Functional Role of Leucine-103 in Myohemerythrin†

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ABSTRACT: Hemerythrins (Hrs) and myohemerythrins (Mhrs) are nonheme iron proteins that function as O_2 carriers in four marine invertebrate phyla. Available amino acid sequences and X-ray structures indicate that a conserved leucine, residue 103 in the *Themiste zostericola* Mhr sequence, occupies a site distal to the Fe−O−Fe center. The side-chain methyl groups of the analogous leucine in *Themiste dyscrita* oxyHr are in van der Waals contact with bound O_2 in the X-ray crystal structure, and this residue may therefore play a role in stabilizing bound dioxygen with respect to autoxidation. In order to test this hypothesis, the gene for *T. zostericola* Mhr was synthesized and expressed in *Escherichia coli*. Two mutant Mhrs, L103V and L103N, were also prepared. Optical spectra and kinetics data for these three proteins are presented. Importantly, neither mutant forms a stable oxy adduct; instead, rapid autoxidation results in formation of the corresponding met forms. In addition, the L103N Mhr displays unusually rapid reduction kinetics, suggesting that the amide functionality of Asn-103 destabilizes most bound ligands and additionally promotes rapid semi-$met_R \rightleftarrows$ semi-met_O isomerization.

Metalloproteins figure prominently (Jameson & Ibers, 1994) in the biological transport of molecular oxygen. The copper-containing hemocyanins and nonheme iron-containing hemerythrins (Hrs)[1] of invertebrates, which function as O_2 transport proteins, have been the subjects of numerous studies that were initially stimulated by the distinctive colors of their respective oxy adducts. Respiratory dioxygen transport by vertebrate heme proteins is, of course, much better understood and is accomplished by a formal one-electron reduction of the ligand upon binding to a ferrous center, producing oxy adducts more appropriately denoted ferric superoxide complexes. In contrast, hemocyanins and hemerythrins carry O_2 by two-electron reduction to the peroxo level.

Hemerythrin, found in four phyla of marine invertebrates, was discovered more than a century ago. During the last 30 years, a large amount of structural, spectroscopic, and reactivity information has been accumulated (Klotz & Kurtz, 1984; Wilkins & Wilkins, 1987; Stenkamp, 1994) for Hrs obtained from sipunculids (marine "peanut" worms), which are oligomers. A 13.9 kDa monomeric analogue, designated myohemerythrin (Mhr), is also found in the retractor muscles of these worms. Hr and Mhr possess binuclear (Fe−O−Fe) centers that are now recognized to be hallmarks of a growing family (Sanders-Loehr, 1989; Vincent et al., 1990; Que & True, 1990; Kurtz, 1990; Wilkins, 1992; Feig & Lippard, 1994) of nonheme diiron-oxo proteins.

Myohemerythrin possesses a four-α-helix bundle motif (Figure 1), first crystallographically characterized by Hendrickson and co-workers (1975), that has also been found (Weber & Salemme, 1980; Kamtekar & Hecht, 1995) in a wide variety of other proteins, such as cytochrome b_{562}, cytochrome *c*′, the ferritin monomer, and the tobacco mosaic virus coat protein. This protein architecture prompted Getzoff and co-workers (1987) to examine the effects of mutations of Mhr surface residues on protein antigenicity. Four-α-helix bundles can be effective scaffolds for metal−ion binding and have recently been employed (Ghadiri & Choi, 1990; Berg, 1993; Betz et al., 1993) in the preparation of *de novo* metalloproteins. A number of naturally occurring multisite metalloenzymes also possess helical bundles in their metal-binding domains, including *Escherichia coli* ribonucleotide reductase (Nordlund & Eklund, 1993), *Lactobacillus plantarum* Mn_2 catalase (Baldwin, 1990), and *Methanobacterium capsulatus* (Bath) methane monooxygenase (Rosenzweig et al., 1993).

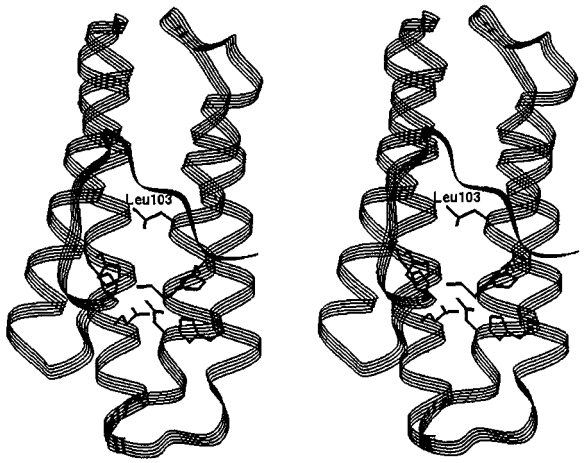

FIGURE 1: Ribbon drawing of the α-carbon backbone of wild-type myohemerythrin, together with the Fe−O−Fe center (designated as a caret), metal ligands, and Leu-103.

