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Histidine ligand variants of a flavo-diiron protein: Effects on structure and activities

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Abstract

Flavo-diiron proteins (FDPs) contain non-heme diiron and proximal flavin mononucleotide (FMN) active sites and function as terminal components of a nitric oxide reductase (NOR) and/or a four-electron dioxygen reductase (O₂R). Despite conservation of most structural, spectroscopic and redox properties, O₂R and NOR activities vary significantly among FDPs. A potential source of this variability is the iron ligation status of a conserved His residue, which provides an iron ligand in all but one known FDP structure, where this His residue is rotated away from iron and replaced by a solvent ligand. In order to test the effect of this His ligation status, we changed this ligating His residue (H90) in *Thermotoga maritima* (Tm) FDP to either Asn or Ala. The wild type Tm FDP shows significantly higher O₂R than NOR activity. Single crystal X-ray crystallography revealed a remarkably conserved diiron site structure in the H90N and -A variants, differing mainly by either Asn or solvent coordination, respectively, in place of H90. The steady state activities were minimally affected by the H90 substitutions, remaining significantly higher for O₂R vs NOR. The pre-steady state kinetics of the fully reduced FDP with O₂ were also minimally affected by the H90 substitutions. The results indicate that the coordination status of this His ligand does not significantly modulate the O₂R or NOR activities, and that FDPs can retain these activities when the individual iron centers are differentiated by His ligand substitution. This differentiation may have implications for the O₂R and NOR mechanisms of FDPs.

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Keywords

non-heme iron; nitric oxide; dioxygen; x-ray crystallography; enzyme kinetics; site-directed mutagenesis

Introduction

Flavo-diiron proteins (FDPs) serve as reductive dioxygen and/or nitric oxide scavengers in many bacteria, archaea, and some protozoans [1–3]. Almost all characterized FDPs function as terminal components of a two-electron nitric oxide reductase (NOR) and/or a four-electron dioxygen reductase (O_2R) according to Scheme 1 [4]. In this scheme, FDP_{ox} refers to the FMN-diferrous form, whereas FDP_{red} refers to the FMNH₂-diferrous form (although participation of intermediate oxidation levels is not excluded). Reducing equivalents from NADH are funneled to FDP typically via an NADH:rubredoxin oxidoreductase (NROR) and rubredoxin (Rd).² FDPs are soluble, non-heme enzymes and are distinct from membrane-bound respiratory NORs or O_2R s.

Fig. 1 illustrates the distinctive and characteristic structural features of FDP. The minimum functional unit consists of a head-to-tail homodimer with flavin mononucleotide (FMN) and non-heme diiron binding domains in each subunit [1, 6–8]. The two symmetry-related active sites each consist of an FMN plus a diiron center across a subunit interface. The diiron domain of FDP shows a metallo-beta-lactamase fold, which may have a counterpart in one recently recognized family of O_2 -activating non-heme diiron enzymes [9]. However, no oxygenase/hydroxylase activity has been reported for any FDP.

Some FDPs appear to function solely as scavenging NORs in vivo [11–13], whereas others apparently function as either scavenging O_2R s exclusively [5, 14–18] or bifunctional scavenging O_2R /NORs [19, 20]. The relative k_{cat} s for O_2R and anaerobic NOR turnover in vitro also vary significantly among FDPs [3, 4, 8, 14, 20–22]. The source of this variability cannot be readily attributed to differences in structure or cofactor redox properties among FDPs [6, 23].

The first reported crystal structure of the FDP family was of a protein referred to as rubredoxin:oxygen oxidoreductase (ROO) [10]. The ROO structure showed a terminal solvent ligand to one of the irons (Fe2), indicated by the red-highlighted OH_x in Fig. 1. All other subsequently reported FDP crystal structures showed a conserved His residue furnishing a ligand to the corresponding Fe2 in place of the terminal OH_x [1, 6–8, 24]. In ROO this His residue is present but rotated away from Fe2. Here we report a test of the hypothesis that the NOR and O_2R activities of FDPs are modulated by the iron coordination status of this His residue. For this test we used a thermostable FDP from *Thermotoga maritima* (Tm) [25]. The X-ray crystal structure of Tm FDP (Protein Data Bank entry, 1VME) shows the diiron site structure labeled as “other FDPs” in Fig. 1, in which the His residue in question (H90) is coordinated to iron. The room temperature O_2R activity of Tm FDP is comparable to that reported for ROO [20], while the NOR activity is significantly lower [4]. These relative O_2R vs NOR activities are consistent with the in vivo evidence of an O_2 scavenging function for Tm FDP [17, 25]. We have produced and characterized Tm FDP variants in which H90 was substituted with either Asn or Ala.

