A Leucine Residue “Gates” Solvent but Not O2 Access to the Binding Pocket of Phascolopsis gouldii Hemerythrin*

Christopher S. Farmer‡, Donald M. Kurtz, Jr.§§, Robert S. Phillips‡, Jingyuan Ai‡, and Joann Sanders-Loehr¶

From the ‡Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602-2556 and the ¶Oregon Graduate Institute for Science and Technology, Portland, Oregon 97291-1000

A leucine residue, Leu-98, lines the O2-binding pocket in all known hemerythrins. Leu-98 in recombinant Phascolopsis gouldii hemerythrin, was mutated to several other residues of varying sizes (Ala, Val), polarities (Thr, Asp, Asn), and aromaticities (Phe, Tyr, Trp). UV-visible and resonance Raman spectra showed that the di-iron sites in these L98X Hrs are very similar to those in the wild type protein, and several of the L98X hemerythrins formed stable oxy adducts. Despite the apparently tight packing in the pocket, all of the L98X Hrs except for L98W, had second order O2 association rate constants within a factor of 3 of the wild type value.

This work was supported by grants GM40388 (D.M.K.), and GM18865 (J.S.-L.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication, February 15, 2000, and in revised form, March 14, 2000
Published, JBC Papers in Press, March 15, 2000, DOI 10.1074/jbc.M001289200

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 22, Issue of June 2, pp. 17043–17050, 2000

A leucine residue, Leu-98, lines the O2-binding pocket in all known hemerythrins. Leu-98 in recombinant Phascolopsis gouldii hemerythrin, was mutated to several other residues of varying sizes (Ala, Val), polarities (Thr, Asp, Asn), and aromaticities (Phe, Tyr, Trp). UV-visible and resonance Raman spectra showed that the di-iron sites in these L98X Hrs are very similar to those in the wild type protein, and several of the L98X hemerythrins formed stable oxy adducts. Despite the apparently tight packing in the pocket, all of the L98X Hrs except for L98W, had second order O2 association rate constants within a factor of 3 of the wild type value. Similarly, the O2 dissociation rate constant was essentially unaffected by substitutions of larger (Phe) or smaller (Val, Thr) residues for Leu-98. L98Y Hr showed a thermally O2 dissociation rates of any known Hr (15–120 s

The structures depicted in Scheme 1 for the deoxy and oxy iron sites have been largely confirmed by x-ray crystallography and various spectroscopies (1). Several lines of evidence (summarized by Brunold and Solomon (Refs. 2 and 3)) show that the O2 binding equilibrium of Hr is best formulated as an internal two-electron/one-proton transfer reaction.

\[
\begin{align*}
[\text{Fe}^1\text{II}(\mu-\text{OH})\text{Fe}^2\text{II}] + \text{O}_2 & \rightleftharpoons [\text{Fe}^1\text{III}(\mu-\text{O})\text{Fe}^2\text{II}\text{O}_2\text{H}] \\
\text{deoxy} & \rightleftharpoons \text{oxy}
\end{align*}
\]

During laser photolysis studies of another oxyHr, opening of a gate was proposed to lead to escape of O2 to solvent following its photodissociation from Fe2 (6). However, this putative “gate opening” occurred on the time scale of microseconds or less, which is much faster than the thermal O2 dissociation rates of any known Hr (15–120 s$^{-1}$; Ref. 12). Furthermore, in order to accurately model the exper-

The O2-binding pocket of Hr is lined with a set of conserved, hydrophobic residues, whose sequential and spatial positions are indicated in Fig. 1. One or more side-chain atoms of residues Ile-28, Phe-55, Trp-97, Leu-98, and Ile-102 are within 4 Å of the coordinated O2 atoms in oxyHr (1). L98C/I is 3.6 Å from atom O2 of the bound dioxygen (cf. Scheme 1 and Fig. 1 for atom numbering). Based on this close distance, it was proposed that Leu-98 could limit ingress or egress of O2 to or from the binding pocket, thereby functioning as a sterically “gate” (11). However, this proposal has not heretofore been subjected to a direct experimental test. Based on laser flash photolysis studies of another oxyHr, opening of a gate was proposed to lead to escape of O2 into solvent following its photodissociation from Fe2 (6). However, this putative “gate opening” occurred on the time scale of microseconds or less, which is much faster than the thermal O2 dissociation rates of any known Hr (15–120 s$^{-1}$; Ref. 12). Furthermore, in order to accurately model the exper-

Hemerythrin (Hr)$^1$ is an oligomeric non-heme iron, O2-carrying protein found mainly in coelomic cells of a few marine invertebrate phyla (1). The octameric Hr from the sipunculid worm, Phascolopsis gouldii, is the most thoroughly characterized of this group. The eight essentially identical subunits are indicated in Fig. 1. One or more side-chain atoms of residues (Thr, Asp, Asn), and aromaticities (Phe, Tyr, Trp). UV-visible and resonance Raman spectra showed that the di-iron sites in these L98X Hrs are very similar to those in the wild type protein, and several of the L98X hemerythrins formed stable oxy adducts. Despite the apparently tight packing in the pocket, all of the L98X Hrs except for L98W, had second order O2 association rate constants within a factor of 3 of the wild type value. Similarly, the O2 dissociation rate constant was essentially unaffected by substitutions of larger (Phe) or smaller (Val, Thr) residues for Leu-98. L98Y Hr showed a 170-fold decrease in the O2 dissociation rate constant and a large D2O effect on this rate, which are attributed to a hydrogen-bonding interaction between the Tyr-98 hydroxyl and the bound O2. Significant increases in autoxidation rates were observed for all of the L98X Hrs other than X = Tyr. These increases in autoxidation rates are attributed to increased solvent access to the binding pocket caused by inefficient packing (Phe), smaller size (Val, Ala), or increased polarity (Thr, Asp, Asn) of the residue 98 side chain. A leucine at position 98 appears to have the optimal size, shape, and hydrophobicity for inhibition of solvent access. Thus, “gating” of small molecule access to the binding pocket of Hr by Leu-98 is not evident for O2, but is evident for solvent.
Facility at the University of Georgia. Escherichia coli cultures were grown either in LB/amp or in LB/amp containing agar. Protein overexpression was monitored by Tricine SDS-polyacrylamide gel electrophoresis (19) on samples of E. coli cultures removed before and after induction (as described below), and on cell lysate fractions.

