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Alteration of the proximal bond energy in the unliganded form of the homodimeric myoglobin from *Nassa mutabilis*

Kinetic and spectroscopic evidence

Massimo Coletta[1], Paolo Ascenzi[2], Giulietta Smulevich[3], Anna Rita Mantini[3], Rosanna Del Gaudio[4], Marina Piscopo[5] and Giuseppe Geraci[5]

[1]*Department of Molecular, Cellular and Animal Biology, University of Camerino, Via Filippo Camerini 2, 62032 Camerino (MC), Italy,* [2]*Department of Pharmaceutical Chemistry and Technology, University of Turin, Via Pietro Giuria 9, 10125 Turin, Italy,* [3]*Department of Chemistry, University of Florence, Via Gino Capponi 9, 50121 Florence, Italy,* [4]*CNR Institute of Protein Biochemistry and Enzymology, Via Guglielmo Marconi 10, 80072 Arco Felice (NA), Italy* and [5]*Department of Genetics, General and Molecular Biology, University of Naples, Via Mezzocannone 8, 80134 Naples, Italy*

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CO binding kinetics to the homodimeric myoglobin (Mb) from *Nassa mutabilis* has been investigated between pH 1.9 and 7.0. Protonation of the proximal imidazole at low pH (≤ 3.0) and the consequent cleavage of the HisF8NE2-Fe proximal bond brings about a ≈ 20-fold increase of the second-order rate constant for CO binding. This process displays a $pK_a = 4.0 \pm 0.2$, significantly higher than that observed in all other deoxygenated hemoproteins investigated up to now. Such a feature underlies a decreased energy for the HisF8NE2-Fe proximal bond in the unliganded form and it also appears supported by resonance Raman spectroscopy in the low frequency region of the Fe(II) deoxygenated hemoprotein. Further, the pH-rate profile of *N. mutabilis* Mb, like that of the homodimeric hemoglobin (Hb) from *Scapharca inaequivalvis* (Coletta, M., Boffi, A., Ascenzi, P., Brunori, M. and Chiancone, E. (1990) J. Biol. Chem. 265, 4828–4830), can be described only by assuming a concerted proton-linked transition with $n = 1.8 \pm 0.1$. Such a characteristic suggests, also on the basis of the amino acid sequence homology between *N. mutabilis* Mb and *S. inaequivalvis* Hb in the region forming the subunit interface, that the interaction mechanism is similar for the two homodimeric proteins, and drastically different from that operative in other hemoproteins.

Myoglobin; *Nassa mutabilis* homodimeric Mb; CO binding kinetics; Resonance Raman spectroscopic property; pH effect

1. INTRODUCTION

Myoglobin is an oxygen-binding hemoprotein which is present in the muscles of most living organisms. Although it is usually formed by a single polypeptide chain [1], dimeric Mb's have been reported during the last few years to be present in the radular muscles of marine gastropods from prosobranchia subclass [2–5]. In particular, the homodimeric Mb from *Nassa mutabilis* has raised interest because of a fairly marked cooperativity in oxygen binding ($n = 1.5$) [3].

The occurrence of cooperative homodimeric Mb's and Hb's in invertebrates [3,6,7] opens the question whether they might all be representatives of an ancestral class of hemoproteins with a similar primitive heme-heme interaction mechanism(s) in spite of their different location and physiological role.

The cooperativity shown by the homodimeric Hb from *Scapharca inaequivalvis*, an Arcid clam recently settled in the Adriatic sea, has recently been suggested to stem from a novel interaction mechanism [8], possibly related to the unusual subunits assembly of this hemoprotein. In this respect, the heme-carrying E and F helices form the subunit interface, bringing the heme groups of the two subunits in a totally different spatial relationship, as compared to human Hb. Thus, the two hemes are significantly closer to each other and are in direct contact with the amino acid residues of the pairing subunits, themselves being part of the subunits' interface [9,10]. Although no three-dimensional structural information is available yet for *N. mutabilis* Mb, it is very important to remark a relevant amino acid sequence similarity between *S. inaequivalvis* Hb and *N. mutabilis* Mb mostly in the region(s) forming the subunit interface (Augusto Parente and Giuseppe Geraci, manuscript in preparation).

These considerations prompted us to carry out a detailed functional and spectroscopic investigation on

Abbreviations: Mb, Myoglobin; Hb, Hemoglobin; *N. mutabilis* Mb, *Nassa mutabilis* homodimeric myoglobin; *S. inaequivalvis* Hb, *Scapharca inaequivalvis* homodimeric hemoglobin

Correspondence address: M. Coletta, Department of Biochemical Sciences, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy. Fax: (39) (6) 4440062.

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N. mutabilis Mb in order to better characterize the heme–heme interaction mechanism operative in this hemoprotein. The results have been analysed in parallel with the molecular properties of *S. inaequivalvis* Hb, human Hb and monomeric Mb's [8,11–14].