²Two archaeal FDPs have been reported to show [reduced factor F₄₂₀]:dioxygen oxidoreductase activity [5]. Presumably this activity does not occur in the majority of known FDP-containing microbes, which are not known to produce or utilize F₄₂₀.

The k_{cat} steps for O₂R and NOR turnover are thought to involve substrate complexes of the diferrous sites [1, 4, 26, 27], but the catalytically competent complexes are not yet well defined. Thus, in addition to testing the aforementioned hypothesis about the His ligand, this approach could allow us to distinguish reactivities of the individual iron centers with O₂ or NO.

Materials and methods

Wild type and H90 variant FDP expression plasmids

The plasmid used to express recombinant Tm FDP was obtained from the Arizona Biodesign Institute repository (<http://dnasu.asu.edu/DNASU/GetCloneDetail.do?cloneid=89997>). This plasmid encodes a 6 × His tag at the N-terminus. (The encoded amino acid sequence including the His tag is reported at the Joint Center for Structural Genomics website, <http://www1.jcsg.org/prod/newscripsts/psca/tagseq.cgi?acc=TM0755&uid=5565147>). The H90A and H90N variant genes were constructed using a QuikChange® protocol (Stratagene) with the wild type Tm FDP expression plasmid as template together with forward and reverse complementary primers overlapping the H90 codon. The nucleotide sequences of the forward primers (variant codons underlined) were 5′ - CCACACCGAGCCGGACGCCAGCGGATCTCTCCCC - 3′ for H90A, and 5′ - CCACACCGAGCCGGACAACAGCGGATCTCTCCCC - 3′ for H90N. The H90 variant genes in the expression plasmids were verified by nucleotide sequencing at the University of Texas Health Science Center San Antonio Nucleic Acids Core Facility.

FDP overexpression and purification

The wild type or H90 variant Tm FDP expression plasmids were heat-shock transformed into *E. coli* TOP-10 competent cells (Invitrogen). The transformed cells were grown at 37 °C in Luria Bertani medium supplemented with 100 mg/L ampicillin. Overexpression of the FDPs was induced in shaking 1-L cultures when OD₆₀₀ reached ~0.6 by addition of 200 mg/L L-(+)-arabinose and 50 mg/L ferrous ammonium sulfate. After either 4 hours at 37 °C or overnight at room temperature, the cells were harvested by centrifugation. The cell pastes were frozen at -80 °C overnight and then resuspended in 50 mM MOPS, 250 mM NaCl, pH 7.4 (buffer A). The resuspended cells were lysed by sonication, and cell debris was removed by centrifugation. The supernatants were applied to a 10-mL column containing Talon® Metal Affinity Resin (Clontech), and the FDPs were eluted with buffer A containing 250 mM imidazole. The eluted FDPs were washed with buffer B (50 mM MOPS, pH 7.4) by several concentration/redilution cycles in 30-kDa MWCO Amicon centrifuge filters (Millipore). The purified proteins, referred to as as-isolated FDPs, were concentrated to 1–2 mM (active site basis) and stored at -80 °C.

Protein, iron and FMN analyses

Protein was quantified by the bicinchonic acid assay, and iron was quantified by either the ferrozine assay [28] or inductively coupled plasma-mass spectrometry in the University of Texas at San Antonio Department of Civil and Environmental Engineering. FMN was quantified using $\epsilon_{462\text{nm}} = 12,000 \text{ M}^{-1}\text{cm}^{-1}$ [21].

