic force of 10 kcal/mol to non-hydrogen atoms of the complexes to allow reorganization of the solvent. Minimized solvated structure has then been subjected to MD simulation at 298 K in the microcanonical ensemble, after a heating run of 10 ps during which the temperature was gradually increased from 0 to 298 K. The simulation time step was set to 0.002 ps. All atoms root mean square deviation reached a plateau after approx. 2.0 ns simulation time (data not shown). The total simulation time was 3.2 ns.

The same procedure described for mini-HHMb was followed for HHMb molecular dynamics simulations, using the HHMb structure corresponding to the PDB ID: 1WLA [10] as the starting structure. In this latter case, all atoms root mean square deviation reached a plateau after approx. 1.3 ns simulation time (data not shown).

For protein tunnels analysis, mini-HHMb structures were sampled every 0.1 ns in the 2.5 ns–3.0 ns MD simulation time interval. Tunnels were calculated using the program Caver [14] which uses the Dijkstra's algorithm [15] to solve a typical single-source (in this case the mini-HHMb heme-Fe atom), shortest path (to solvent) problem in weighted graphs. The parameters probe size and number of tunnels were 0.8 Å and 3, respectively. Throughput values have been calculated according to ref. [16]. Tunnels have been visualized using PyMol molecular graphics software (DeLano Scientific LLC).

In order to allow a clear description of the comparative structural properties of mini-HHMb and HHMb, the residue numbering and the α-helices nomenclature of the full-length HHMb has also been used for mini-HHMb.

2.3. Imidazole binding to mini-HHMb(III) and HHMb(III)

Rapid-mixing stopped-flow experiments have been carried out at 20 °C employing an SX.18 MV apparatus equipped with a diode array detector (Applied Photophysics, Salisbury, UK). The absorption spectra were collected with a time resolution of 1.5 ms. The dependence of imidazole binding kinetics on the ligand concentration has been investigated mixing either mini-HHMb-Fe(III) or HHMb-Fe(III), dissolved in 1.0×10^{-1} M phosphate buffer at pH 7.0, with the same buffer solution containing varying amounts of imidazole ranging between 1.0×10^{-3} M and 2.0×10^{-1} M.

Kinetics and thermodynamics of imidazole binding to mini-HHMb(III) and HHMb(III) were analyzed in the framework of minimum reaction mechanism depicted in Scheme 1 [17]:

where P-L is the ferric protein binding the endogenous ligand (L) at the heme-metal center, P is the ferric protein with the distal side "free", P-Im is the ferric protein binding imidazole (Im) at the heme-Fe(III) atom, k_L is the first-order rate constant for the dissociation of the endogenous ligand, k'_L is the bimolecular association rate constant for the binding of the endogenous ligand, k'_{lm} is the bimolecular association rate constant for imidazole binding, and k_{lm} is the first-order rate constant for imidazole dissociation.

3. Results

3.1. Mini-HHMb and HHMb molecular dynamics simulations

The initial mini-HHMb structural model has been obtained by trimming the *N*-terminal residues 1–31 (corresponding to α-helices A and B) and the *C*-terminal residues 140–153 (corresponding to the terminal part of α-helix H) of HHMb [10] to match the mini-HHMb primary structure. In detail, residues 1–31 and 140–153 of HHMb were deleted to generate the new Leu32 and Arg139 *N*- and *C*-termini, respectively.

The mini-HHMb model has been subsequently refined by MD simulations in explicit solvent. The value of the backbone atoms rmsd from the initial structure reached convergence after approximately 2 ns to yield the structure shown in Fig. 1. This procedure mimics what happens during the preparation of mini-HHMb (see Materials and Methods and [1,8]), but, given the timescale explored by molecular dynamics simulations, it follows only the first main structural consequences of the trimming; structural modifications, which actually occur at much later times, cannot be reproduced. In fact, after about 1 min from heme addition to apo-mini-HHMb a significant portion of the initial penta-coordinated species shifts toward a hexa-coordinated form [8].