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Contribution of the heme propionate groups to the electron transfer and electrostatic properties of myoglobin

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Abstract

The role of the heme propionate groups in determining the electron transfer and electrostatic properties of myoglobin have been studied by thermodynamic, kinetic, and spectroscopic studies of horse heart myoglobin in which the heme propionate groups are esterified. Spectroelectrochemical analysis has established that the $E_{m,7}$ of dimethylester heme-substituted Mb (DME-Mb) ($E_{m,7} = 100.2(2)$ mV *vs.* NHE (Normal Hydrogen Electrode) (25 °C) is increased ~40 mV relative to that of the native protein with $\Delta H° = -12.9(2)$ kcal/mol and $\Delta S° = -51.0(8)$ cal/mol/deg (pH 7.0, $\mu = 0.1$ M (phosphate)). The second order rate constant for reduction of DME-metMb by $Fe(EDTA)^{2-}$ is increased > 400-fold relative to that for reduction of native metMb to a value of $1.34(2) \times 10^3\ M^{-1}\ s^{-1}$ with $\Delta S^{\ddagger} = -13(1)$ cal/mol/deg and $\Delta H^{\ddagger} = 9.2(3)$ (pH 7.0, $\mu = 0.1$ M (phosphate)). Analysis of the pH dependences of the reduction potential and rate constant for reduction by $Fe(EDTA)^{2-}$ demonstrates that heme propionate esterification introduces significant changes into the electrostatic interactions in myoglobin. These changes are also manifested by differences in the pH dependences of the ^{1}H NMR spectra of native and DME-metMb that reveal shifts in pK_a values for specific His residues as the result of heme propionate esterification. In sum, the current results establish that heme propionate esterification not only affects the electron transfer properties of myoglobin but also influences the titration behavior of specific His residues.
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Keywords: Myoglobin; Spectroelectrochemistry; $Fe(EDTA)^{2-}$; Kinetics; Propionate; NMR; Electrostatics

1. Introduction

Myoglobin (Mb) is a monomeric protein (MW ~17,000) that possesses a single, non-covalently bound heme prosthetic group. The physiological function of Mb has been regarded for some time to be facilitation of the diffusion of dioxygen in skeletal and cardiac muscle tissue [1–3] though discovery that a mouse devoid of myoglobin exhibits a surprisingly mild phenotype [4] has stimulated further consideration of this subject [5–8]. Mb is readily autoxidized to metMb in an aerobic environment (reviewed in [9]), and enzymatic reduction of myoglobin to the reduced, Fe(II) state was reported over twenty years ago [10] though the subsequent literature has produced inconsistent results regarding the nature of this enzymatic system (reviewed in [11]). The literature concerning the oxidation–reduction properties and electron transfer kinetics of myoglobin is extensive (a review of the older literature is provided in [12]) and in more recent years has emphasized the use of myoglobin as (i) a model for studies of intramolecular electron transfer (e.g., [13,14]), (ii) a participant in protein–protein electron transfer reactions (e.g., [15–18]), a model for ligand binding (e.g., [19–23]) and (iv) a protein scaffold for genetic engineering of new functionalities (e.g., [19,24–28]).

In previous studies, we demonstrated that the heme propionate groups of cytochrome b_5 are involved in determination of the reduction potential of this protein and influence the kinetics of its reduction by $Fe(EDTA)^{2-}$ [29] and its interaction with cytochrome *c* [30], and we have studied the contribution that the heme propionate groups

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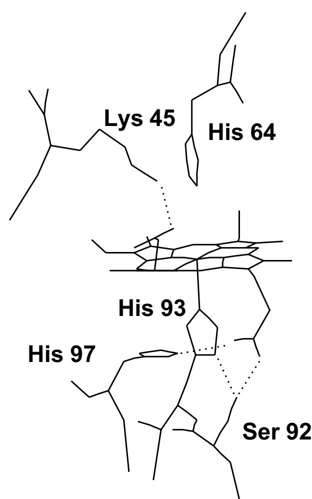

Fig. 1. Structural diagram of the hydrogen bonding interactions of the heme propionate groups as identified in the crystallographically determined structure for horse heart metMb [34].

of horse heart myoglobin make toward stabilizing the interaction of the heme prosthetic group with the apo-protein [31,32]. In view of the structural relationship that has been noted to exist between cytochrome b_5 and myoglobin [33], it seemed reasonable to expect that the role of heme propionate groups observed in the case of cytochrome b_5 are also operative in the oxidation–reduction behavior of myoglobin. Consequently, we have investigated this possibility through comparison of the reduction potentials and reduction kinetics of native horse heart myoglobin with a derivative of the protein in which the native heme prosthetic group has been replaced with dimethylester heme IX. The results are interpreted in light of the three-dimensional structure of the protein [34,35] (Fig. 1).