Owing to its monomeric nature and low molecular weight, Mhr is an attractive candidate for mutagenic structure−reactivity studies. Perhaps the most compelling reason for studies of recombinant Mhrs lies in the reversible dioxygen-

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[1] Abbreviations: Mhr, myohemerythrin; Hr, hemerythrin; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; CD, circular dichroism; LMCT, ligand-to-metal charge-transfer.

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binding reactivity of the wild-type protein: diferrous deoxyMhr reacts with molecular oxygen to form diferric oxyMhr, wherein the exogenous ligand is bound as hydroperoxide. In addition to O_2, a variety of other di- and triatomic ligands have been observed to bind *in vitro*; some of these, particularly azide, form chromophoric adducts that provide useful spectroscopic signatures.

OxyMhr can also decay by an autoxidation process: H_2O_2 dissociates, with the concomitant formation of diferric metMhr. This process, while orders of magnitude slower than O_2 release, is nonetheless physiologically significant because metMhr is unable to bind O_2. A NADH−cytochrome b_5 reductase system (Utecht & Kurtz, 1988) most likely functions as the physiological reductant in *Phascolopsis gouldii* coelomocytes, converting metHr to deoxyHr via two semi-met intermediates. An analogous system presumably exists for Mhr as well. Curiously, the reduction of metMhr by simple reagents (e.g., dithionite) is usually very slow, and the rate-limiting step of the observed kinetics (Harrington et al., 1981) of deoxyMhr formation is independent of reductant concentration. This rate-limiting step in the *in vitro* met → deoxy interconversion could be viewed as an isomerization involving the two mixed-valent (i.e., semi-met) forms. This isomerization also limits the rate of deoxy oxidation to the met form.

A high-resolution (1.3/1.7 Å) X-ray structure of *Themiste zostericola* azidometMhr (Sheriff et al., 1987) contains several noteworthy features: (a) the endogenous iron ligands are derived from all four α-helices; (b) azide binds to one iron (Fe_2) and is oriented roughly parallel to the Fe_2−O(bridge) axis; and (c) there is a large, hydrophobic, interhelical cavity distal to the Fe−O−Fe active site. Leucine-103 (ca. 4 Å from the active site) lines the bottom end of this cavity, as indicated in Figure 1, and is conserved in all known (Stenkamp, 1994; Negri et al., 1994) Hr and Mhr sequences. In the refined *Themiste dyscrita* oxyHr and azidometHr structures (Holmes et al., 1991; Holmes & Stenkamp, 1991), as in the *T. zostericola* azidometMhr structure, the methyl groups of the analogous leucine residue are in van der Waals contact with the bound exogenous ligand. Resonance Raman studies (Shiemke et al., 1984; Shiemke et al., 1986) and the polarized crystal spectrum (Reem et al., 1989) of oxyHr indicate that the bound peroxide is protonated (i.e., hydroperoxide) and that the ligand is likely hydrogen-bonded to the μ-oxo bridge. Sanders-Loehr and co-workers (Shiemke et al., 1984; Stenkamp et al., 1985) have proposed that the proton involved in this hydrogen bond is derived from a bridging hydroxyl group in deoxyHr.

In order to assess the importance of Leu-103 to the proper functioning of Hrs and Mhrs, we report the results of a comparative study of the optical spectra and reactivities of three recombinant *T. zostericola* Mhrs: wild-type, L103V, and L103N. Both mutant Mhrs exhibit little O_2 transport capability, due to a rapid autoxidation reaction that ensues after oxyMhr formation. As an alternate probe of exogenous ligand binding, azide binding results are presented. Finally, the kinetics of the reduction of the metMhrs to their deoxy forms are contrasted. These observations indicate that this distal leucine plays a key role in controlling both the ligand-binding and electron-transfer reactivities of this class of O_2 carriers.

MATERIALS AND METHODS

Materials. DNA modifying enzymes and molecular biology grade reagents were purchased from U.S. Biochemical, New England Biolabs, Pharmacia-LKB, Stratagene, and Promega. Dideoxy sequencing was carried out using the Circumvent DNA sequencing kit (New England Biolabs) and Amersham [^{35}S]ATP. All other reagents, culture media, electropohoresis media, and chromatography resins were obtained from Sigma, Qiagen, Amersham, Difco, Pharmacia-LKB, U.S. Biochemical Corp., and Whatman, unless indicated otherwise. Oligodeoxynucleotides were prepared, using an Applied Biosystems Model 480B DNA synthesizer and deprotected at the University of Utah Protein-DNA Core facility.