**Cloning of the P. gouldii Hr Gene—**Specimens of P. gouldii were obtained live from the Marine Biological Laboratory (Woods Hole, MA). Hemerythrocytes (the hemerythrin-containing eukaryotic cells) were isolated from the combined eukaryotic fluid of approximately a dozen specimens by low speed centrifugation and washing in artificial seawater. Total RNA was isolated from ~ 2 ml of packed hemerythrocytes using a total RNA isolation kit from Stratagene, Inc., and assuming that 1 ml of packed hemerythrocytes corresponded to 1 g of tissue. The precipitated RNA was stored at ~20 °C. The recombinant P. gouldii Hr gene was cloned from the total RNA by reverse transcription-PCR using a Perkin-Elmer Cetus GenAMP® RNA PCR kit. The reverse transcription-PCR mix contained, in addition to the standard ingredients, ~1 μg of the P. gouldii hemerythrocyte total RNA (dissolved in 2 μl of 10 μM Tris and 1 mM EDTA, pH 8) and 0.25 μM each of two degenerate primers, DMK2 (5′-GCTGCGAGTAAAGGAGTGTACATGGGNTTYCCNATHC CNGA-Y-3′) and DMK3 (5′-ATGCGAGTATADATYTTNCYTTYRATYTT-3′), in a total volume of 50 μl. The degenerate portions of the nucleotide sequences of DMK2 and DMK3 were based on the published N- and C-terminal amino acid sequences, respectively, of P. gouldii Hr (1), and one was designed for HindIII restriction sites (underlined) at the 5′ ends of the primers. The following thermal cycling sequence was used in the PCR: once for 2 min at 95 °C, 35 times for (1 min at 95 °C and 1 min at 42 °C), once for 7 min at 60 °C. The resulting PCR product was ligated into the PstI and HindIII restriction sites of pBluescript KS+ (Stratagene, Inc.). The resulting plasmid was sequenced to confirm insertion of the Hr gene, which was then isolated and purified from a PstI/HindIII digest of the plasmid. This purified, PstI/HindIII-digested Hr gene was used as template in a PCR with ~0.5 μM primers DMK6 (5′-TATAACTAGTTCCGAGTCTCCGG-3′) and DMK3. The nucleotide sequence of DMK6 duplicated that of the N-terminal end of the cloned P. gouldii Hr gene with the start codon indicated in lowercase, and an Ndel restriction site (underlined) incorporating the start codon was added to the 5′ end. The thermal cycling sequence used for the PCR was: 30 times for (2 min at 95 °C, 2 min at 60 °C, and 3 min at 72 °C), and once for 5 min at 72 °C. The PCR product was ligated into the Ndel/HindIII restriction sites of pT7–7 (20). Nucleotide sequencing of the resulting pT7–7 derivative, designated pDK4–1, confirmed the correctness of the inserted Hr gene sequence. The cloned Hr gene sequence in pDK4–1 was deposited as GenBank accession no. AF220659.

**Construction of L98X-mutated Genes—Site-directed mutagenizations in the Hr gene were obtained by the method of splicing by overlap extension (21), which requires two rounds of PCRs for each mutation. The first round consisted of two PCRs, both using BglII-digested pDK4–1 as template. One first-round PCR used the oligonucleotide T7 (5′-TAACGACTCACTATAGGG-3′), and one oligonucleotide, L98X, with the sequence 5′-ATCACTAGTTCCGAGTCTCCGG-3′ as primers. The nucleotide sequence, L98X, consisted of one of the following sequences (5′-3′) for each L98X mutation: F = X = Y = T; X = W, G; G = C, T; GA; X = Y, V; G; T = AGT; X = W, CCA; X = D, GTC; X = N, AAC; X = A, GCA. The other first-round PCR used oligonucleotide T7R (5′-TCAGACCAAGTTTACTACA-3′) and the corresponding reverse complement of the L98X oligonucleotide as primers. T7 and T7R duplicate nucleotide sequences in pT7–7 upstream of the Ndel restriction site and downstream of the HindIII restriction site, respectively (20). The second round of PCR used the two combined, purified PCR products from the first round as template with T7 and T7R as primers. For both rounds the PCR reaction mixtures contained ~5 ng of template DNA, ~0.08 μM amounts of oligonucleotide primers, 0.25 μM amounts of each of the dNTPs, and 0.5 unit of Taq polymerase in PCR reaction buffer (10 mM Tris–HCl, pH 8.9, 50 mM KCl, 2.5 mM MgCl2) to a final volume of 50 μl. The PCR temperature-cycling sequence was: 1 × 5 min at 95 °C, 30 × 1 min at 94 °C, 55 °C for 1 min, and 2 min at 72 °C. The second-round PCR products were restriction-digested with Ndel and HindIII and ligated into the corresponding restriction sites of pT7–7. Those plasmids containing the correctly mutated L98X Hr gene sequences were used to transform E. coli BL21(DE3) (22).

