Control of Myoglobin Electron-Transfer Rates by the Distal (Nonbound) Histidine Residue

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Abstract: Changing the distal histidine (H64) of sperm-whale myoglobin into any of the following residues—valine, leucine, methionine, glycine, or phenylalanine—causes a dramatic improvement in the reversibility (electron-transfer kinetics) and reproducibility of the direct electrochemistry. Cyclic voltammograms of native or wild-type recombinant myoglobin are irreversible (in the electrochemical sense) and critically dependent on the condition of the sample and electrode. By contrast, the H64 mutants display quasi-reversible electrochemistry much more typical of results obtained with true electron-transfer proteins. The difference in activity correlates sharply with alterations to the distal-pocket H-bonding network, which in the native protein comprises the H_2O that is coordinated to Fe(III), the N^{ϵ} of H64, and the "lattice" extending from arginine-45 to the heme periphery. It is proposed that this H-bond network increases the electron-transfer activation energy by coupling the displacement of Fe(III)-coordinated H_2O to higher reorganization requirements, including that of solvent H_2O molecules near the heme periphery. The poor reproducibility and extreme sensitivity of the electrochemical response to experimental conditions is rationalized by the microscopic model for protein electrochemistry which predicts that the waveshape and potential positions for inherently irreversible (sluggish) systems will be critically dependent on the state of the electrode surface.

Introduction

Myoglobin (Mb)[1] provides a superb example of how a common active site, the heme group, has been optimized to accomplish a single task—reversible O_2 binding—to the apparent exclusion of other possible functions, such as electron transfer. Comparisons with established electron-transfer proteins such as cytochrome *c*[2,3] indicate that Mb displays poor redox kinetics, whether studied by chemical[3,4] or direct electrochemical methods.[5−9] Significantly, autoxidation—reaction of Fe(II) Mb with O_2 to give the physiologically-inactive Fe(III) form—is also very slow.[10] Studies of intramolecular electron transfer in Ru-modified Mb suggested[3,4] that the poor redox kinetics are the result of a high reorganization energy. Hawkridge and co-workers further proposed[6] that the ill-defined and irreversible cyclic voltammetry of horse skeletal Mb arises because the redox process is gated by dissociation and re-association of the bound H_2O molecule in the Fe(II) form, i.e. that the mechanism is of the "EC" coupled type.[6] However, the situation is complicated immensely by observations that the voltammetry is extremely sensitive to sample purity and the condition of the electrode surface.[7−9] The best results have been reported for horse Mb. Rusling and co-workers found that the electrochemical response of horse skeletal Mb at a graphite electrode was enhanced in the presence of a surfactant.[7] Taniguchi and co-workers achieved quasi-reversible voltammetry of horse heart Mb with a peak separation (ΔE_p) of ca. 100 mV at a scan rate of 20 mV s^{-1} using an ultra-clean and hydrophilic indium oxide electrode.[8,9] Notably, under identical conditions, the electrochemistry of sperm whale Mb was still observed to be much less reversible, with ΔE_p exceeding 250 mV.[9]

Almost all myoglobins contain a distal histidine (H64) which assists the binding of O_2 to Fe(II) heme by forming a H-bond between the protonated N^{ϵ} atom of the imidazole ring and the remote O-atom.[1,11] Histidine-64 also forms a H-bond with the H_2O that is coordinated to the Fe(III) form; this interaction, importantly, forms part of a highly ordered H-bonding "lattice" extending to the heme periphery and bulk solvent.[12] While reduction of the Fe(III) form leads to rupture of the $Fe-OH_2$ bond, the H_2O molecule remains tightly held in the pocket by H-bonding to the imidazole N^{ϵ} atom.

Using structurally or spectroscopically characterized H64 mutants it becomes possible to elucidate the role of this residue in greater detail. Previous studies have shown that H64 mutants

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display weaker O_2 binding and faster rates of ligand exchange and autoxidation.[10,13,14] We have now studied the cyclic voltammetry of several H64 variants at a pyrolytic graphite edge (PGE) electrode in order to make direct comparisons of their *electron-transfer* activities with native or recombinant wild type (WT) forms. Since the H64 variants have similar *external* structures[12] they are not expected to differ significantly in interactions with the electrode surface. Direct electrochemical methods thus provide an excellent way to compare intrinsic redox activities without the need to correct for driving force variations arising from changes in reduction potentials.

Methods

Recombinant wild-type and mutant sperm whale myoglobins were expressed and purified using the synthetic gene constructed by Springer and Sligar.[15] Wild-type Mb is identical to native Mb, except that a methionine initiator is attached to the amino terminus and aspartate-122 is replaced by asparagine. Native myoglobin was purified as described previously.[16] Almost all experiments used WT rather than native protein. Mb samples were re-purified immediately prior to experiments using Fast Protein Liquid Chromatography (Pharmacia) with a Mono-S column.