Iron enrichment of the H90A variant

The as-isolated H90A variant typically contained ~1 Fe per protein monomer. In order to increase the iron content, the H90A variant (1.5 mL of ~1 mM as-isolated protein in buffer B) was anaerobically incubated with 2 molar equivalents per protein monomer of ferrous ammonium sulfate for 30 minutes at room temperature. The mixture was then exposed to air by gentle pipetting in order to oxidize the iron. The air-exposed protein was washed by

concentration/redilution with 50 mM MOPS, 5 mM EDTA pH 7.4 to remove the excess iron followed by concentration/redilution cycles with buffer B in order to remove the EDTA.

FMN enrichment

The as-isolated wild type and H90 variant FDPs contained 5–20% of the expected FMN content of 1 per protein monomer. FMN was inserted into the as-isolated FDPs essentially as previously described [4]. One milliliter of ~1 mM FDP (monomer basis) in buffer B was incubated with ~5 mol FMN:mol FDP monomer at 70 °C for 30 minutes. Excess FMN was removed by repetitive concentration/dilution cycles in buffer B until the filtrate was colorless. The FMN:iron mol ratio using this method was reproducibly ~1:2 for the wild type and H90 variants (after the iron enrichment procedure in the case of H90A).

O₂R and NOR activity assays

Recombinant Tm rubredoxin (Rd), and Tm NADH:rubredoxin oxidoreductase (NROR) were obtained as previously described [14]. Activities of the FMN-enriched FDPs were measured at room temperature (~23 °C) in buffer B by monitoring initial rates of NADH consumption ($\epsilon_{340\text{nm}} = 6220 \text{ M}^{-1}\text{cm}^{-1}$) with an Ocean Optics USB 2000 spectrometer. Assays were carried out in rubber septum-capped UV-Vis quartz cuvettes, which, for NOR activity, contained an N₂ atmosphere. Assay solutions contained 150 μM NADH, 1.5 – 20 μM Rd, 0.6 μM NROR and 1–2 μM FDP listed in the order of injection from concentrated anaerobic stock solutions. NO gas from (Praxair) was bubbled through aqueous 1 M KOH solutions to remove higher oxides of nitrogen. NOR activity was initiated by injecting 1 atm of NO into the evacuated headspace and rapidly inverting the cuvette. O₂R activity was initiated by addition of the FDP into the otherwise complete air-saturated assay mixture. Small O₂ or NO-dependent background NADH consumptions in the absence of FDP were subtracted from those obtained in the complete assay mixtures. NADH/O₂ consumption stoichiometry was monitored by repeatedly injecting small measured volumes of air-saturated buffer via syringe into cuvettes containing 1-mL anaerobic assay mixtures and measuring the magnitude of NADH consumption upon each O₂ injection.

Pre-steady state kinetics

Pre-steady state UV-vis absorption spectral time courses for reactions of FDP_{red} with O₂ were performed on an Applied Photophysics Ltd. Model SX20 stopped-flow spectrophotometer in either the single-wavelength (450 nm) or spectral collection modes. FDP_{red}s were obtained in buffer B by anaerobic incubation of 200 μM FMN-enriched FDPs with NADH (800 μM) Rd (1 μM) and NROR (1 μM) overnight at room temperature. The stopped-flow reactions were carried out by mixing the resulting FDP_{red} solutions 1:1 (v/v) with O₂-saturated buffer B at 3 °C. Rate constants were obtained by fitting the single wavelength time courses to exponential functions using the Origin program (OriginLab, Northampton, MA). More details are provided in the Fig. 3 legend.

Crystallization, structure determination, and refinement

As-isolated H90 variant FDPs (iron-enriched in the case of H90A) were concentrated to at least 30 mg/mL in buffer B. Crystallizations experiments were performed using the hanging drop vapor diffusion method at 4 °C using conditions similar to those for wild type Tm FDP (Protein Data Bank entry, 1VME). One microliter of protein solution was mixed with an equal volume of reservoir solution and equilibrated against 1 mL of reservoir solution. The most suitable crystals were grown from a solution containing 0.1 M sodium acetate, pH 3.9 – 4.2, 30%–40% 2-methyl-2,4-pentanediol. Cubic shaped crystals typically appeared after 3 days and grew to dimensions of about 0.4 × 0.4 × 0.3 mm in 2 weeks.