Gene Construction and Protein Expression. The Mhr gene was constructed (Raner, 1994) from six single-stranded oligodeoxynucleotide fragments (66−99 bases in length). Standard procedures (Sambrook et al., 1989; Ausubel et al., 1995) were used to produce and sequence the full-length gene, which was cloned into the expression vector pSPT-19 (Pharmacia-LKB), which is under the control of a T7 promoter. The resulting plasmid was used to transform *E. coli* BL-21 (DE3), which was kindly provided by Prof. Dennis Winge (University of Utah). Plasmids used for the expression of L103V and L103N Mhrs were prepared by cassette mutagenesis of the wild-type plasmid. Small inoculating cultures of transformed cells were grown overnight at 37 °C in LB media containing ampicillin (0.10 g/L) and then transferred to a New Brunswick Microgen fermentor (10 L working volume) for larger scale growth (37 °C) to an o.d. of 1 at 600 nm. Following the addition of IPTG (0.05 mM final concentration) to induce Mhr biosynthesis, cells were grown at 37 °C to an o.d. of ca. 3 and then harvested. Protein expression was monitored by SDS−PAGE of lysed *E. coli* cell fractions.

Protein Purification. All operations were carried out at 4 °C. Wet cell paste, typically ca. 80 g, containing wild-type Mhr, was suspended in 0.05 M Tris (pH 8.0) and stirred for 8−10 h. This suspension was sonicated on ice for six 1 min cycles and then centrifuged for 1 h at 18 000 rpm. The supernatant was subjected to a 0−60% $(NH_4)_2SO_4$ precipitation and centrifuged at 18 000 rpm for 20 min to remove insoluble impurities. The resulting red supernatant was concentrated to ca. 30 mL and dialyzed against the suspension buffer. The sample was then loaded onto a DE-52 ion-exchange column pre-equilibrated with the dialysis buffer. Fractions containing wild-type Mhr were pooled, concentrated, and then loaded onto a Sephacryl S-100 HR column. The purity of the Mhr preparation after gel filtration chromatography was >90%. After dialysis against 10 mM borate buffer (pH 9.0), the protein was loaded onto a pre-equilibrated (pH 9.0) 10 cm DE-52 column, to which the remaining impurities adhered. The recovered wild-type Mhr (ca. 100 mg) was homogeneous by SDS−PAGE.

For comparative purposes, native *T. zostericola* Mhr was also purified. *T. zostericola* worms were purchased from Pacific Biomarine Supply (Venice, CA), bled, and then dissected to remove the retractor muscles. The muscle tissue was homogenized using a hand-operated ground glass homogenizer and then subjected to a 0−80% $(NH_4)_2SO_4$ precipitation to remove most of the contaminants. A subsequent 80−100% ammonium sulfate cut was centrifuged

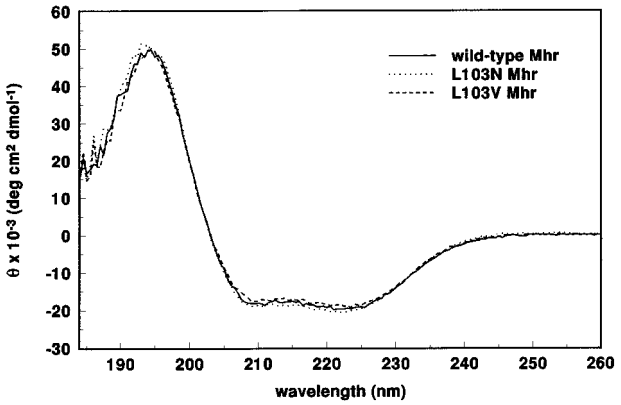

FIGURE 2: Far-UV circular dichroism (20 mM phosphate, pH 8.0; 25.0 °C) spectra of metMhrs.

and dissolved in pH 8.0 buffer (50 mM Tris, 0.2 M KCl). Following gel filtration on a Sephacryl S-100 HR column, the protein was greater than 97% pure.

The L103V and L103N Mhr mutants both appeared in the insoluble fractions of lysed cell extracts, necessitating a modified purification protocol. Following the initial centrifugation, insoluble material was taken up in a solution containing 6 M urea, 0.2 M glycine, 10 mM EDTA, 5 mM DTT, and 50 mM Tris (pH 8.0). The subsequent suspension was centrifuged for 1 h at 18 000 rpm to clarify the solution. The supernatant was then loaded onto a DE-52 column and eluted with 0.05 M Tris (pH 8.0). The recovered fractions were analyzed by SDS−PAGE to locate the Mhr. The Mhr-containing fractions were centrifuged at 18 000 rpm for 45 min, concentrated by ultrafiltration (Amicon YM-3 membrane), filtered, and then loaded onto a 120 cm Sephacryl S-100 HR gel filtration column (0.05 M Tris, 0.2 M KCl, pH 8.0) in 10 mL aliquots. Fractions containing Mhr were pooled and dialyzed against 0.05 M Tris (pH 8.0) and then ion-exchanged using a DE-52 column (0.05 M Tris, 0.2 M KCl, pH 8.0). In both cases, the recovered Mhr mutants were found to be unfolded apoproteins. The active sites in the mutant apoproteins were reconstituted using a procedure similar to that described by Kurtz and co-workers (Zhang et al., 1991). Contaminating metal ions were first removed by repeated incubation with 6 M guanidinium hydrochloride, 10 mM EDTA, 10 mg of $Na_2S_2O_4$, and 10 mM 2,2′-bipyridine in 50 mM Tris (pH 8.0). Following gel filtration to remove small contaminants, a 10-fold excess of ferrous ammonium sulfate was then used to reconstitute the apoproteins, which were degassed, reduced with β-mercaptoethanol, and stirred under nitrogen for 3 h. Contaminants were removed from the reconstituted holoproteins by passage through a Sephadex G-25 column (50 mM Tris, pH 8.0), followed by buffer exchange and DE-52 ion-exchange chromatography (10 mM borate, pH 9.0), to yield homogeneous mutant Mhr preparations.