2. MATERIALS AND METHODS

N. mutabilis Mb was prepared as previously reported [3]. Horse heart Mb was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and further purified as previously reported (see ref. [1]). Carbon monoxide was obtained from Caracciolossigeno S.p.A. (Rome, Italy). All other reagents were from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification.

The absorption spectrum of the deoxygenated derivative of *N. mutabilis* Mb was recorded, at pH 7.0 (in 0.2 M phosphate buffer) and 20°C, on a Varian Cary 219 double beam spectrophotometer. The concentration of *N. mutabilis* Mb was determined on the basis of $\varepsilon = 11.7\ mM^{-1} \cdot cm^{-1}$ for the unliganded derivative [3]. The transient absorption spectrum of the deoxygenated derivative of *N. mutabilis* Mb was obtained at pH 3.1 (in 0.2 M phosphate buffer) and 20°C, before the occurrence of the hemoprotein denaturation ($t_{1/2} \approx 1$ s), from the initial optical density changes after rapidly mixing the deoxygenated derivative of *N. mutabilis* Mb with degassed buffer to bring the pH to 3.1 [11]. Values of the second-order rate constant for CO binding to the deoxygenated derivative of *N. mutabilis* Mb were obtained, between pH 1.9 and 7.0 (in 0.2 M phosphate buffer) at 20°C, by the rapid-mixing stopped-flow technique [11]. All kinetic observations were carried out using a Durrum–Gibson stopped-flow apparatus.

The resonance Raman spectra of the deoxygenated derivative of *N. mutabilis* and horse heart Mb's were obtained at pH 7.0 (in 0.2 M phosphate buffer) and 20°C, with excitation from the 406.7 nm line of a Kr^+ laser (Coherent Radiation, USA). The backscattered light from a slowly rotating NMR tube was collected and focussed into a computer-controlled double monochromator (Jobin–Yvon HG-2S) equipped with a cooled photomultiplier (RCA) and photon counting electronics. The spectra were calibrated with the indene and CCl_4 bands.

3. RESULTS AND DISCUSSION

The second-order rate constant for CO binding to *N.mutabilis* Mb is strongly pH-dependent and its value increases at low pH (Fig. 1A). At pH 3.1 the spectrum of the deoxygenated derivative of *N. mutabilis* Mb is clearly changed and it is characterized by two bands at 530 and 560 nm (Fig. 1B). These findings indicate that the proximal HisF8NE2-Fe bond is cleaved (as a result of the protonation of the proximal imidazole), as previously observed for hemoproteins and heme model compounds [8,11,12,14]. Similar to that observed for the *S. inaequivalvis* Hb [8] and different from human Hb and monomeric Mb's [11,12], the pH-rate profile for CO binding to *N. mutabilis* Mb requires the involvement of at least two protons in a concerted fastion ($n = 1.8 \pm 0.1$). This peculiar behaviour suggests that the heme–heme interaction mechanism invoked for *S. inaequivalvis* Hb (see above) might be operative also in the case of *N. mutabilis* Mb. This possibility appears to be supported by the observation that the amino acid sequence of *N. mutabilis* Mb (Augusto Parente and Giu-

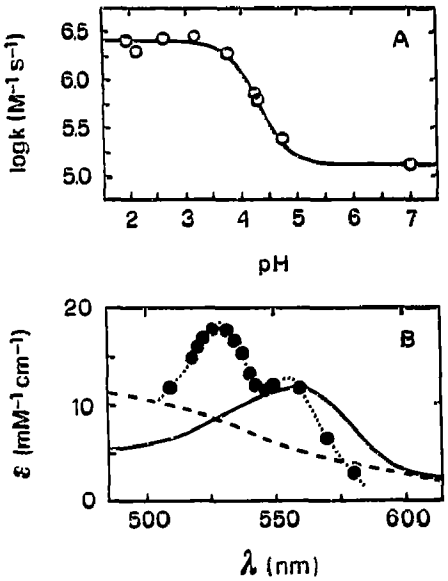

Fig. 1. (Panel A) pH dependence of the second-order rate constant for CO binding to *N. mutabilis* Mb. The continuous line is the best fitting curve calculated according to the following equation: $k = k_a (1/(1 + K_a [H^+]^n + k_f (K_a [H^+]^n)/(1 + K_a [H^+]^n)$; with the following set of parameters: $k_a = 1.40 (\pm 0.17) \times 10^5$; $k_f = 2.63 (\pm 0.16) \times 10^5$; $\sqrt{K_a} = 1.0 (\pm 0.1) \times 10^4$; $n = 1.8 \pm 0.1$. Values of the second-order rate constants were obtained in 0.2 M phosphate buffer at 20°C. The concentration of *N. mutabilis* Mb was 2 μM heme. For additional experimental details, see text. (Panel B) Absorption spectra of the deoxygenated derivative of ferrous *N. mutabilis* Mb at pH 3.1 (dotted line) and at pH 7.0 (continuous line). The dashed line corresponds to the absorption spectrum of the denatured deoxygenated derivative of ferrous *N. mutabilis* Mb at pH 3.1, long time after mixing. The experimental points (filled circles) represent the initial optical density changes after rapid mixing of ferrous deoxygenated *N. mutabilis* Mb with degassed buffer to bring the pH to 3.1. The absorption spectra have been collected at 20°C. The concentration of *N. mutabilis* Mb was 25 μM heme. For additional experimental details, see text.