Crystal specimens in space group $P2_1$ suitable for single crystal diffraction were flash frozen in liquid nitrogen prior to data collection. Diffraction data were acquired on a Rigaku MicroMax 007-HF X-ray generator equipped with X-Stream cryogenic cooling systems and RAXIS HTC image plate detectors at the *X-ray Crystallography Core Laboratory* at the University of Texas Health Science Center at San Antonio. All data were processed using HKL-2000 [29]. The structures of the H90 variants were determined by the molecular replacement method implemented in PHASER [30] using coordinates from Protein Data Bank entry 1VME (Joint Center for Structural Genomics, unpublished) as the search model. Coordinates for each model were refined using PHENIX [31], including simulated annealing with torsion angle dynamics, alternating with manual rebuilding in the program COOT [32]. Data collection and refinement statistics are shown in Table 1. The PDB entry codes are 4DIK and 4DIL for the H90A and H90N variants, respectively.

Results

Structures of the H90 variants

The wild type and H90N variant FDPs contained ~2Fe per subunit in their as-isolated forms (Table 2). The as-isolated H90A variant typically contained sub-stoichiometric iron (~1Fe per protein subunit) but could be readily enriched to ~2Fe per subunit (Table 2). All data on H90A reported in this work are for this iron-enriched form. The as-isolated wild type and H90 variant Tm FDPs all contained sub-stoichiometric FMN (0.05–0.2 FMN per subunit). As previously reported for the wild type Tm FDP structure (PDB entry 1VME), the H90 variant structures reported here are of the deflavo forms, which apparently crystallize preferentially from solutions of the as-isolated proteins. The as-isolated H90 variant FDPs could be readily enriched to contain stoichiometric FMN (1FMN:2Fe:protein subunit) using a procedure previously described for the wild type protein [4]. Attempts to crystallize the FMN-enriched forms were unsuccessful. The proteins were crystallized under air-exposed conditions in the absence of added reducing agents, and the structures, therefore, most likely represent diferric sites.³

Representations of the wild type and H90 variant Tm FDP crystal structures are superimposed in Fig. 2. Fig. S1 depicts electron density contours of the side chain and solvent atoms of the H90A and -N variant diiron sites. The superpositions in Figure 2 were generated pairwise from the wild type and each of the H90 variant coordinates using the PyMOL super algorithm, which yielded root mean square deviations of 0.292 Å (5096 atoms) for H90A and 0.555 Å (4883 atoms) for H90N. The structures of the deflavo H90 variants, including the diiron sites, are remarkably similar to that of the wild type protein. The only significant structural differences in the variants are at the residue 90 coordination position on Fe2, where either N90 (presumably its OD1) in H90N or solvent in H90A has replaced the H90NE2. The Fe2-OD1(N90) distances are 2.3 and 2.4 Å in the two subunits of the asymmetric unit. The same numerical distances are coincidentally observed for the corresponding Fe2-OH_x in the H90A variant subunits. These distances are essentially identical to that of Fe2-NE2(H90) (2.4 Å) in the wild type protein.

The putative substrate binding pocket surrounds the “pocket OH_x”s above the diiron site. The positions of the residues lining this pocket (not shown) are quite similar among the wild type and H90 variant structures. With the exception of one additional solvent molecule in the H90N pocket (not shown), no electron densities assignable to other exogenous atoms were visible in these pockets.

³The diffraction data were collected using a conventional in-house X-ray generator and not a high-intensity synchrotron X-ray source, which can sometimes cause reduction of ferric iron sites in protein crystals [34].

The bridging solvent ligand ($\mu\text{-OH}_x$) in the wild type diiron site is also retained in the H90 variants. Previous spectroscopic and structural results on diferric FDPs [1, 4, 21] are consistent with either a hydroxo or aqua bridging ligand serving as a hydrogen bond donor to OD1 of the D89 ligand. (This hydrogen bonding interaction is shown schematically in Fig. 1). The $\mu\text{-O}\cdots\text{OD1(D89)}$ distance is 2.7–2.8 Å for all three of the Tm deflavo-FDP structures depicted in Fig. 2, indicating conservation of this hydrogen bonding interaction in the H90 variants.