2. Experimental

2.1. Preparation of DME-Mb

Horse heart myoglobin (Sigma Type III) was purified as described by Tomoda et al. [36]. Apomyoglobin was prepared by the method of Teale [37] and reconstituted with DME-heme by a modification of the method described by Tamura et al. [38,39] as follows. Apomyoglobin (150 mg) was concentrated by ultrafiltration (Amicon YM-5 membrane) to about 400 μM. DME-heme (Porphyrin Products, Logan UT (now Frontier Chemical); 1.25-fold excess) was dissolved in a minimal volume (<5% of the protein solution) of hot methanol, centrifuged to remove any insoluble material, and added in a dropwise manner to ice-cold apoprotein with gentle stirring. The mixture was allowed to equilibrate at 4 °C for ~2 h. During this time, reconstitution was followed by measuring the A_{407}/A_{280} absorption ratio (ca. 2.2 in 0.1 M sodium phosphate buffer (pH – 6.0 at 25 °C) after 2 h). A second portion of DME-heme dissolved in hot methanol (1.25-fold excess in a volume <5% of the initial apoprotein solution) was added to the mixture as described above and allowed to equilibrate for 2 h (4 °C). The final methanol concentration was kept below 10%. The A_{409}/A_{280} ratio after this second addition was 2.8. The protein/DME-heme solution was then reduced with concentrated $Fe(EDTA)^{2-}$ solution and dialyzed against two changes of cold $NaHCO_3$ solution (4 L, 50 mg/L) (4 °C). The mixture was centrifuged at 9200*g* for 30 min (4 °C), the pellet was discarded, and the supernatant fluid was concentrated to its original volume. Two more portions (1.0 and 0.5 equiv, respectively) of DME-heme solution were then added to the protein solution as described above after which the A_{409}/A_{280} ratio was ~3.5. The protein solution was fully reduced with concentrated $Fe(EDTA)^{2-}$ solution, dialyzed against two changes of cold (4 °C) Tris–HCl buffer (15 mM, pH 8.4), centrifuged at 9200*g* (30 min), and the supernatant fluid loaded onto a column (70 × 2.5 s) of Sephadex G-75 superfine (Amersham) that had been equilibrated with 20 mM sodium phosphate buffer (pH 7.2 at 4 °C). The protein was eluted with this same buffer (4 °C). A green band of unbound heme eluted before the reddish-brown band of reconstituted myoglobin. Protein fractions with A_{407}/A_{280} ratio >5.0 were pooled and concentrated by ultrafiltration. The myoglobin reconstituted with DME-heme (DME-Mb) was recovered with a yield of 72–80% ($A_{407} = 168$ mM cm^{-1}) and stored in liquid nitrogen. DME-MbO_2 was oxidized to DME-metMb with ferricyanide, and excess oxidizing agent was removed by ultrafiltration followed by passage through Dowex X-8 ion exchange resin to remove bound oxidant [40]. Molar absorptivities were determined by the pyridine hemochromagen method [41].

2.2. Electrochemical and kinetic measurements

Electronic absorption spectra were obtained with a Cary 219 spectrophotometer (slit width, 1 nm). Potentiometric titrations were performed with an optically transparent thin-layer electrode (OTTLE) and an electrode configuration described previously [29] with $[Ru(NH_3)_5imidazole]Cl_3$ [42] as mediator. Laccase from *Rhus vernicifera* (a gift from Prof. Harry B. Gray) was added (0.1 μM) to the protein solution to assure anaerobiasis during the potentiometric titration. Catalase (Sigma Type C-100; ~200 Sigma units) was also required to obtain well-defined isosbestic points. Nernst plots were constructed from absorbance readings made at 407 nm, and the resulting midpoint potentials were converted to the Normal Hydrogen Electrode (NHE) [43]. Kinetic measurements were made with a modified Durrum-Gibson stopped-flow spectrophotometer (Dionex, Sunnyvale, CA) interfaced to a microcomputer (OLIS, Bogart, GA). Data reduction techniques have been described previously [29,44].

2.3. ^1H NMR spectroscopy

Protein solutions (20 mg in 0.5 mL) were exchanged 3–4 times into 0.1 M $NaCl/D_2O$ (99.7%) by centrifugal

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