**Overexpression, Isolation, and Purification of Recombinant P. gouldii Hrs—** Fifty-milliliter cultures of E. coli BL21(DE3) transformed with either pDK4–1 or one of its L98X-mutated derivatives were grown overnight at 37 °C in LB/amp with shaking at 250 rpm. Four of these 50-ml overnight cultures were used to inoculate four 1-liter batches of LB/amp, and these 1-liter cultures were incubated under the same conditions of temperature and shaking. When the OD600 of the cultures
Gating of Access to the O$_2$-binding Pocket in Hemerythrin

17045

reached ~1.0, either isopropl-$\beta$-d-thigalactoside to 0.4 mM final concentration or 4.0 g of $\beta$-naphthol was added to induce overexpression of the Hr gene. (Either isopropl-$\beta$-$\beta$-d-thigalactoside or lactose gave comparable levels of Hr overexpression.) The induced cultures were incubated for another 2–4 h at the same temperature and shaking speed. All aspects of cell processing were performed under anaerobic conditions. The organisms were pelleted for centrifugations and pressure ultrafiltrations, which were at 4°C. The harvested, combined cells from four 1-liter cultures were resuspended in ~100 ml of 50 mM HEPES, 150 mM Na$_2$SO$_4$, pH 7.5 (buffer), and then lysed by sonication. The lysed cell suspension was centrifuged at 30,000 $\times$ g for 30 min. The overexpressed Hr was found to be exclusively in the pellet, which was washed with buffer and then re-suspended in ~12 ml of 6 M guanidine-HCl in buffer. To this suspension was added 200 $\mu$l of $\beta$-mercaptoethanol, and the mixture was stirred vigorously for 5–24 h aerobically at room temperature to facilitate dissolution of the inclusion bodies. The green-brown suspension was then centrifuged at 30,000 $\times$ g for 30 min, and the supernatant was discarded. The insoluble protein pellet was re-dissolved in guanidine-HCl/$\beta$-mercaptoethanol as before; only several minutes of gentle stirring were required to re-dissolve the pellet at this stage. The solution (~10 ml) was transferred to a 500-ml Schlenk-type flask fitted with a pressure-equalizing addition funnel, which contained 100 ml of buffer. This was sealed, and then was transferred to a vacuum manifold, and alternately evacuated and flushed with argon while stirring until the protein solution was negligible. The addition funnel was briefly removed from the flask under a positive pressure of argon, and ~0.1 g of solid ferrous ammonium sulfate was rapidly added to the protein solution. The addition funnel was replaced, anaerobically re-established, and the 100 ml of buffer was added dropwise at a constant rate over a period of ~10 h with gentle stirring. After complete addition of the buffer, the protein solution was centrifuged aerobically at 30,000 $\times$ g for 30 min. The resulting pink/yellow solution, which contained soluble, iron-containing Hr, was concentrated by ultrafiltration (Amicon, YM30 membrane, 30-kDa cut-off) under argon pressure to ~5 ml. Two 50- to 5-ml dilution/reconcentration cycles with buffer were performed to remove residual reagents. To ensure homogeneity, the Hr was converted to the met form by addition of ~5–10 small crystals of potassium ferricyanide. This solution was stirred overnight at 4°C. The ~5 ml of protein solution was then loaded onto a Superdex 200 16/60 gel filtration column (Amersham Pharmacia Biotech) pre-equilibrated with buffer. The Hr was eluted with buffer at a flow rate of 0.5 ml/min as a single peak, and was concentrated by centrifugal ultrafiltration (Centricon 30, 30-kDa cut-off). Both wild type and L98X Hrs were similarly overexpressed, isolated, and purified. The purified Hrs were stored at ~80°C. Amounts and purities of the recombinant Hrs were estimated by UV-visible spectrophotometry ($\epsilon$$_{500}$ = 6400 M$^{-1}$ cm$^{-1}$ subunit) and A$_{280}$/A$_{300}$ ratio (typically 4–4.5) (23). SDS-polyacrylamide gel electrophoresis showed a single protein band for the recombinant Hrs after this purification procedure. Four liters of E. coli culture typically yielded 50 mg of purified soluble recombinant Hr.

Preparation of Deoxy- and OxyHrs—MetHr in buffer, prepared as described above, was used as the starting point. The deoxy forms of the recombinant Hrs were prepared by anaerobic dialysis of samples of metHr against ~5 mM sodium dithionite solutions in buffer for ~24 h at room temperature either in a Co$_2$ anaerobic chamber or in a Schlenk-type flask attached to a vacuum manifold. The protein was then dialyzed three times against 100 volumes of deoxygennated buffer to remove excess dithionite. The resulting protein solution was centrifuged at 10,000 $\times$ g for 5 min inside the Co$_2$ chamber in order to remove any precipitate. The o xoforms were prepared by briefly bubbling air through a freshly prepared solution of deoxyHr. Aliquots (~1 ml) of the oxyHrs were frozen rapidly in liquid nitrogen and stored at ~80°C. Measurement of O$_2$ Affinities of Hrs—O$_2$ affinities for wild type and L98X Hrs were measured by spectrophotometric titration. The method has been previously described for native P. gouldii Hr (24). Solutions of the deoxyHr to be examined (1.0–1.5 ml, 50–100 $\mu$g in subunit, 1 mM sodium dithionite, 150 mM Na$_2$SO$_4$, 50 mM HEPES, pH 7.5 in buffer) were transferred to a freshly prepared solution of deoxyHr. Aliquots (~1 ml) of the oxyHrs were frozen rapidly in liquid nitrogen and stored at ~80°C. Measurement of O$_2$ Affinities of Hrs—O$_2$ affinities for wild type and L98X Hrs were measured by spectrophotometric titration. The method has been previously described for native P. gouldii Hr (24). Solutions of the deoxyHr to be examined (1.0–1.5 ml, 50–100 $\mu$g in subunit, 1 mM sodium dithionite, 150 mM Na$_2$SO$_4$, 50 mM HEPES, pH 7.5 in buffer) were transferred to a freshly prepared solution of deoxyHr. Aliquots (~1 ml) of the oxyHrs were frozen rapidly in liquid nitrogen and stored at ~80°C.

The derived amino acid sequence was identical to that published for one of the minor amino acid variants of native P. gouldii Hr in which a threonine is substituted for a glycine at position 79 (26). Native P. gouldii Hr consists of a total of five amino acid substitution variants in varying proportions; none of these variants is known to affect any spectroscopic or functional properties of the di-iron site (27). Both the recombinant wild type and L98X P. gouldii Hrs were expressed in E. coli and purified as single species using the T7 polymerase promoter over-expression system (20, 22). Based on a previously described procedure used to reconstitute native P. gouldii Hr from the apoprotein (23, 28), a method was developed for resolubilizing the recombinant wild type P. gouldii apoHr from the inclusion bodies and for incorporation of iron such that a di-iron site with properties (see below) essentially indistinguishable from that in native P. gouldii Hr was obtained. The same method was used to obtain a series of soluble di-iron-containing recombinant L98X P. gouldii Hrs. All of these recombinant Hrs were octameric, as expected. Activation parameters were determined as described above. Autoxidation half-lives at 25°C were estimated by monitoring the distinctive changes between oxyHr and metHr absorption spectra (23) on a Shimadzu UV2101PC scanning spectrophotometer. For autoxidation half-times that were shorter than a few seconds, the rates were determined on the Ols stoped-flow spectrophotometer by mixing the deoxy form with O$_2$ solution as described above for the O$_2$ association rates. 