Optical Spectroscopy. Electronic absorption spectra (25 °C) were obtained with a Cary 2215 spectrophotometer. Far-UV circular dichroism spectra (25 °C) were recorded on an Aviv 60DS spectrometer equipped with a nitrogen purge. The CD amplitude was calibrated with (+)-camphor sulfonic acid (Aldrich), and the data scaled accordingly. Mhr samples were ca. 10 μM in 20 mM phosphate buffer (pH 8.0) and were filtered through a Gelman 0.45 μm filter prior to spectral acquisition. Spectra were scanned at a rate of 0.2 nm/s and are shown in Figure 2 as averages of five scans each. Resonance Raman spectra were recorded with a SPEX Triplemate Raman spectrometer, using 514.5 nm excitation from a Coherent INNOVA 300 Ar^+ laser (100 mW output) and a CCD detector (45−120 s acquisition times). Protein samples for the Raman experiments were 1−2 mM. All spectra were processed using SpectraCalc (Galactic Industries Corp.) software.

Kinetics and Ligand Binding. The kinetics (25 °C) of azide binding to metMhrs were measured spectrophotometrically at 490 nm for wild-type Mhr, and 444 nm for the L103V and L103N Mhrs. Solutions contained 0.05−0.10 mM met protein in 50 mM Tris buffer (pH 8.0). Excess NaN_3 was added, in the form of a buffered stock solution, to initiate the reactions, which were followed for at least four half-lives under pseudo-first-order conditions. Azide binding constants were determined spectrophotometrically, at the above wavelengths, for these proteins by incubation (25 °C, 20 h) with aliquots of a concentrated sodium azide stock solution (0.05 mM Tris, pH 8.0). The data were then subjected to a Scatchard analysis (Connors, 1987) to obtain the reported binding constants. The autoxidation rate (25 °C, pH 8.0, Tris) for wild-type oxyMhr was measured by monitoring the loss in absorbance at 500 nm for two half-lives, due to the slowness of the reaction. The rates of L103V and L103N oxyMhr autoxidation were too fast to accurately measure by manual mixing. Reductions of the metMhrs to their corresponding deoxy forms were studied (50 mM Tris, pH 8.0) by monitoring the loss in absorbance at 380 nm upon the anaerobic addition of buffered solutions of sodium dithionite. Protein solutions were degassed for these studies by repeated cycles of gentle evacuation and purging with purified argon on a vacuum manifold. Data were analyzed using SigmaPlot (Jandel Scientific).

Miscellaneous Analytical Procedures. Metal contents were measured using a Perkin-Elmer 305 A atomic absorption spectrometer. Extinction coefficients were determined by measurement of sample absorbances at 280 nm and normalizing these against the sample concentrations (obtained by amino acid analysis using a Beckman System 6300 High Performance Analyzer).

RESULTS

Recombinant Mhrs are produced in good yields only when cloning vectors containing the T7 promoter are used. Our results with vectors (e.g., pUC-19) containing the *tac* promoter were unsatisfactory: transformation rates were low, coupled with high mutation rates, indicating that the gene product may be cytotoxic. The use of the T7 promoter, coupled with IPTG-induction of protein biosynthesis, routinely produced ca. 150 mg quantities of crude wild-type Mhr per 10 L of fermentation. While the wild-type Mhr was recovered from *E. coli* extracts as the properly folded holoprotein, both mutant proteins were found in inclusion bodies in lower yields. Amino-terminal sequencing and amino acid analysis (data not shown) indicates that the leader fMet was cleaved from the nascent recombinant proteins. The far-UV CD spectra (Figure 2) of all three recombinant metMhrs are virtually superimposable, indicating that iron reconstitution of L103V and L103N apoMhrs yields properly folded holoproteins. Determinations of the iron contents of these three proteins, by atomic absorption spectrometry, gave 2.0 ± 0.1 equiv of iron per mole of metMhr. Due to the

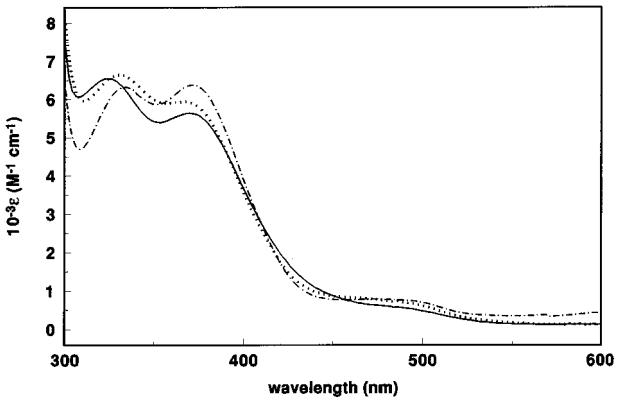

FIGURE 3: Ligand-to-metal charge-transfer spectra (50 mM Tris, pH 8.0; 25.0 °C) of metMhrs: solid line, wild-type; dotted line, L103V; and dot-dashed line, L103N.