Steady state O_2R and NOR activities

All spectroscopic and kinetics results reported here are for FMN-enriched FDPs containing ~1 FMN and ~2Fe per protein monomer (after iron-enrichment in the case of the H90A variant), as listed in Table 2. Tm FDP is a homodimer in solution, but for the results presented here, FDP concentrations are expressed as protein monomers, which is equivalent to active sites.

The O_2R steady state activities reported in Table 2 for the wild type Tm FDP were measured under saturating substrate and redox components (NADH, NROR and Rd) previously determined from Michaelis-Menten plots [4]. The activities reported in Table 2 for wild type Tm FDP are very similar to the k_{cat} values determined from those plots. The same assay conditions were used for the H90 variants and resulted in activities very similar to those for the wild type protein, with slightly higher activity for the H90N variant. Increasing the concentrations of the NROR or Rd in the assay mixtures above those listed in Materials and Methods did not lead to significantly higher activities. Injections of O_2 from air-saturated solutions into anaerobic assay mixtures resulted in consumption of ~2 mol NADH/mol injected O_2 for both the wild type and H90 variant proteins, as expected for the O_2R stoichiometry in Scheme I.

The analogous steady state activities were measured with NO as substrate. The NOR activity of the wild type Tm FDP listed in Table 2 is quite similar to the previously reported k_{cat} value [4] and, similarly to the O_2R activities, was not greatly affected by H90 substitution. In all cases the NOR activities remained 50- to 100-fold lower than the O_2R activities.

Pre-steady state reactions of FDP_{red} with O_2

Stopped-flow spectroscopy was used to examine whether H90 substitutions affected the pre-steady state reaction of Tm FDP_{red} with O_2 . Reactions of wild type and H90 variant FDP_{red} s with excess O_2 resulted in oxidation of the majority of the FMN at rates near the upper limit measurable by our stopped-flow methods, even at 3 °C. These data are represented as Phase I in Table 3 and the Fig. 3, left panel time courses. Two successively ~10-fold slower phases (Phase II and Phase III) accounted for a very minor percentage of the FMNH_2 oxidations in the cases of wild type and H90N and a somewhat larger but still minor percentage for H90A. In all cases the oxidations appeared to proceed from FMNH_2 to FMN with little or no accumulation of the FMN semiquinone. The respective k_{obs} for all phases of O_2 oxidation were very similar among wild type and the H90 variants. Fig. 3 also shows a broad, relatively weak absorption feature between 550 and 700 nm, which formed within the mixing dead time, largely prior to Phase I, and which decayed concomitantly with FMNH_2 oxidation.

Discussion

Our results demonstrate that a diiron site is retained in Tm FDP variants in which a His ligand residue is substituted by a potentially more weakly coordinating (Asn) or non-coordinating (Ala) residue and that these substitutions have very modest effects on the diiron

site structure. The only significant difference is either Asn or solvent coordination, respectively, in place of the substituted His residue. Native Asn ligation is relatively rare but not unprecedented in non-heme iron proteins. We have found eight other examples in the Protein Data Bank, all of which show the Asn as ligating via OD1. (The Electronic supplementary material contains PDB entries and literature citations for these examples). The *trans* disposition of the terminal solvent ligands in the H90A variant may be even rarer. We have found no other examples in non-heme iron proteins. A pair of solvent molecules, at least one of which coordinates to iron, occupy the putative substrate binding pocket in the wild type and H90-substituted Tm FDPs. Structures of other FDPs typically show either solvent or other small molecules occupying this pocket [1, 8]. In the ROO crystal structure, a diatomic molecule (presumed to be dioxygen) occupies this pocket [10].

These H90 substitutions resulted in very modest effects on O₂R or NOR activities; the steady state O₂R activities remained significantly higher than the NOR activities. While the effects of the H90 substitutions on K_m were not examined in detail, they did not appear to be drastically higher than those of the wild type protein ($\sim 2 \mu\text{M}$ O₂ and $\sim 100 \mu\text{M}$ NO [4]). The pre-steady state UV-vis absorption time courses for reactions of the FDP_{red} with O₂ were also minimally affected by the H90 substitutions, in all cases resulting in rapid conversion of FMNH₂ to FMN. These minimal effects lead us to conclude that the H90 ligation status is not a significant modulator of O₂R or NOR activities of Tm FDP. Presumably this conclusion also applies to the homologous His residue in other FDPs.