RESULT

Isolation of the Recombinant P. gouldii Hrs—The P. gouldii Hr gene was cloned from cDNA of hemerythrin-containing coelomic cells. The derived amino acid sequence was identical to that published for one of the minor amino acid variants of native P. gouldii Hr in which a threonine is substituted for a glycine at position 79 (26). Native P. gouldii Hr consists of a total of five amino acid substitution variants in varying proportions; none of these variants is known to affect any spectroscopic or functional properties of the di-iron site (27). Both the recombinant wild type and L98X P. gouldii Hrs were expressed in E. coli and purified as single species using the T7 polymerase promoter over-expression system (20, 22). Based on a previously described procedure used to reconstitute native P. gouldii Hr from the apoprotein (23, 28), a method was developed for resolubilizing the recombinant wild type P. gouldii apoHr from the inclusion bodies and for incorporation of iron such that a di-iron site with properties (see below) essentially indistinguishable from that in native P. gouldii Hr was obtained. The same method was used to obtain a series of soluble di-iron-containing recombinant L98X P. gouldii Hrs. All of these recombinant Hrs were octameric, as expected.
judged by gel filtration, as is native P. gouldii Hr (1).

Spectral Properties of the Recombinant P. gouldii Hrs—After resolubilization, iron incorporation, and oxidation with ferri-
cyanide, all of the recombinant wild type, and L98Y, -F, -V, -W, -T, -D, -N, and -A P. gouldii Hrs contained di-iron sites whose absorption spectra (cf. Fig. 2) were very similar to that of native P. gouldii metHr at pH 7.5 (23). The absorption features at ~320 and ~360 nm, and shoulder at ~480 nm, are due to \( \mu \)-oxo \( \rightarrow \) Fe(III) LMCT transitions, and this set of features serves as a fingerprint for \( \mu \)-oxo-di-iron(III) sites with supporting carboxy-
late bridges (29, 30). The absorption spectrum of the recom-
brinant L98Y metHr was found to have a weak, broad absorp-
tion between 500 and 700 nm, which was not present in the wild type or other L98X metHr spectra. This feature showed variable intensity in multiple preparations of L98Y metHr, and neither its intensity nor position was affected by pH between 6.0 and 9.0. The L98Y metHr spectrum shown in Fig. 1 contains a very minor contribution from this feature. Resonance Raman spectra, discussed below, indicate that this absorption is due to a portion of the protein in which a phenolate, presumably from Tyr-98, is ligated to Fe(III). Resonance Raman spectra of the recombinant wild type metHr (data not shown) exhibited the characteristic \( \nu(Fe-O-Fe) \) and \( \nu_{\nu}(Fe-O-Fe) \) stretching frequencies of the oxo-bridged di-iron(III) site at 508 and 758 cm\(^{-1}\) (weak), respectively. These frequencies mimic those of the native P. gouldii metHr (510 and ~753 cm\(^{-1}\), respectively; Ref. 31), which indicates conservation of the bent Fe-O-Fe angle (~125°) enforced by the two additional carboxy-
late bridges (32). An extensive and detailed analysis of the resonance Raman spectra of the recombinant metHrs can be found elsewhere (33).

Upon anaerobic reduction with sodium dithionite, and sub-
sequent re-exposure to air, the wild type and L98Y, -F, -Y, and -T P. gouldii Hrs formed oxy adducts that were sufficiently stable at room temperature to obtain their characteristic UV-
visible absorption spectra (cf. Fig. 3). In addition to the peak at 330 nm and shoulder at ~360 nm (both due to oxo-Fe(III) LMCT), all of these latter Hrs displayed a broad absorption
centered near 500 nm, which is due to the hydroperoxo→Fe(III) LMCT transition (30). Either very small or negligible pertur-
bations of the wild type oxyHr spectrum are evident in the spectra of the L98X oxyHrs. These spectra completely bleach in the deoxy forms, which thereby provides a convenient spectro-
scopic monitor of \( O_2 \) binding.

Laser excitation into the hydroperoxo→Fe(III) LMCT tran-
sition of native P. gouldii oxyHr is known to enhance the \( \nu(O-O) \) and \( \nu(Fe-O_2) \) Raman-active stretching frequencies of the bound \( O_2 \) (31). The analogous Raman experiments on the recombi-
nant oxyHrs produced the spectra shown in Fig. 4. The frequencies for the recombinant wild type oxyHr at 844 and 503 cm\(^{-1}\) are identical to those previously reported for \( \nu(O-O) \) and \( \nu(Fe-O_2) \), respectively, of native P. gouldii oxyHr (31). Small (~2 cm\(^{-1}\)) upshifts in these frequencies are observed in the L98F oxyHr. In L98Y oxyHr, the \( \nu(O-O) \) frequency has shifted 3 cm\(^{-1}\) downward to 841 cm\(^{-1}\). The \( \nu(Fe-O_2) \) frequency region of the L98Y oxyHr Raman spectrum is complicated by contribu-
tions from a minor portion of the met form that invariably contaminates concentrated samples of oxyHrs. In the case of L98Y Hr, the met frequencies are apparently enhanced to a greater extent than for wild type or L98F met forms. Compar-
isons to resonance Raman spectra of L98Y metHr (data not shown; Ref. 33) revealed that the Raman features at 498 and 515 cm\(^{-1}\) are due mostly to \( \nu(Fe-O-Fe) \) of two different met forms, one of which has a tyrosinate, presumably Tyr-98, coor-
dinated to Fe(III). The feature at 573 cm\(^{-1}\) in Fig. 4 is attributed to \( \nu(Fe-O) \) of this coordinated tyrosinate in the met form (34). These comparisons also indicate some residual intensity from the \( \nu(Fe-O_2) \) of L98Y oxyHr near 505 cm\(^{-1}\). In any case both the resonance Raman and UV-visible absorption spectra indicate that, in those L98X Hrs that form a stable oxy adduct, the bound \( O_2 \) is a peroxo ligand resulting from the same inter-
med redox reaction (Reaction 1) as for the wild type Hr.

