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Insights into the anomalous heme pocket of rainbow trout myoglobin

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ABSTRACT

Rainbow trout myoglobin (Mb) is characterized by an unusually low affinity for oxygen, having a P_{50} of 4.92 ± 0.29 mm Hg at 25 °C which is the highest ever reported for any vertebrate Mb at the same temperature (Helbo and Fago, (2011) *Am. J. Physiol. Regul. Integr. Comp. Physiol. 300*, R101-R108). In order to gain insight into the structural factors of the heme pocket that may be important determinants for this atypical oxygen affinity, we have carried out an electronic absorption and resonance Raman characterization of the ferric and ferrous protein with and without exogenous ligands (O_2, CO, F^-) and compared the results with those of other Mbs. While the ν(Fe–His) stretch appears at a frequency similar to other vertebrate Mbs, the resonance Raman frequencies of the Fe-ligand stretching modes reveal significant variations in the interaction of iron-bound ligands with distal residues. In particular, the spectroscopic characterization highlights two exceptional properties of rainbow trout Mb, a significantly higher level of reversed heme and reduced hydrogen bonding interactions between ligands and the distal HisE7 residue compared with other Mbs. The weakening of the hydrogen bond interaction is proposed to be the primary cause of the significantly reduced oxygen affinity.

1. Introduction

Myoglobin (Mb), the O_2 carrier of heart and skeletal muscle of vertebrates, is one of the most widely used models for the study of stereochemical interactions basic to heme protein function. In mammalian Mbs, O_2 binding affinities are almost invariably identical, with a P_{50} (O_2 tension at half-saturation) of 1 mm Hg at 25 °C [1], whereas fish Mbs show a broad range of affinity values [2–4], probably in order to mediate intracellular O_2 transport under more variable conditions than in mammalian species.

It has recently been shown that rainbow trout Mb is characterized by an unusually low affinity for oxygen, having a P_{50} of 4.92 ± 0.29 mm Hg at 25 °C, which is the highest ever reported for any vertebrate Mb at the same temperature [5]. In addition, O_2 binding to rainbow trout Mb, although being an exothermic reaction as in Mbs from other vertebrates, liberates less heat upon oxygenation, with an overall ΔH of -12.03 kcal/mol O_2 [5] that is lower than that of other Mbs, including fish Mbs ($\Delta H \sim -18$ kcal/mol O_2) [3]. These functional properties, taken together, suggest a weaker bond between the heme iron and the O_2 molecule. On the basis of the amino acid sequence of trout Mb, it was provisionally suggested [5] that the low O_2 affinity of this Mb may not derive from weak interactions between the O_2 ligand and the distal residues, as these are highly conserved in this Mb (LeuB10, PheCD4, HisE7, ValE11), but could be a trans-axial effect. In fact, on the proximal side of the heme, trout Mb is unique in having a Leu in position F3 rather than a Pro, which may affect the orientation of the heme-carrying F helix containing the proximal HisF8, and potentially weaken the strength of the iron-O_2 bond.

In order to determine the structural factors of the distal or proximal side of the heme pocket that may be responsible for this atypically low O_2 affinity, we have undertaken a spectroscopic characterization of the ferric and ferrous protein with and without exogenous ligands (O_2, CO, F^-) and compared the results with those of other Mbs.

The application of resonance Raman (RR) spectroscopy to the study of the structure, function, folding and dynamics of heme-containing proteins has been exceptionally fruitful during the past several decades. In particular, the combination of electronic absorption and RR spectroscopies allows detailed information of the active site to be obtained at the molecular level. Furthermore, the combined study of ferric and ferrous proteins, at different pHs and in the presence of various exogenous ligands enables the key functional residues in the heme cavity to be identified and their structure–function properties elucidated [6,7]. In fact, in the high-frequency region of the spectrum, the frequency and intensity of the core size marker bands, that are sensitive to the heme-coordination and spin states, are further modulated by the protein environment surrounding the heme and, therefore, provide useful structural information. In the low-frequency region, the RR frequencies of the Fe-ligand stretching

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modes provide information on the interaction of heme iron-bound ligands with distal residues.

In the case of trout Mb, while the vibrational frequency of the Fe–His stretching mode of the heme iron and the proximal HisF8 residue appears at a frequency similar to that of other vertebrate Mbs, the RR frequencies of the Fe-ligand stretching modes are extremely informative in revealing variations in the distal cavity. In particular, the spectroscopic characterization of rainbow trout Mb reported herein highlights two exceptional properties when compared with other Mbs: a significantly higher level of reversed heme and reduced hydrogen bonding interactions between ligands and the distal HisE7 residue compared with other Mbs. The relevance of these unusual characteristics for a myoglobin protein in determining the exceptionally low oxygen affinity is discussed.

2. Materials and methods

2.1. Materials

Gaseous ^{12}CO and ^{13}CO were purchased from Rivoira and FluoroChem, respectively. Sodium dithionite was obtained from Fluka Biochemika and Biogel P-6DG from Bio-Rad Laboratories, U.S. Fluoride salts and isotopically enriched water D_2O (99.8%) were obtained from Merck AG (Darmstadt, Germany). All the other chemicals and swMb were obtained from Aldrich, Steinheim, Germany. All chemicals were analytical or reagent grade and were used without further purification.

2.2. Purification of myoglobin

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Funderholme trout farm, Denmark. Fish were kept in well-aerated tanks at room temperature and fed daily with commercial fish food. Mb isolated from the fish hearts was purified as previously reported [5].