DC cyclic voltammograms were measured using either an Ursar Instruments potentiostat (Oxford) or an Autolab analyzer (EcoChemie, Holland). All experiments were carried out in an anaerobic glovebox (Belle Technology, Poole, England) with O_2 levels <1 ppm. Solutions of proteins, typically 50−200 μM, in 0.1 M Hepes (Na), pH 7.0, were thoroughly degassed on a Schlenk line before introducing to the glovebox. Solutions of reduced (Fe(II)) Mb were prepared by anaerobic reaction with a small excess of sodium dithionite, followed by chromatography on a small column of Sephadex G-25 (Pharmacia Biotech). The electrochemical cell, electrode preparation, and solution preparations have been described previously.[17] All potentials (measured using a Saturated Calomel Electrode (SCE)) have been corrected to correspond with the Standard Hydrogen Electrode (SHE) scale (E_{SCE} = 243 mV vs SHE at 22 °C).

Results and Discussion

Cyclic voltammograms of native and WT-Mb at a polished PGE electrode are characterized by poorly defined waves having the large potential separations normally associated with electrochemical irreversibility.[9] By contrast, the H64 mutants display remarkable improvements in sharpness and reversibility, as well as in stability and reproducibility. Figure 1 shows typical slow-scan voltammograms (solid traces) obtained for the H64L and H64G mutants, along with the best result that we have been able to obtain for WT-Mb. The leucine mutant H64L has a hydrophobic distal pocket in which the Fe is five-coordinate in both oxidation states, whereas H64G has a H_2O coordinated to Fe(III) but not Fe(II), as found for WT Mb.[12] Electrochemical data and other information are compiled in Table 1. For native and wild-type Mb, the reduction potential obtained by taking the average of forward and reverse voltammetric peaks is in excellent agreement with the previously published value of 59 mV vs SHE determined by thin-layer spectroelectrochemistry.[18]

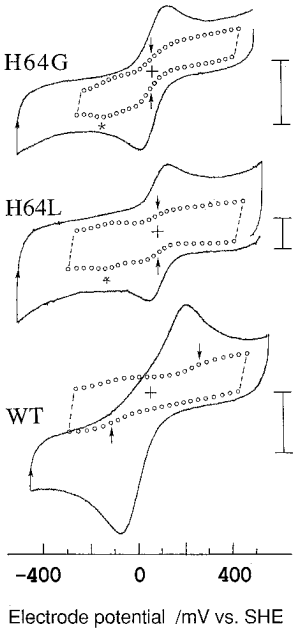

Figure 1. Cyclic voltammograms (CVs) of recombinant Myoglobins obtained at a PGE electrode. Temperature 23 °C, 0.10 M Na Hepes at pH 7.0. **H64G** (distal pocket polar)—Solid line: CV measured at 5 mV s^{-1} (current scale 0.25 μA). Circles: CV measured at 50 mV s^{-1} and trimmed to remove charging component (current scale 1.25 μA). Some adsorption of heme is evident, as indicated by the asterisk; the potential corresponds to the value obtained independently for adsorption from a dilute solution of hemin. **H64L** (distal pocket hydrophobic)—Solid line: CV measured at 5 mV s^{-1} (current scale 0.1 μA). Circles: CV measured at 100 mV s^{-1} and trimmed to remove charging component (current scale 1.3 μA). Some adsorption of heme is evident, as indicated by the asterisk (see above). **WT** (wild type)—Solid line: CV measured at 1 mV s^{-1} (current scale 0.1 μA). Circles: CV measured at 50 mV s^{-1} and trimmed to remove charging component (current scale 2.7 μA).

Table 1. Comparison of the Electrochemical Parameters for Mb and H64 Mutants, and Correlation with the Presence of Coordinated H_2O and the Distal Ligand Hydrogen Bond to H_2O[a]

			H_2O coordinated[c]		H_2O H-bonded[c]	
protein	$\Delta E_p(\nu)$[b]/mV	$E°'$/mV[d]	Fe(III)	Fe(II)	Fe(III)	Fe(II)
native	280 (1)	59	yes	no	yes	yes
WT	270 (1)	59	yes	no	yes	yes
H64G	95 (5)	65	yes	no	no	no
H64L	70 (5)	84	no	no	no	no
H64V	115 (2)	76	no	no	no	no
H64M	94 (10)	98	no[e]	no	no	no
H64F	100 (5)	109	no[e]	no	no	no

[a] Protein concentrations were 50 μM in 0.1 M Na Hepes, pH 7.0, 22 °C. [b] ν is the scan rate, in mV s^{-1}. [c] Unless otherwise stated, the presence of H_2O in the distal pocket is based upon crystal structure analysis according to ref 12. [d] Reduction potentials vs standard hydrogen electrode (SHE). [e] Crystal structure not determined, but the position of the Soret band is identical to that of H64L in which the heme pocket is hydrophobic (unpublished results).