**O\(_2\)** Binding Kinetics and Equilibria of Recombinant Wild Type and L98X Hrs—The change in absorbance of the 500-nm feature was used to measure both the \( O_2 \) affinities and the \( O_2 \) binding kinetics of the recombinant Hrs. Using the previously
described O₂-scavenging method (4), the O₂ dissociation rates of the recombinant P. gouldii oxyHrs could be conveniently monitored by stopped-flow spectrophotometry. Within a relatively narrow range of deoxyHr concentrations and excess O₂ (cf. “Experimental Procedures”), the O₂ association kinetics of the recombinant P. gouldii Hrs could also be reliably measured by stopped-flow spectrophotometry between 5 and 35 °C. Autoxidation half-times (Reactions 2 and 3) were also estimated. In those cases where the autoxidation rates were on the stopped-flow time scale (L98A, -N, and -D, cf. Table I), the initial formation of the 500-nm feature characteristic of oxyHr was observed, followed by a decrease in absorbance toward the met form, which is the product of autoxidation (Reactions 2 and 3). L98W Hr was an exception, and is discussed separately. The O₂ association and dissociation kinetics were best fit to monophasic processes in all cases (except L98W). Even when the O₂ association reactions were monitored in the wavelength-scanning mode of the stopped-flow spectrophotometer with higher protein concentrations under non pseudo first order conditions, no intermediate chromophores were evident. The results of these O₂ affinity and kinetics measurements are collected in Table I. The O₂ affinity and rate constants for the recombinant wild type P. gouldii Hr closely parallel those previously reported for native P. gouldii Hr (4, 24).

Given the relatively narrow temperature range, we were able to examine, the small temperature dependence of the O₂ association rates, and the large extrapolations used to obtain ΔS° from the Eyring plots, quantitative comparisons among the activation parameters of the various Hrs listed in Table I is not warranted. However, a few qualitative points are noteworthy. Although the values of ΔS° on determined for the recombinant P. gouldii Hrs (Table I) are significantly more negative than previously reported for native P. gouldii Hr (4), they are not inconsistent with a second order associative reaction, such as occurs in O₂-carrying proteins (e.g., Coletta et al. (Ref. 35)). A ΔS° on of −11 cal/mol-K was reported for Tz myoHr (5). Previous kinetic and calorimetric studies on native P. gouldii Hr have shown the overall O₂ association reaction of native P. gouldii Hr to be exothermic by −9 to −12 kcal/mol (4, 36). The calculated ΔH = ΔH° on − ΔH° off values for the O₂ association equilibria of the recombinant P. gouldii Hrs (cf. Table I) are also exothermic, but, once again, somewhat more so than reported for the native P. gouldii Hr.

Effects on O₂ Association—Perhaps the most striking aspect of the data in Table I is the similarities in k on among the wild type and several L98X Hrs. The rates for O₂ association are all within the range of 1–3 × 10⁶ M⁻¹ s⁻¹ (except for L98W). These high rates, and the low activation enthalpies, are incompatible with an O₂ association mechanism in which bond formation or ligand substitution occurs in the rate-limiting step. Rather, these parameters suggest an open coordination site for O₂ binding, as is indeed observed for Fe₂ (cf. Scheme 1) in the x-ray crystal structure of deoxyHr (1). The O₂ association parameters listed in Table I are consistent with a diffusion-controlled associative mechanism (6, 37–39). According to this mechanism, two effects could lower the second order reaction constants below that expected for a purely diffusion-controlled encounter (−10⁹ M⁻¹ s⁻¹). First, the time scales required for structural fluctuations of the protein to create transient internal cavities are longer than that for collisional encounters, and second, favorable interactions of the small molecule exist with only a fraction of the protein surface and/or internal matrix. Perhaps due to modest steric inhibition by the larger side chain, L98F Hr shows an approximately 3-fold reduction in k on, which is reflected in its approximately 3-fold lower O₂ affinity, i.e. −3-fold higher P₅₀ (cf. Table I). However, the Leu-98 mutants with aliphatic residues smaller than leucine did not show an increase in k on or O₂ affinity, which would be expected if steric restrictions at Leu-98 regulated O₂ association. Thus, a narrow range of O₂ association rate constants is observed for Hrs having a wide range of sizes and polarities at residue 98. We, therefore, conclude that Leu-98 does not limit the rate of O₂ access to the binding pocket of P. gouldii Hr. The fact that L98N and -D Hrs have O₂ association parameters similar to the other L98X Hrs indicates that substitution of Leu-98 with polar residues do not significantly affect their O₂ association kinetics.