2.3. Sample preparation

Ferrous deoxy samples were prepared by addition of a 5% volume freshly prepared sodium dithionite (20 mg/mL) solution to the ferric forms previously degassed with nitrogen. The oxy sample was prepared by gel filtration of a deoxy sample on a Biogel P-6DG column equilibrated with 0.1 M Tris–HCl, 0.5 mg/mL dithiothreitol (DTT), pH 7.5. The CO complexes were prepared by degassing the met protein solution by flushing firstly with nitrogen, then with CO or ^{13}CO and reducing the heme by addition of a 5% volume freshly prepared sodium dithionite (20 mg/mL) solution. The fluoride complexes were prepared by adding a 0.5 M buffered solution of NaF to the ferric samples, giving a final concentration of 0.46 M. The hydroxyl complex in isotopically enriched water was prepared by adding 5 μL of Mb, in 0.02 M natural abundance buffer, to 50 μL of deuterated 0.1 M glycine buffer at pD 10.1.

Protein concentrations in the range 30–90 μM were used for the electronic absorption and RR samples. The protein concentration was determined on the basis of the molar absorptivity, $\varepsilon = 128\ mM^{-1}\ cm^{-1}$ at 414 nm [1].

Sperm whale Mb was dissolved in 0.1 M phosphate at pH 7.0 and the CO complex prepared as described above for trout Mb.

2.4. Spectroscopy

Electronic absorption spectra were measured with a double-beam Cary 5 spectrophotometer (Varian, Palo Alto, CA), using a 5-mm NMR tube or a 1-cm cuvette, and a 600 nm/min scan rate. The RR spectra were obtained using a 5-mm NMR tube and by excitation with the 413.1 nm line of a Kr^+ laser (Coherent, Innova 300C, Santa Clara, CA) and the 441.6 nm line of a HeCd laser (Kimmon IK4121R-G, Tokyo Japan). Backscattered light from a slowly rotating NMR tube was collected and focused into a triple spectrometer (consisting of two Acton Research SpectraPro 2300i and a SpectraPro 2500i in the final stage with a 1800 or 3600 grooves/mm grating) working in the subtractive mode, equipped with a liquid nitrogen cooled CCD detector.

All RR measurements were repeated several times under the same conditions to ensure reproducibility. To improve the signal/noise ratio, a number of spectra were accumulated and summed only if no spectral differences were noted. The RR spectra were calibrated with indene, CCl_4, dimethyl sulfoxide, acetone, and acetonitrile as standards to an accuracy of $\pm 1\ cm^{-1}$ for intense isolated bands. To determine peak intensities and positions a curve-fitting program (Lab Calc, Galactic) was used to simulate the spectra using a Lorentzian line shape.

3. Results

3.1. Ferric trout Mb

The electronic absorption spectrum of ferric trout Mb at pH 6.0 is characterized by a Soret band at 406 nm, Q_1 and Q_0 bands at 502 and 543 nm, respectively, and the charge transfer band (CT1) at 633 nm (Fig. 1A). The spectrum is very similar to that of swMb [8] suggesting an aquo high-spin (HS) coordination. Accordingly, the RR high-frequency spectrum, recorded in polarized (Fig. S1, Supplementary material) and non-polarized light (Fig. 1B), indicates the presence of a hexacoordinate HS species (ν_3 1481, ν_2 1563, ν_{37} 1580, ν_{10} 1609 cm^{-1}) [9]. Hence, the heme cavity of the ferric form does not display major differences compared with other Mbs. However, in the low-frequency region (Fig. 1C, trace a), while the RR spectrum is mainly characterized by the ν_8 (344 cm^{-1}), propionyl (374 cm^{-1}) and vinyl bending modes (409 cm^{-1}), and out-of-plane modes as hhMb [9], it markedly differs from the latter due to the intensity enhancement of the γ_6 at 338 cm^{-1} and the appearance of a new intense band at 426 cm^{-1} in the region of the vinyl bending modes. The assignment of the low-frequency region is reported in Table 2.

3.2. Anion bound ferric complexes

At alkaline pH the electronic absorption spectrum of ferric trout Mb is characteristic of a mixture of a hexacoordinate low-spin species (6cLS) (maxima at 412, 543, 577 nm) and a 6cHS species [charge transfer (CT) bands at 490 (CT2) and 605 nm (CT1)] (Fig. 1A) resulting from a hydroxyl group bound to the heme iron atom. This behavior is similar to that of hhMb except for a 5 nm upshift of both CT bands [10]. In agreement with the presence of a 6cHS and a 6cLS species, the high-frequency RR spectrum at pH 10.0 (Fig. 1B; Fig. S1, Supplementary material) shows two ν_3 bands at 1478 and 1504 cm^{-1}, respectively. The corresponding low frequency RR spectrum shows two new isotope sensitive bands at 492 and 559 cm^{-1} (Fig. 1C, trace b), which downshift to 476 and 543 cm^{-1} in D_2O (Fig. 1C, trace c), clearly evident in the difference spectrum. Therefore, they are assigned to the high-spin and low-spin Fe–OH stretching modes (ν(Fe–OH)), respectively. Hence, as observed for other Mbs and heme proteins [10], at room temperature and alkaline pH rainbow trout Mb exists as an equilibrium between high- and low-spin forms. The frequency of the high-spin band (492 cm^{-1}) is very similar to that of other Mbs, whereas the low-spin band frequency (559 cm^{-1}) is significantly higher (Table 1) suggesting a weaker or no H-bond interaction between the iron-bound hydroxyl group and the distal HisE7 residue in rainbow trout Mb. In fact, two structural/electronic effects generally contribute to the Fe–OH bond strength, and therefore, to the bond-stretching frequency. In general, it is