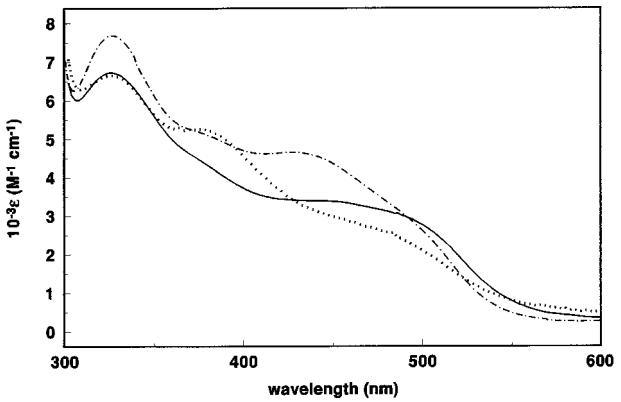

FIGURE 4: Ligand-to-metal charge-transfer spectra (50 mM Tris, pH 8.0; 25.0 °C) of azidometMhrs: solid line, wild-type; dotted line, L103V; and dot-dashed line, L103N.

insolubility of the Mhrs at their isoelectric points (7.6−7.7), all data are reported for pH 8.0. All spectra reported here for the wild-type recombinant Mhr are identical to those obtained for Mhr isolated from *T. zostericola* retractor muscles.

Electronic spectra of the Mhrs are displayed in Figure 3 for the met forms. In all cases, the two oxo → Fe(III) charge-transfer transitions (300−400 nm) expected for diferric Fe−O−Fe centers are present. The higher energy transition, centered at 324 nm in the wild-type protein, shifts to 334 nm the in L103N mutant, and the intensity of the 370 nm band increases by ca. 13% relative to that for the wild-type protein. Charge-transfer spectra of the azidometMhrs are presented in Figure 4. These orange proteins display an absorption maximum at 326 nm [oxo → Fe(III) LMCT] and a broader azide → Fe(III) LMCT envelope, containing Gaussian components centered at 504 nm for wild-type Mhr, 492 nm for L103V Mhr, and 497 nm for L103N Mhr. In addition, spectra for both azidomet Mhr mutants contain more pronounced shoulders at 373 nm. Prominent resonance Raman bands (514.5 nm excitation) are reported for the chromophoric azidometMhrs in Table 1, which also summarizes electronic spectral features for the met and azidomet proteins. As indicated below, the azide affinity of the L103N metMhr is sufficiently weak that it is not possible to isolate pure L103N azidometMhr; consequently, electronic and resonance Raman spectra are reported for the azidomet form of this protein in the presence of a 10^2-fold excess of sodium azide.

Table 1: Spectral Properties (25.0 °C, pH 8.0) of Recombinant Azidometmyohemerythrins

	electronic absorption		resonance Raman (cm^{-1})	
protein	λ (nm)	ϵ(M^{-1} cm^{-1})	ν_s(Fe−O−Fe)	ν(Fe−N_3)
wild-type Mhr	326	6720	507	374
	362	4890		
	440	3390		
L103V Mhr	326	6650	507	362
	374	5260		
	448	3000		
L103N Mhr	326	7680	508	360
	372	5230		
	425	4660		

Table 2: Summary of Rate and Equilibrium Data[a] for Azidometmyohemerythrin Formation

protein	k (M^{-1} s^{-1})	K_{eq} (M^{-1})
wild-type Mhr	6.8 ± 0.2	$2.3\ (\pm 0.7) \times 10^5$
L103V Mhr	12.6 ± 0.6	$2.6\ (\pm 0.2) \times 10^4$
L103N Mhr	1.1 ± 0.2	500 ± 20

[a] 25.0 °C, pH 8.0.

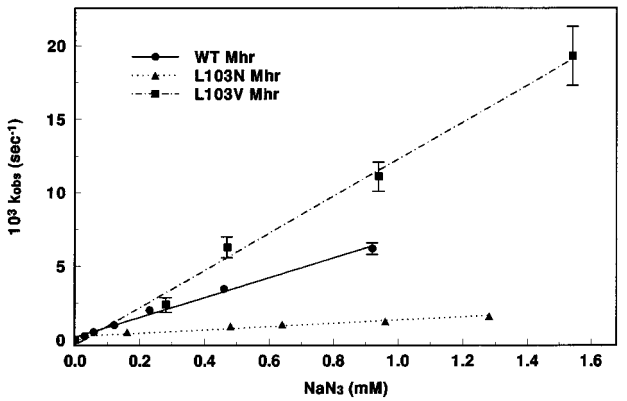

FIGURE 5: Pseudo-first-order kinetics (50 mM Tris, pH 8.0; 25.0 °C) of azide binding to recombinant metMhrs.