![Resonance Raman spectra of recombinant wild type, L98F, and L98Y oxyHrs in the Fe-O and O-O stretching regions. Hrs were ~1 mg in di-ion sites in 50 mM HEPES and 150 mM Na₂SO₄ (pH 7.5). Spectra were acquired at ice temperature using 514-nm laser excitation. Other spectral conditions are given under "Experimental Procedures."](http://www.jbc.org/)

### Table I

| Hr         | 10⁻⁶ kₐ | ΔH° on | ΔS° on | kₐ     | ΔH° off | ΔS° off | P₅₀   | Autodation (t½) |
|-----------|---------|--------|--------|--------|---------|--------|-------|-----------------|
| Wild type | 3.3     | 3      | −35    | 51     | 29      | 47     | 4.8   | 20 h            |
|           | (7.4)   | (8)    | (+1)   | (51)   | (21)    | (19)   | (3.6) | (19 h)          |
| L98V      | 2.0     | 2      | −42    | 53     | 24      | 29     | 5.8   | 3.7 h           |
| L98F      | 1.0     | 2      | −42    | 52     | 22      | 25     | 15    | 2 h             |
| L98Y      | 1.7     | 3      | −38    | 0.30   | 24      | 19     | 0.32  | <40 h           |
| L98T      | 2.7     | 5      | −32    | 54     | 26      | 38     | ND    | 30 min          |
| L98W      | 0.07    | 6      | −34    | ND     | ND      | ND     | ND    | 0.07 s          |
| L98A      | 2.3     | 2      | −41    | ND     | ND      | ND     | ND    | 0.2 s           |
| L98N      | 1.2     | 4      | −36    | ND     | ND      | ND     | ND    | 0.8 s           |
| L98D      | 3.3     | 4      | −35    | ND     | ND      | ND     | ND    | 1.1 s           |

* Numbers in parentheses were previously reported for native Pg Hr at pH 8.2 and 25 °C (4).

* Previously reported for native Pg Hr at pH 7.1 and 24 °C (24).

* ND, not determined.
residues does not lead to significant binding of solvent to Fe2 (cf. Scheme 1) in the deoxy form. Such solvent binding would be expected to alter the kinetics of O2 association.

Effects on O2 Dissociation—The $\Delta H^\ddagger$ values listed in Table I are consistent with significant Fe-O2 bond weakening or breakage during the step limiting O2 dissociation. This conclusion had been reached previously for native Hrs and myoHr based on both temperature and pressure dependences of the dissociation rates (8, 39). Since one C6 atom of Leu-98 is reported to be 3.6 Å from a bound O2 atom in the crystal structure of oxyHr (1), this residue could in principle sterically hinder expansion of the Fe2-O2 coordination sphere. However, changing Leu-98 to either smaller (Thr, Val) or larger (Phe) residues did not affect $k_{\text{off}}$ (cf. Table I). Thus, Leu-98 does not appear to modulate the rate of O2 dissociation from P. gouldii Hr.

The O2 dissociation rate constant for L98Y Hr is 170 times slower than that of wild type Hr. The approximately 15-fold higher O2 affinity (i.e. lower $P_{50}$) of L98Y Hr is qualitatively consistent with the rate constants. Such high O2 affinities (<0.5 mmHg) are difficult to accurately measure by spectrophotometric tonometry. Since the similarly sized and shaped phenyl side chain in L98F oxyHr produced no detectable perturbation in $k_{\text{off}}$, steric inhibition is unlikely to be responsible for the much slower dissociation rate in L98Y oxyHr. An alternative explanation is that the Tyr-98 hydroxyl forms a hydrogen-bond with O-1 (or possibly O-2) of the bound O2 (cf. Scheme 1), thereby stabilizing the oxy adduct. As a test of the hydrogen-bonding explanation, the O2 dissociation rate of L98Y oxyHr was measured in buffered D2O. The O2 dissociation rate of wild type P. gouldii Hr is approximately 20% slower in D2O than in H2O (40). However, we found that the O2 dissociation rate of L98Y P. gouldii Hr was ~4 times slower in D2O (0.092 s$^{-1}$) than in H2O (0.30 s$^{-1}$) at 25 °C. This much larger isotope effect is consistent with a proton transfer event during the rate-limiting step of O2 dissociation in L98Y oxyHr. The 841-cm$^{-1}$ ν(O-O) Raman-active stretching frequency of L98Y oxyHr shifts upward by 1 cm$^{-1}$ in D2O (data not shown) compared with a 4-cm$^{-1}$ upshift in D2O for the wild type $\nu$(O-O) stretching frequency (31). This latter difference is also consistent with an altered hydrogen bonding pattern in L98Y oxyHr. Given the very slow autoxidation rate of L98Y oxyHr (even slower than for wild type), it is unlikely that solvent introduction into the binding pocket is responsible for these altered isotope effects.

Effects on Autoxidation—Wild type, L98V, L98T, L98N, L98D, and L98A Hrs autoxidize successively more rapidly, even though their O2 association rates are all very similar (cf. Table I). This comparison indicates that both the size and hydrophobicity of the residue 98 side chain contribute barriers to solvent access in the binding pocket when bound O2 is also present. The importance of side chain volume is illustrated by the fact that L98A Hr was the most rapidly autoxidizing of the series. The importance of hydrophobicity can be illustrated by the relative autoxidation rates of L98V and L98T oxyHrs. The Val and Thr side chains are approximately the same size and shape, but the less hydrophobic substitution mutant, L98T, autoxidizes 8 times faster. L98N and -D oxyHrs autoxidize ~4,000 times faster than does L98T, further emphasizing the importance of pocket polarity in enhancing autoxidation rates. Thus, Leu-98 plays a significant role in inhibiting autoxidation of P. gouldii Hr, due both to its size and its hydrophobicity. The very slow autoxidation rate of L98Y Hr, even slower than for wild type, is presumably related to the same stabilizing interactions giving rise to its very slow O2 dissociation rate. The L98F mutation resulted in a 10-fold increase in autoxidation rate relative to wild type. Since this mutation did not affect $k_{\text{off}}$ or significantly perturb the oxyHr absorption and Raman spectra, the increased autoxidation rate of L98F cannot be due to a direct perturbation of the Fe2-O2 adduct structure. Therefore, the increased autoxidation rate of L98F must be due to structural perturbations in the binding pocket without any concomitant stabilizing interactions, as occurs in L98Y Hr.

L98W Hr—The rate of O2 association for L98W deoxyHr is ~50 times slower than for wild type Hr. The inset to Fig. 5 shows that the chromophoric species formed on the stopped-flow time scale for the L98W deoxyHr reaction with O2 is different from that of wild type (or any of the other L98X Hrs). The absorption maximum of the first detectable species is at ~650 rather than 500 nm. This particular experiment was not performed under pseudo first order conditions in order to facilitate detection of this intermediate. No absorption feature having a maximum near 500 nm was observed subsequent to the traces shown in Fig. 5. Only the spectrum attributable to the met form developed. Autoxidation of L98W Hr is extremely rapid, with a half-time of 70 ms. The spectrum of the end product of the reaction between L98W deoxyHr and O2 is shown in Fig. 4. Comparison with the spectra of Fig. 1 shows that this product has an oxo-bridged di-iron site typical of metHrs.