Most proposed mechanisms for the O₂R and NOR activities of FDPs invoke substrate complexes of the diferrous sites [1, 4, 26, 27], but the nature of these complexes and the participation of FMNH₂ in substrate turnover are ill-defined. Our results have some implications for these mechanisms. The two pocket solvent molecules shown in Fig. 2 mimic likely NO and O₂ binding sites. The conservation of the diiron site structure, the solvent occupied sites, and the surrounding pocket structure is likely one reason why the activities of the H90 variants are similar to those of the wild type protein. The pre-steady state, stopped-flow spectral time courses for reactions of the wild type and H90 variant FDP_{red}s with O₂ all showed evidence for a rapid process preceding two-electron FMNH₂ oxidation. This rapid process is manifested as formation and decay of a weak, broad absorption feature between 550 and 700 nm (Figure 3), which is not known to be associated with flavin redox changes in FDPs [21, 23, 35]. This spectral time course closely resembles that reported previously for another FDP, whose crystal structure shows the homologous H90 residue coordinated to iron [8] (although the spectral process preceding FMNH₂ oxidation was not explicitly noted). We have not found any corresponding pre-steady state time course reported for ROO. In the case of Tm FDP the 550–700 nm absorption feature is essentially unperturbed by the H90 substitution at Fe2, which could conceivably indicate initial reaction of dioxygen with Fe1 (the iron proximal to FMN). An alternative, but to us, less likely, explanation is that H90 and N90 dissociate from iron upon reduction to the diferrous state and remain dissociated during the redox changes in both the pre-steady state and steady state, thereby mimicking the H90A coordination environment. We have so far been unsuccessful in obtaining crystals of the reduced Tm FDP. However, X-ray crystal structures of another FDP showed that the bis(His-ligated) Fe2 and the diiron site structure labeled “other FDPs” in Fig. 1 were retained upon anaerobic reduction of the oxidized protein crystals with sodium dithionite [24]. In the case of NO, our recent studies in collaboration with the Moenne-Loccoz lab have provided evidence for both mono- and dinitrosyl adducts of the diferrous site in Tm FDP [4, 36]. The involvement of these adducts in NOR turnover remains to be determined. Endogenous iron ligand variants such as those reported here may assist in characterizations of intermediates in both the O₂R and NOR reactions of FDPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FDP	flavo-diiron protein
FDP_{ox}	FMN-diferric FDP
FDP_{red}	FMNH ₂ -diferrous FDP
FMN	flavin mononucleotide
FMNH₂	FMN hydroquinone
NOR	nitric oxide reductase
O₂R	dioxygen reductase
NROR, NADH	rubredoxin oxidoreductase
Rd	rubredoxin
ROO, rubredoxin	oxygen oxidoreductase
PDB	Protein Data Bank
Tm	<i>Thermotoga maritima</i>
MOPS	3-(N-morpholino)propanesulfonic acid

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Fig. 1. (Top) Structure of a representative FDP homodimer drawn in PyMOL (Delano Scientific) using coordinates in Protein Data Bank entry, 1E5D [10]. Protein backbone is shown in cartoon mode with individual subunits colored cyan and green. FMN (yellow) and diiron sites (orange) are shown as atom spheres. (Bottom) Schematic renderings of active site structures in the FDP from *Desulfovibrio gigas*, given the trivial name, rubredoxin:oxygen oxidoreductase (ROO) [10] (left) compared to that in other FDPs (right). OH_x indicates a bridging hydroxo ($x = 1$) or aqua ($x = 2$) ligand. Differences in iron ligands in ROO vs other FDPs are red-highlighted.

Fig. 2.

Depictions of the crystal structures of the deflavo forms of wild type (1VME) and H90 variant (this work) Tm FDPs. The homodimer protein backbone subunit pairs are color-coded green/cyan for wild type, blue/magenta for H90N and orange/red for H90A. Black circles surround the two non-crystallographically related diiron site locations within each homodimer. An expanded view of one set of these diiron sites is shown color-coded green for wild type, blue for H90N and orange for H90A. The diiron sites are depicted in an orientation equivalent to that in the Fig. 1 schematic drawing. The pocket OH_xs closest to Fe1 are not depicted as bonded in the wild type and H90A drawings because their Fe1--OH_x distances are 2.6 Å. The corresponding distance in H90N is 2.3 Å.