Recombinant wild-type oxyMhr displays the broad electronic absorption, centered at 500 nm, expected for a peroxo → Fe(III) LMCT. This band, and the consequent purple color of the oxy protein, slowly disappears as the met form is produced by autoxidation. At pH 8.0 and 25 °C, the half-life of this reaction is ca. 18.5 h. In contrast, exposure of either deoxy L103V or L103N Mhr to O_2 resulted in rapid oxy formation, followed by unexpectedly rapid autoxidations that are ca. 10^2 and 10^5 faster, respectively, than that of the wild-type oxyMhr (Lloyd, 1996).

Since we are unable to report O_2 affinities for either of the mutants, we present results for azide binding to the met proteins. The results of Scatchard analyses of azide binding equilibria are listed in Table 2. Azide binding to the L103N mutant is sufficiently weak that gel filtration chromatography of the azidomet protein results in recovery of the met form. The observed rates (pH 8.0, 25 °C) of azide binding to the metMhrs are plotted in Figure 5. Second-order rate constants are listed in Table 2. While the reaction for the L103V metMhr is somewhat faster than that of the wild-type protein, that of the L103N metMhr is surprisingly slower. We have not included calculated rates of azide dissociation because other kinetic evidence (Lloyd et al., 1995; Lloyd, 1996) indicates that ligand binding to wild-type metMhr involves consecutive steps.

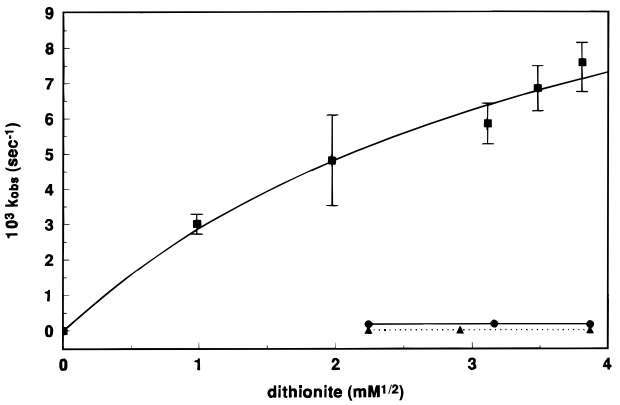

FIGURE 6: Pseudo-first-order kinetics of the reduction (50 mM Tris, pH 8.0; 25.0 °C) of metMhrs by sodium dithionite. Data for wild-type Mhr (●) and L103V Mhr (▲) are independent of the reductant concentration. In contrast, the reduction of L103N Mhr (■) exhibits saturation kinetics, and the data were accordingly fit to a hyperbolic equation (described in the Results).

Table 3: Kinetics of the Reduction[a] of Metmyohemerythrins by $Na_2S_2O_4$

protein	rate
wild-type Mhr	$1.76\ (\pm 0.03) \times 10^{-4}\ s^{-1}$
L103V Mhr	$1.34\ (\pm 0.04) \times 10^{-5}\ s^{-1}$
L103N Mhr[b]	$1.49\ (\pm 0.60) \times 10^{-2}\ s^{-1}$

[a] Data (25 °C, pH 8.0) are for the rate-limiting step in the met → deoxy conversion (Figure 7). [b] The observed kinetics (Figure 6) are characterized by a pre-equilibrium step involving SO_2^- binding, for which $K = 0.24 \pm 0.03\ mM^{-1/2}$.

The observed rates of reduction of the metMhrs by sodium dithionite are plotted against $(S_2O_4^{2-})^{1/2}$ in Figure 6. The reason for the choice of the *x*-axis lies in the fact that the active reductant (Creutz & Sutin, 1974) in these reactions is the SO_2^- radical anion, which equilibrates with $S_2O_4^{2-}$ in aqueous solutions rapidly in comparison with the rates reported here. As previously observed, the rate-limiting step in the sequential one-electron reduction of wild-type metMhr to deoxyMhr is independent of dithionite concentration. We also observe this behavior for L103V metMhr (Table 3). However, L103N metMhr exhibits a pronounced, nonlinear dependence on $[S_2O_4^{2-}]^{1/2}$. We interpret this nonlinearity to signify saturation kinetics, and have therefore fit the data points to the following equation: $k_{obs} = kK[S_2O_4^{2-}]^{1/2}/(1 + K[S_2O_4^{2-}]^{1/2})$. Our model for the behavior of the L103N metMhr includes a pre-equilibrium SO_2-binding step, where *K* is the equilibrium binding constant, followed by electron transfer ($k = 1.49 \times 10^{-2}\ s^{-1}$) from bound SO_2^- to the diferric center.