**DISCUSSION**

Although the structure and function of the di-iron site in Hr is well established, the role of conserved amino acid residues, other than those providing metal ligands, is less well characterized. The essentially concerted redox and proton transfer process that occurs upon O2 binding to Hr (Reaction 1 and Scheme 1) is self-contained within the di-iron site (3), and the conserved pocket residues in Hr are all hydrophobic (cf. Fig. 1). Therefore, if these pocket residues influence the O2 binding process, they must do so by relatively indirect means.

The UV-visible and resonance Raman data (Figs. 2–4) indicate that the essential features of the native oxyHr di-iron site are preserved in the L98X oxyHrs. Thus, any effects of these

---

**Fig. 5. UV-visible absorption spectra of recombinant L98W metHr and six stopped-flow traces obtained following mixing L98W deoxyHr with O2 ( inset).** All solutions were in 50 mm Hepes and 150 mm Na2SO4 (pH 7.5). Stopped-flow spectrophotometry was at 7 °C, and concentrations after mixing were 40 μM L98W Hr and 140 μM O2. Each stopped-flow spectrum was collected over 1 ms, and succeeding spectra were acquired at 5-ms intervals from lowest to highest absorbance. Arrow indicates direction of absorbance changes with time.
mutations on the kinetics or thermodynamics of O₂ binding cannot be due to significant alterations in the ground state of the oxy di-iron site. The “steric gate” function previously proposed for Leu-98 in Hr (14), is not supported by the kinetics of O₂ association and dissociation for a series of L98X Hrs. Despite the apparently tight packing in the pocket, substitutions of Leu-98 with larger, smaller, or more polar residues had little or no effect on the second order O₂ association rate constant. Similarly, larger and smaller residues could be substituted for Leu-98 with no effect on the O₂ dissociation rate constant. Thus, Leu-98 does not affect expansion of the Fe₂-O₂ coordination sphere, assuming that is the rate-limiting step in O₂ dissociation (39). L98Y was the only mutation we tested that showed a large effect on kₐ. This mutation also gave a much larger D₂O effect on kₐ than did wild type. Therefore, a hydrogen-bonding interaction that has been either introduced or enhanced relative to the wild type could be responsible for the much lower O₂ dissociation rate of L98Y Hr. The hydroxyl of Tyr-98 would not interact favorably with the other hydrophobic residues lining the binding pocket, thereby favoring its localization near the polar Fe₂-O₂ moiety. The hypothetical structure shown in Scheme 2 is consistent with our data for L98Y oxyHr, assuming that the proton between O₂ and the oxo bridge is transferred during the rate-limiting step of O₂ dissociation (40).

The x-ray crystal structure of L98Y metHr has been solved, and the position of the Tyr-98 hydroxyl indicates that the structure shown in Scheme 2 is feasible. Details of the L98Y metHr crystal structure will be reported separately. Substitutions of Leu-98 with successively smaller or more polar residues significantly increased the autoxidation rate of P. gouldii oxyHr. Due to rapid fluctuations of protein matrices in solution, it has been proposed that hydrophobicity rather than volume limits water occupancy in buried cavities within proteins (37). This proposal, in fact, rationalizes the hydrophobic side chains around the Fe₂-O₂ moiety. This packing minimizes pocket volume and, thereby, limits occupancy of the pocket by solvent. A leucine side chain at position 98 appears to have the optimal combination of size, shape and hydrophobicity for this role. This combination of properties may have led to its conservation in all known Hrs (46). Nevertheless, Leu-98 does not modulate the rate-limiting steps of O₂ entry into or exit from the binding pocket of P. gouldii Hr. Thus, “gating” by pocket residues in Hr is evident for solvent but not for O₂, and the gate for solvent entry appears to consist of both hydrophobic and steric barriers. Our recent investigations of corresponding pocket residue-mutated myoHrs led us to similar conclusions.

The increases in autoxidation rates observed for the L98X Hr mutants in the absence of large effects on O₂ association/dissociation kinetics suggest an important and specific role in inhibition of autoxidation for this residue. Our results suggest that the conserved residues lining the binding pocket kinetically inhibit autoxidation by efficient packing of their hydrophobic side chains around the Fe₂-O₂ moiety. This packing minimizes pocket volume and, thereby, limits occupancy of the pocket by solvent. A leucine side chain at position 98 appears to have the optimal combination of size, shape and hydrophobicity for this role. This combination of properties may have led to its conservation in all known Hrs (46). Nevertheless, Leu-98 does not modulate the rate-limiting steps of O₂ entry into or exit from the binding pocket of P. gouldii Hr. Thus, “gating” by pocket residues in Hr is evident for solvent but not for O₂, and the gate for solvent entry appears to consist of both hydrophobic and steric barriers. Our recent investigations of corresponding pocket residue-mutated myoHrs led us to similar conclusions.

Acknowledgments—We thank Dr. Robert L. Robson for experimental assistance in the initial cloning of the P. gouldii Hr gene and Shirin Arastu for experimental assistance in isolation and characterization of some of the L98X Hrs.