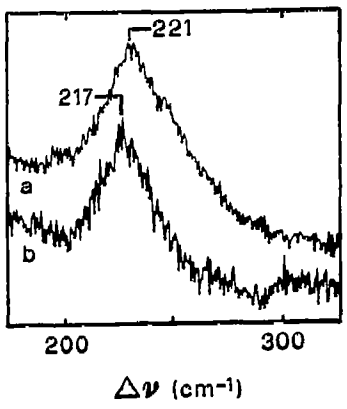

Fig. 2. Resonance Raman spectra of the deoxygenated derivative of ferrous horse heart (a) and *N. mutabilis* (b) Mb's obtained with 406.7 nm laser excitation at 20°C. Conditions: 5 cm^{-1} resolution, (a): 20 mW laser power at the sample, 0.5 cm^{-1}/10 s collection interval: (b): 40 mW laser power at the sample, 0.5 cm^{-1}/4 s collection interval. The concentration of *N. mutabilis* and horse heart Mb's was ≈ 250 μM heme. For additional experimental details, see text.

seppe Geraci, manuscript in preparation) conserves critical residues, forming the subunit interface of *S. inaequivalvis* Hb [10].

However, some differences in the kinetic behaviour between the two homodimeric hemoproteins should be remarked. In particular, it must be outlined that the pK_a(= 4.0 ± 0.2) for the pH dependence of CO binding to *N. mutabilis* Mb is significantly higher than that observed for unliganded *S. inaequivalvis* Hb (pK_a = 3.2 ±0.2, [8]), as well as for the deoxygenated derivative of monomeric and tetrameric hemoproteins (average pK_avalues of 3.1 ± 0.3, [11,12]). Such a difference indicates a decrease by almost 1 kcal/mol of the bond energy involved in the protonation kinetic effect. It suggests that in *N. mutabilis* Mb the proton competes with the proximal HisF8NE2-Fe bond more efficiently than in other hemoproteins, underlying a different stereo chemistry for the proximal side of the heme pocket in *N.mutabilis* Mb with respect to other hemoproteins investigated up to now [8,11,12].

The interaction between the Fe atom and the proximal imidazole can be monitored directly via the frequency of the Fe-N (His) stretching vibration, which gives rise to a prominent band between 200 cm^{-1} and 250 cm^{-1} in the resonance spectra of ferrous heme proteins. Shifts of its frequency reflect a considerable sensitivity of the Fe-imidazole bond strength to the status of the proton on the bound imidazole [15–17].

The low frequency region resonance Raman spectrum of the deoxygenated derivative of *N. mutabilis* Mb shows that the strongest band at 217 cm^{-1} shifts down by 4 cm^{-1} with respect to the band observed for horse heart Mb (Fig. 2), and assigned to the stretching mode between the Fe atom and the HisF8NE2 proximal ligand [15,16]. This shift is attributed either to a weaker bond strength between the Fe atom and the proximal imidazole in *N. mutabilis* Mb or to an easier access of the proton to the proximal site. Both interpretations agree with the increased pK_a value observed for the protonation of the proximal bond in the deoxygenated derivative of this Mb, as derived from the pH dependence of CO binding kinetics (see Fig. 1A).

In conclusion, the results presented here show that *N. mutabilis* Mb indeed displays qualitative CO binding features which suggests that the heme–heme interaction mechanism responsible for the expression of the ligand binding cooperativity might be closely similar to that postulated for *S. inaequivalvis* Hb [8], and significantly different from that of tetrameric human Hb [12]. This finding could be related to the likely similar assembly of the two subunits in these homodimeric hemoproteins, that allows a direct communication between the two hemes. On the other hand, the pH rate profile of the CO binding kinetics suggests significant structural differences for the proximal side of the heme pocket between *N. mutabilis* Mb on the one side and monomeric Mb's, *S. inaequivalvis* Hb and human Hb on the other side.

Altogether these observations suggest that from the evolutionary standpoint *N. mutabilis* Mb (and possibly *S. inaequivalvis* Hb) might represent an interesting case of the intermediate situation between monomeric Mb's and multimeric cooperative Hb's.

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