DISCUSSION

Alexander and co-workers (1991) reported the synthesis and expression of a *T. zostericola* wild-type Mhr gene. However, their wild-type azidomet LMCT spectrum does not agree with that reported here. All of our recombinant wild-type spectra are quantitatively in agreement with those obtained for authentic Mhr from *T. zostericola* retractor muscles. It is interesting to note that the L103V and L103N mutants (as well as others not reported here) are isolated from inclusion bodies as apoproteins, in contrast to the appearance of the wild-type Mhr as the soluble holoprotein in *E. coli*. The fact that these mutations do not involve iron ligands or gross changes in packing makes this observation somewhat surprising. Nondenaturing PAGE and far-UV CD spectra of the apoproteins indicate that they are largely unfolded (Martins, 1994; results not shown), implying that Fe−O−Fe assembly and protein folding are synergistic processes.

The CD spectra (Figure 2) of the three metMhrs are virtually superimposable and reflect a high α-helical content, ca. 60−70%, in keeping with the X-ray structures reported for Hrs and *T. zostericola* azidometMhr. The values of the ν_s(Fe−O−Fe) mode (Table 1) obtained from resonance Raman spectra of the more chromophoric azidomet proteins are also in good agreement. This symmetric vibrational stretch has been previously reported (Freier et al., 1980) to occur at 507 cm^{-1} for *P. gouldii* azidometHr and was shown (Sanders-Loehr et al., 1989) to monotonically increase with the Fe-O-Fe angle (135° for Hr) for a series of diiron-oxo proteins and model complexes. Taken together, these spectral results clearly indicate that the recombinant proteins are properly folded and contain assembled Fe−O−Fe active sites.

The electronic absorption spectra in the charge-transfer region indicate that there are subtle structural perturbations in the mutant proteins. While the wild-type and L103V metMhr spectra are in general agreement, the L103N metMhr spectrum (Figure 3) contains two oxo → Fe(III) bands (300−400 nm region) whose intensity ratio is reminiscent of the hydroxometHr spectrum (Garbett et al., 1971; Bradić & Wilkins, 1983). Further spectroscopic studies are underway to clarify the nature of the exogenous ligand (i.e., aquo or hydroxo) in the L103N metMhr. The following paper in this issue (Martins et al., 1997) describes a refined X-ray structure of this protein in which the ligand was modeled as hydroxide. The ca. 500 nm band in azidometHrs has been assigned to an azide → Fe(III) LMCT transition (Garbett et al., 1969; Shiemke et al., 1984). The LMCT spectra (400−500 nm region) of both azidometMhr mutants are somewhat different from that of the wild-type azidometMhr. Solomon and co-workers (Solomon et al., 1981; Reem et al., 1989) have reported an analysis of polarized crystal spectra of azidometMhr, noting transitions at 390 and 490 nm for parallel polarization (with respect to the Fe−Fe axis), and at 450 and 490 nm for perpendicular polarization. Using these reported spectra as a frame of reference, we suggest that the L103V azidometMhr spectrum indicates that the azide is bound at a less acute N(azido)−Fe−O(bridge) angle. The shift to higher energy of the low-energy side of the azide → Fe(III) LMCT envelope suggests that the ligand is a poorer excited-state reductant in the L103N mutant.

Of the three recombinant deoxy Mhrs, only the wild-type protein reacts with O_2 to produce a stable oxy analogue. As Figure 7 indicates, oxyMhr can also decay by an autoxidation reaction, producing metMhr. The autoxidation rate of this protein is, as expected, quite slow at pH 8.0 ($9.9 \times 10^{-6}\ s^{-1}$). In contrast, both deoxy L103V and deoxy L103N Mhrs were observed to autoxidize so rapidly, even at 4 °C, that we are unable to report accurate spectra of the oxy forms of these proteins. This observed behavior for the L103V mutant is especially surprising, since valine is hydrophobic and only one -CH_2- linkage shorter than leucine. Wilkins has proposed an autoxidation mechanism involving the following three steps:

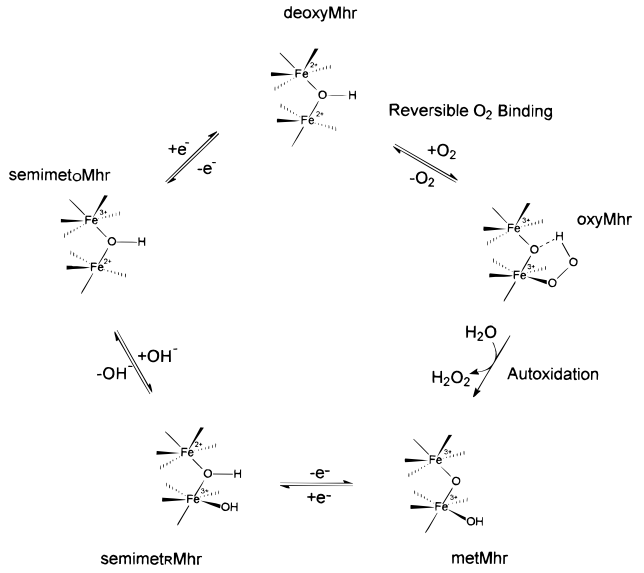

FIGURE 7: Five-state scheme for Mhr, illustrating the relationships between dioxygen binding and release, autoxidation, and active-site redox processes. Ligated amino acids are omitted for clarity.