Fig. 3.

Stopped-flow UV-vis absorption spectral time courses for Phase I (left panels) and Phases II and III (right panels) of the reactions of wild type or H90 variant Tm FDP_{red} with excess O₂ in 50 mM MOPS pH 7.3 at 3 °C. Rate constants for the phases are listed in Table 3, and corresponding single-wavelength time courses are shown in Fig. S2. Concentrations after mixing were: 100 μM FDP and ~1 mM O₂. Indicated times are after the mixing dead time (~2 msec).

Scheme 1.

Table 1

Data collection and refinement statistics

	H90A	H90N
Data collection		
Space group	$P2_1$	$P2_1$
Cell dimensions		
a, b, c (Å)	55.2, 96.0, 90.3	54.6, 91.9, 86.3
α, β, γ (°)	90, 95.8, 90	90, 96.8, 90
Wavelength (Å)	1.5418	1.5418
Resolution (Å) *	30 – 1.75 (1.81 – 1.75)	30 – 2.0 (2.07 – 2.00)
R_{sym}	0.041 (0.486)	0.072 (0.508)
$I\sigma_1$	24.5 (2.4)	23.0 (3.4)
Completeness (%)	99.7 (100)	99.8 (99.8)
Redundancy	3.5 (3.4)	6.6 (6.0)
Wilson B value	28.2	36.6
Refinement		
Resolution (Å)	29.1 – 1.75 (1.77 – 1.75)	28.9 – 2.0 (2.03 – 2.00)
No. Reflections	94191	57492
$R_{\text{work}}/R_{\text{free}}$	0.191/0.215	0.198/0.235
Monomers per asymmetric unit	2	2
No. Atoms		
Protein	6608	6414
Heteroatom [§]	9	9
Solvent	430	185
B -factors (Å ²)		
Protein	32.5	37.5
heteroatoms [§]	25.7	34.2
Solvent	39.3	37.6
R.m.s deviations		
Bond lengths (Å)	0.009	0.007
Bond angles (°)	1.185	1.090
Ramachandran statistics (%)		
Most favored regions	88.0	89.2
Additional allowed regions	11.1	9.6
Generously allowed regions	0.1	0.4
Disallowed regions	0.7	0.7

* Values in parentheses are for the highest-resolution shell.

[§] Heteroatoms include bound Fe, μ -OH_X, and Cl ions.

Calculated using PROCHECK [33].

Table 2

Iron/protein mol ratios and steady state O₂R and NOR activities for wild type and H90 variant Tm FDPs.

FDP	Iron/protein ^a	activities (s ⁻¹) ^b	
		O ₂ R	NOR
wild type	2.3 ± 0.2	2.6 ± 0.2	0.03 ± 0.01
H90N	2.1 ± 0.2	3.8 ± 0.1	0.09 ± 0.02
H90A	2.4 ± 0.2	2.5 ± 0.3	0.05 ± 0.01

^aMol Fe/mol protein monomer. All FDPs contained 1FMN:protein monomer.

^bMol substrate equivalents consumed/sec-mol FDP calculated from initial rates of NADH consumption at saturating substrate and electron donor. Detailed conditions are described in Materials and Methods.

Table 3Rate constants for oxidation of Tm FDP_{red}s by excess O₂.^a

FDP	Phase I	Phase II	Phase III
wild type	167 ± 9	2.3 ± 0.3	0.15 ± 0.01
H90N	184 ± 9	1.7 ± 0.4	0.17 ± 0.03
H90A	213 ± 15	1.8 ± 0.1	0.12 ± 0.02

^a k_{obs} (s⁻¹) determined from exponential fits to the rate of increase in A₄₆₂ nm vs time via stopped-flow spectrophotometry under the conditions described in the Fig. 3 legend and Materials and Methods. Representative A₄₆₂ nm vs time traces are contained in Fig. S2.