Design, synthesis, and characterization of a photoactivatable flavocytochrome molecular maquette

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ABSTRACT We report the construction of a synthetic flavo-heme protein that incorporates two major physiological activities of flavoproteins: light activation of flavin analogous to DNA photolyase and rapid intramolecular electron transfer between the flavin and heme cofactors as in several oxidoreductases. The functional tetra- α -helix protein comprises two 62-aa helix-loop-helix subunits. Each subunit contains a single cysteine to which flavin (7-acetyl-10-methylisoalloxazine) is covalently attached and two histidines appropriately positioned for *bis*-his coordination of heme cofactors. Both flavins and hemes are situated within the hydrophobic core of the protein. Intramolecular electron transfer from flavosemiquinone generated by photoreduction from a sacrificial electron donor in solution was examined between protoporphyrin IX and 1-methyl-2-oxomesoheme XIII. Laser pulse-activated electron transfer from flavin to meso heme occurs on a 100-ns time scale, with a favorable free energy of approximately -100 meV. Electron transfer from flavin to the lower potential protoporphyrin IX, with an unfavorable free energy, can be induced after a lag phase under continuous light illumination. Thus, the supporting peptide matrix provides an excellent framework for the positioning of closely juxtaposed redox groups capable of facilitating intramolecular electron transfer and begins to clarify in a simplified and malleable system the natural engineering of flavoproteins.

Nature selects from a battery of redox active cofactors and assembles them within a protein matrix to facilitate essential functions such as substrate binding, electron transfer, energy conversion, and chemical catalysis. The cofactors often are juxtaposed closely within a single structure with the physical chemical properties adjusted by the protein environment to perform the desired function satisfactorily and with high fidelity. Studies aimed at understanding the molecular basis of catalytic fitness in enzymes often are confounded by the sheer complexity of natural proteins. It is our goal to uncover the engineering of natural oxidoreductase protein design by simplifying the problem and synthesizing the minimalistic protein structures that assemble working arrays of cofactors and reproduce native-like function. We call these working structures “molecular maquettes.”

To date there has been much success with this approach. The peptide scaffold of choice is a tetra- α -helix-bundle that assembles and is stable over a wide range of conditions (1, 2). This is exemplified by the remarkable utility of the simple scaffold for successful incorporation of up to four hemes as a maquette for the cytochrome *b* subunit of the cytochrome *bc*₁ complex (3–8), the covalent attachment of a pendant porphyrin dimer as the fundamental component of a photosynthetic reaction center maquette (9, 10), and the incorporation of an iron–

sulfur cluster into the loop region to generate a ferredoxin–heme maquette (11). All of the aforementioned systems exhibit key properties of their natural counterparts with respect to the physical chemical characteristics of the cofactors and the influence that the peptide environment exerts on their properties. However, none of these systems was designed to have any inherent function associated with them. In this paper, we describe a molecular maquette that is capable of supporting one of the most fundamental of all biological processes—light activatable electron transfer. Unique to this maquette is its stable assembly in aqueous solution and the ability to incorporate flavin and heme as native-like cofactors within the protein hydrophobic core, resulting in significant modification of their spectral and redox properties. Ultimately, we anticipate that maquettes will be able to support elementary functions, such as classic “dark” oxidoreduction reactions that reflect the action of many natural enzymes.

In biology, the most common function of flavin is as a cofactor with versatile redox chemistry in an array of oxidoreductases (12, 13). This is exemplified by flavocytochrome *b*₂, where the flavin is involved in both catalytic dehydrogenation of l-lactate to pyruvate and the subsequent electron transfer to a heme cofactor (14). Flavins also can be used as an efficient photoreceptor and mediator of light-induced electron transfer by DNA photolyase (15) and the plant blue light photoreceptor (16). Irradiation of flavin in solution with blue photons generates the excited triplet state, which is a powerful oxidant. In the presence of a sacrificial electron donor (irreversible reaction) such as EDTA, singly reduced flavosemiquinone is generated, which subsequently can react further with protein-bound cofactors (17–19). Flavins are commonly used as a flash-activatable intermolecular source of electrons for investigating protein-mediated electron transfer (20) and recently has been used as an *in situ* intraprotein electron donor, where it was covalently attached to a cysteine residue on the surface of cytochrome *c* to generate semisynthetic flavocytochrome *c* (21). Thus, we chose to use a flavin chromophore as the light-activatable species in our system. The actual flavin of choice was 7-acetyl-10-methylisoalloxazine (Fig. 1A), which although not naturally occurring, exhibits the same chemistry as natural flavins (22, 23). From a thorough investigation of existing flavoprotein structures, we concluded that engineering a flavin binding site into our existing maquette framework would be premature and would pose considerable design problems. It is evident that flavoproteins, including even the simple flavodoxins, possess a completely different architecture to that offered by tetra- α -helix-bundles (24). However, many flavoproteins contain flavin covalently bound to the polypeptide (25). Therefore, there exists a precedent for localizing the flavin within the helix-loop-helix framework by covalently attaching it to an amino acid side chain situated within the