REFERENCES

1. Stenkamp, R. E. (1994) Chem. Rev. 94, 715–726
2. Brunold, T. C., and Solomon, E. I. (1999) J. Am. Chem. Soc. 121, 8277–8287
3. Brunold, T. C., and Solomon, E. I. (1999) J. Am. Chem. Soc. 121, 8288–8295
4. deWaal, D. J. A., and Wilkine, R. G. (1997) J. Biol. Chem. 251, 2339–2345

J. Xiong, D. M. Kurtz, Jr., R. S. Phillips, J. Ai, and J. Sanders-Loehr, unpublished results.
Gating of Access to the O₂-binding Pocket in Hemerythrin

5. Petrou, A. L., Armstrong, F. A., Sykes, A. G., Harrington, P. A., and Wilkins, R. G. (1981) Biochim. Biophys. Acta 670, 377–384
6. Lavalette, D., and Tetreau, C. (1988) Eur. J. Biochem. 177, 97–108
7. Alberding, N., Lavalette, D., and Austin, R. H. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2307–2309
8. Lloyd, C. R., Eyring, E. M., and Ellis, W. R., Jr. (1995) J. Am. Chem. Soc. 117, 11993–11994
9. Bates, G., Brunori, M., Amiconi, G., Antonini, E., and Wyman, J. (1968) Biochemistry 7, 3016–3020
10. Sayle, R., and Milner-White, E. J. (1995) Trends Biochem. Sci. 20, 374–376
11. Steiner, W. F., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) BioTechniques 8, 518–530
12. Feig, A. L., and Lippard, S. J. (1994) Chem. Rev. Biochemistry 114, 36, 583–589
13. Sayle, R., and Milner-White, E. J. (1995) Trends Biochem. Sci. 20, 374–376
14. Raner, G. M., Martins, L. J., and Ellis, W. R., Jr. (1997) Biochemistry 36, 7044–7049
15. Wilkins, R. G., and Harrington, P. C. (1983) Adv. Inorg. Biochem. 5, 51–85
16. Bradic, Z., Conrad, R., and Wilkins, R. G. (1977) J. Biol. Chem. 252, 6069–6075
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1990) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1990) Current Protocols in Molecular Biology, Green Publishing/Wiley-Interscience, New York
19. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
20. Tabor, S. (1990) in Current Protocols in Molecular Biology (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 16.2.1–16.2.11, Green Publishing/Wiley-Interscience, New York
21. Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1989) BioTechniques 8, 528–530
22. Studier, W. F., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
23. Zeng, J. H., Kurtz, D. M., Jr., Xia, Y. M., and Debrunner, P. G. (1991) Biochemistry 30, 586–589
24. Robinette, P. M., and Kurtz, D. M., Jr. (1988) Biochemistry 27, 4458–4465
25. Garbett, K., Darnall, D. W., Klotz, I. M., and Williams, R. J. P. (1969) Arch. Biochem. Biophys. 135, 419–434
26. Klippenstein, G. L. (1972) Biochemistry 11, 372–380
27. Ward, K. B., Hendrickson, W. A., and Klippenstein, G. L. (1975) Nature 257, 818–821
28. Zhang, J. H., Kurtz, D. M., Jr., Xia, Y. M., and Debrunner, P. G. (1992) Biochim. Biophys. Acta 1122, 293–298
29. Kurtz, D. M., Jr. (1990) Chem. Rev. 90, 585–606
30. Solomon, E. I., Tucek, F., Root, D. E., and Brown, C. A. (1994) Chem. Rev. 94, 827–856
31. Shiemke, A. K., Loehr, T. M., and Sanders-Loehr, J. (1989) J. Am. Chem. Soc. 110, 4951–4956
32. Sanders-Loehr, J., Wheeler, W. D., Shiemke, A. K., Averill, B. A., and Loehr, T. M. (1989) J. Am. Chem. Soc. 111, 8084–8089
33. Ai, J. (1998) Characterization of the Oxygen Binding Sites in Stearoyl-ACP Desaturase and Hemerythrin by Resonance Raman Spectroscopy. Ph.D. thesis, Oregon Graduate Institute, Portland, OR
34. Averill, B. A., Davis, J., Burman, S., Zirino, T., Sanders-Loehr, J., Loehr, T., Sage, J. T., and Debrunner, P. (1987) J. Am. Chem. Soc. 119, 5760–5767
35. Coletta, M., Gambacurta, A., Clementi, M. E., Erba, F., Polizzi, F., Falconi, M., and Ascoli, F. (1999) J. Biol. Inorg. Chem. 4, 678–683
36. Langerman, N., and Sturtevant, J. M. (1971) Biochemistry 10, 2809–2815
37. Feher, V. A., Baldwin, E. P., and Dahlquist, F. W. (1996) Nat. Struct. Biol. 3, 516–521
38. Olsen, J. S., and Phillips, G. N., Jr. (1996) J. Biol. Chem. 271, 17593–17596
39. Projekt, H.-D., Schindler, S., van Eldik, R., Fortier, D. G., Andrew, C. R., and Sykes, A. G. (1995) Inorg. Chem. 34, 5935–5941
40. Armstrong, G. D., and Sykes, A. G. (1986) Inorg. Chem. 25, 3135–3139
41. Creighton, T. (1993) Proteins: Structure and Molecular Properties, 2nd Ed., W. H. Freeman and Co., New York
42. Kim, K., and Lippard, S. J. (1996) J. Am. Chem. Soc. 118, 4914–4915
43. Pereira, A. S., Small, W., Krebs, C., Tavares, P., Edmondson, D. E., Theil, E. C., and Huynh, B. H. (1998) Biochemistry 37, 9871–9876
44. Cohen, J. D., Payne, S., Hagen, K. S., and Sanders-Loehr, J. (1997) J. Am. Chem. Soc. 119, 2960–2961
45. Wilkins, R. G., and Harrington, P. C. (1983) Adv. Inorg. Biochem. 5, 51–85
46. Negri, A., Gedeshi, G., Bonomi, F., Zhang, J.-H., and Kurtz, D. M., Jr. (1994) Biochim. Biophys. Acta 1208, 277–285
A Leucine Residue "Gates" Solvent but Not O₂ Access to the Binding Pocket of Phascolopsis gouldii Hemerythrin

Christopher S. Farmer, Donald M. Kurtz, Jr., Robert S. Phillips, Jingyuan Ai and Joann Sanders-Loehr

J. Biol. Chem. 2000, 275:17043-17050.
doi: 10.1074/jbc.M001289200 originally published online March 15, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001289200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 4 of which can be accessed free at http://www.jbc.org/content/275/22/17043.full.html#ref-list-1