Voltammograms of H64G, H64L, and H64M were measured with various protein concentrations. Both H64L and H64G exhibited a stable and well-defined peak-type voltammetric response at a scan rate of 5 mV s^{-1} with myoglobin concentrations up to 200 μM. Little variation in reduction potential was

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observed as a function of the protein concentration. To determine that the improved electrochemistry was not due to there being significantly lower levels of proteinaceous impurities in the mutant samples (which would thus alleviate fouling of the electrode surface), studies were carried out on solutions having equal concentrations of WT-Mb and H64L. The resulting voltammograms showed the more reversible waves of H64L *in addition to* the poorly defined, irreversible response of WT Mb. Adsorbed heme was often observed at a potential of approximately −140 mV, but this was less noticeable with WT-Mb than with the mutants.

In all cases, scan rate dependences of waveforms and currents conformed to the microscopic model[19] for protein diffusional voltammetry, i.e. reaction at partially blocked electrode surfaces for which the diffusional geometry changes from linear (yielding the familiar peak-type waveform[20,21]) to radial (yielding a sigmoidal waveform) as the scan rate is increased. On the one hand, the mutant Mbs display reproducible quasireversible voltammograms with a linear dependence of peak current on (scan rate)$^{1/2}$ up to 20 mV s^{-1}.[20] At higher scan rates, the waves eventually transform to a sigmoidal appearance and the faradaic current becomes independent of scan rate. Essentially identical values of $E_{1/2}$ are obtained for oxidation and reduction waves and these are in good agreement with formal reduction potentials $E^{\circ\prime}$ measured from the average of peak positions at slow scan rates. However, for WT-Mb the transition to a sigmoidal waveform is much more abrupt, and even at 5 mV s^{-1} the waves are almost sigmoidal but display widely differing and erratically varying $E_{1/2}$ values for either direction. Similar voltammetry throughout the entire range of scan rates was observed for experiments in which a solution of native Mb was introduced to the electrochemical cell in the reduced form.

The convoluted waveform means that ΔE_p values give only a *comparative* guide to electrochemical kinetics, and rate constants calculated by the Nicholson method[21] will err on the low side. With such a model, rate constants (cm s^{-1}) for the examples in Figure 1 would be as follows: H64L, 1.3×10^{-3}; H65G, 4×10^{-4}; WT, 1.4×10^{-5}. More realistically, inspection of the sigmoidal responses (circles) obtained at higher scan rates shows unequivocally that H64L and H64G mutants display electrochemistry that is effectively reversible, i.e. $k > 10^{-2}$ cm s^{-1}, and reinforces the conclusion that redox transformation of WT-Mb is sluggish.

The greatly increased electron-transfer activities of H64 mutants correlate with structural differences that are essentially limited to the vicinity of the mutated residue and the distal pocket.[12] Considered alone, the electrochemical reversibility of mutants with *hydrophobic* distal pockets (H64L, H64V, H64F, and H64M) could be interpreted as reflecting the lack of H_2O exchange activity normally coupled to (and perhaps controlling) the redox transformation. However, more subtle factors are revealed with H64G. The most significant difference between WT-Mb and H64G (and which must therefore underlie the increased activity of H64G) is the ability of H64 to H-bond with the coordinated H_2O and link this interaction to the ordered H-bonded network of the distal pocket.[12] The inference is that Mb redox kinetics are determined not only by redox-coupled coordination and dissociation of H_2O but also by the ease with which any such displacement is *communicated* to bulk H_2O at the heme periphery, i.e. (for WT) via H64 N^{ϵ} and the ordered H-bonding lattice. Redox activity of the heme in WT-Mb is thus coupled to this lattice and to solvent reorganization itself.

The possibility that the mechanism is of the gated (i.e. "EC") type as opposed to a concerted process[6] has not been addressed, but distinctions may not be too significant.[22] The electrochemical activities of the H64 mutants match their higher rates of autoxidation[10] and suggest that the same reorganization barrier retards this physiologically detrimental reaction, which is dominated by an outer-sphere (bimolecular) mechanism at low O_2 concentrations.[10] Experimentally, the restriction provided by H64 appears also to sensitize the system to *extrinsic* factors, i.e. the condition of the electrode surface.[9] This observation is now explained by the microscopic model[19] since for an inlaid disc microelectrode, *irreversible* electrode reactions give a voltammetric response for which the half-wave potential as well as the current depends on the *size of the disc*, i.e. of the unblocked, electroactive sites on the electrode.[23] Consequently the reproducibility of protein electrochemistry at electrodes that become partially blocked (fouled) by adsorption of inactive material (such as apoprotein) is expected to be particularly poor whenever the reaction is sufficiently irreversible (sluggish).

To summarize, hydrogen bonding by H64 not only controls ligand binding but also restricts the ability of the heme group to undergo facile electron transfer. Participation of H64 in the H-bonding network between the active site and heme periphery renders it equivalent to an interfacial (surface) residue, in that displacement of H_2O at the active site may be effectively coupled to the more demanding reorganization of bulk H_2O. The effects produced by a single distal pocket mutation serve both to illustrate subtleties of electron transfer and to help unravel the complexities involved in achieving and interpreting protein voltammetry.

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