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Abbreviations: Fl, flavin (7-acetyl-10-methylisoalloxazine); H, protoheme IX; OMH, 1-methyl-2-oxomesoheme XIII; α -L- α , helix-loop-helix peptide; Fl- α -L- α , helix-loop-helix peptide with flavin attached. ‡To whom reprint requests should be addressed. e-mail: dutton@mail.med.upenn.edu.

cause most absorb light in the same wavelength region as that of the flavin and confuse analysis. We relied entirely on the weak interaction of flavin with the electrode, and the system was left for much longer than usual to establish equilibrium among the electrodes, the solution, and the flavin species after each potential adjustment (30 min). This proved possible because the overall system was reversible and without background oxidative or reductive drift. The second method used the technique introduced by Massey (27). This involves equilibration with an indicator dye whose midpoint potential is known and is within 30 mV of the $F_{\text{ox}}/F_{\text{red}}$ couple to be measured. Reduction was initiated and maintained by use of the xanthine/xanthine oxidase system. The dyes that satisfied the selective requirement were 5-5'-indigodisulfonate ($E_{\text{m7}} = -140$ mV) for free flavin and 5, 5'-indigotrisulfonate ($E_{\text{m7}} = -90$ mV) for $\text{Fl-}\alpha\text{-l-}\alpha$.

Fluorescence Spectroscopy. Fluorescence spectra were acquired on an ISS-2 fluorimeter. The quantum yields of flavin fluorescence were determined by comparing the integral of the emission spectra with that obtained for flavin mononucleotide under identical excitation conditions. Spectra were acquired at room temperature in 50 mM Tris and 100 mM NaCl (pH 8.0) buffer. Quenching constants of flavin and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ fluorescence by sodium iodide were determined from the Stern-Volmer equation (28): $F_0/F = (1 + K_{\text{SV}}[I]) \exp([I]V)$, where F_0 and F are the intensities in the absence and presence of quencher, respectively. K_{SV} (M^{-1}) is the quenching constant, $[I]$ quencher concentration and V is the volume of the quenching sphere of action (M^{-1}).

Photoreduction. Continuous light photoreduction data were acquired on a Beckman DU-550 diode array spectrophotometer. A Cuda 1-150 light generator was used as a white light source for photoexcitation, and the light was directed onto the sample with a fiber optic light guide. The sample was placed in a sealed glass cuvette and rendered anaerobic by flushing with argon gas while stirring. The buffer in all of the photoreduction experiments was 50 mM phosphate, 100 mM NaCl, and 10 mM EDTA (pH 8.0). Laser flash-induced transient absorption difference spectra in the nanosecond to millisecond time scale were performed with a Quantum Ray DCR-11 YAG laser. The exciting laser pulse was at 355-nm, 20-mJ, 8-ns pulse width, operating at 10 Hz. Detection by a photodiode array was gated at selected intervals following the laser flash and was mediated by a fiber optically guided white light source directed into the sample normal to the incident laser light. The sample was maintained under anaerobic conditions by flushing with argon and was contained in a sealed quartz flow cell connected to a reservoir, such that the sample exposed to laser irradiation was replenished continually. The transient difference spectra were generated by simultaneously subtracting the acquired sample (laser exposed) and reference (no incident laser light). All experiments were performed at room temperature.