$$\text{oxyMhr} \rightleftarrows \text{deoxyMhr} + O_2 \quad (1)$$

$$\text{deoxyMhr} + X^- \rightarrow \text{deoxyMhrX}^- \quad (2)$$

$$\text{deoxyMhrX}^- + O_2 \rightarrow \text{metMhrX}^- + H_2O_2 \quad (3)$$

Wilkins and co-workers (Bradić et al., 1977) noted that the autoxidation rate of wild-type oxyMhr is much slower than the rate of O_2 release and suggested that either step 2 or step 3 limits the observed rate, depending upon the nature of X^-. This mechanism requires that molecular oxygen serve as a two-electron oxidant (step 3), which seems unlikely in view of the fact that the reaction would have to proceed via an outer-sphere redox pathway. A comprehensive study of this reaction has been recently completed (Lloyd, 1996) and will be reported elsewhere.

The rates of azide binding to the metMhrs, reported in Table 2, are only ca. 2× faster for the L103V mutant than the wild-type Mhr. On the other hand, the relative decrease in the L103N rate suggests that there may be a steric impediment to formation of the Fe−N bond, possibly involving the release of coordinated hydroxide and/or water from the distal pocket. The equilibrium constants for azide binding clearly indicate that the mutants possess a lower affinity for azide than the wild-type Mhr. For the latter, binding is sufficiently strong that the ligand cannot be removed unless the protein is denatured. At the other extreme, azide can be removed from the L103N azidomet-Mhr simply by gel filtration or dialysis.

The preparation of coordination complexes that are accurate structural and functional models of the Fe−O−Fe active site of Mhr is a much sought after goal. Two groups reported (Armstrong & Lippard, 1983; Wieghardt et al., 1983) the preparation of diferric complexes in 1983 that are reasonable structural models of metMhr. However, the diferrous forms of model Fe−O−Fe complexes prepared to date have not proven to be robust, and the available peroxo adducts of binuclear iron complexes (Kitajima et al., 1990; Hayashi et al., 1992; Ookubo et al, 1996; Dong et al., 1996; Kim & Lippard, 1996; Eulering et al, 1996) appear to involve bridging peroxide, rather than end-on binding of peroxide. Our work underscores the need for an unusually well-packed, solvent-inaccessible Mhr cavity in exogenous ligand binding and further suggests that the synthesis of low molecular weight iron complexes that accurately model the room-temperature O_2-binding activity of Mhr (and Hr) will remain a challenge for some time to come.

In order to recover O_2 transport activity, metMhr must first be recycled to the deoxy form, as illustrated in Figure 7. Wilkins and co-workers (Babcock et al., 1980; Muhoberac et al., 1980; Harrington et al., 1981) have identified two semimet intermediates that slowly interconvert in Hr and Mhr redox reactions. In the reduction of metMhr, this process is believed (Armstrong & Sykes, 1986; McCormick et al., 1991) to involve the protonation of bound hydroxide in semimet$_O$Mhr, followed by dissociation of water from semimet$_R$Mhr. This semi-met$_O$ $\rightleftarrows$ semi-met$_r$ step limits the rates of met $\rightleftarrows$ deoxy Mhr conversions with simple inorganic or organic reagents. Similar considerations apply to Hrs, but the observed kinetics are more complex, possibly due to intramolecular disproportionation (Pearce et al., 1987). The reductions of both wild-type and L103V metMhrs are slow, 1.76×10^{-4} s^{-1} and 1.34×10^{-5} s^{-1}, respectively. Our observed (Table 3) lack of dependence on dithionite concentration is in accordance with previous results for *P. gouldii* Hr, which indicate that the initial reduction step, met $\rightarrow$ semimet$_R$, is fast, ca. 10^6 M^{-1} s^{-1} (Bradić et al., 1986). Surprisingly, the reduction kinetics (Figure 6) of L103N metMhr do exhibit a marked dependence on dithionite concentration, suggesting that the reduction behavior of this mutant is qualitatively different. The data are not well described by an equation linear in $[S_2O_4]^{1/2}$ because the rate begins to saturate at higher reductant concentrations. Hence, our kinetic model includes a SO_2-binding equilibrium step prior to the second electron transfer. The primary significance of the Leu-103 $\rightarrow$ Asn-103 mutation lies in this distinctive reduction behavior: this met protein can be fully reduced many orders of magnitude faster than either the L103V or wild-type metMhrs. A structural rationale for the kinetic behavior of the L103N mutant Mhr is discussed in the following paper in this issue (Martins et al., 1997) reporting the refined X-ray crystal structures of the wild-type and L103N metMhrs.

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