RESULTS AND DISCUSSION

Design of $[\alpha\text{-l-}\alpha]_2$. The design was based on our prototype series of heme binding protein maquettes (2-5). The histidines in each 62-aa helix-loop-helix subunit ($\alpha\text{-l-}\alpha$) of the tetra- α -helix-bundle $[\alpha\text{-l-}\alpha]_2$ are at positions 22 and 41, appropriate for *bis*-histidine coordination of bound heme (Fig. 1B). The unique cysteine at position 48 is one complete heptad repeat above that of histidine 41, chosen to place the flavin attachment site at a hydrophobic α position of the helix and is expected to direct the flavin toward the interior of the bundle (4), sterically close to the hemes (Fig. 1C). The flavin of choice was 7-acetyl-10-methylisalloxazine because it is straightforward to synthesize and has a methyl group in place of the bulky ribose, ribophosphate, or ribophosphate adenine dinucleotide present in naturally occurring flavins (22). Two types of iron protoporphyrin were used in this investigation: protoheme IX

(H) and 1-methyl-2-oxomesoheme XIII (OMH) (7). The oxomeso substituents of the porphyrin macrocycle elevate the intrinsic midpoint potential of OMH compared with H and allows the effect of altering the driving force for electron transfer to be probed.

Maquette Architecture and Cofactor Environment. Size exclusion chromatography confirmed that the *apo* maquette, $[\alpha\text{-l-}\alpha]_2$, and variants eluted with retention times consistent with tetra- α -helix-bundle aggregation states at micromolar concentrations. This was based on column calibration with globular proteins and comparison with the chromatographic behavior of previously characterized tetra- α -helix-bundles (2, 3).

All of the maquettes possess $\approx 70\%$ α -helical secondary structure. The $\Theta_{222}/\Theta_{208}$ ratio for $[\alpha\text{-l-}\alpha]_2$ in aqueous buffer is close to 0.95, as opposed to 0.85 in 50% trifluoroethanol. A $\Theta_{222}/\Theta_{208}$ ratio of 1.0 is considered diagnostic of a coiled-coil structure (29, 30). The decrease in the $\Theta_{222}/\Theta_{208}$ ratio in high concentrations of trifluoroethanol (> 4 M) known to disrupt hydrophobic core packing but maintain secondary α -helical structure is representative of the formation of extended or monomeric helices (9, 31, 32).

Fig. 2A shows the absorption spectra of flavin compared with that of $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ in aqueous buffer. Attachment of flavin to $[\alpha\text{-l-}\alpha]_2$ causes a shift of the blue absorption peak from 427 to 430 nm, but otherwise the spectra are qualitatively similar. Fig. 2B shows that addition of two hemes per tetra- α -helix-bundle to generate $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$ produces a typical *bis*-histidine ligated ferric *b*-heme spectrum superimposed on the flavin absorption. On reduction with sodium dithionite, the typical ferrous *b*-heme spectrum is obtained.

The thermodynamic stability of $[\alpha\text{-l-}\alpha]_2$ and the effect of introducing the flavin and heme cofactors on bundle stability was probed by the use of chemical denaturants. Addition of guanidine hydrochloride (Gdn.HCl) to $[\alpha\text{-l-}\alpha]_2$ resulted in a loss of ellipticity at Θ_{222} nm in the CD spectrum, consistent with denaturation to a disordered structure with a $[\text{Gdn.HCl}]_{1/2}$ value of 5.5 M. The free energy for unfolding ΔG_{H2O} was 18.1 ± 0.8 kcal·mol $^{-1}$, and the cosolvation term m was 2.4 ± 0.2 kcal·mol $^{-1}$ ·M $^{-1}$. Placing flavin in the hydrophobic core of $[\alpha\text{-l-}\alpha]_2$ to form $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ causes the $[\text{Gdn.HCl}]_{1/2}$ value to decrease to 3.8 M, indicating that attachment of flavin destabilizes the bundle ($\Delta\Delta G = 6.3$ kcal·mol $^{-1}$). The cooperativity of the unfolding transition is also lowered in $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ ($m = 1.5 \pm 0.2$ kcal·mol $^{-1}$ ·M $^{-1}$) as compared with $[\alpha\text{-l-}\alpha]_2$, probably because of weakened packing of the hydrophobic core due to

FIG. 2. UV-visible spectra of maquettes in various states of construction: (A) free flavin, $[\alpha\text{-l-}\alpha]_2$ and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$; (B) oxidized $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$ (γ_{max} at 411 and 535 nm, corresponding to the Soret and unresolved α/β bands, respectively), and reduced $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$ (γ_{max} at 423, 560, and 531 nm, corresponding to the Soret α and β bands, respectively). All concentrations were 10 μM , the buffer used was 50 mM Tris and 100 mM NaCl (pH 8.0), and the spectra were acquired at room temperature.

the presence of a bulky flavin chromophore. Despite this, the $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ still forms a stable tetra- α -helix-bundle ($\Delta G_{\text{H}_2\text{O}} = 11.8 \pm 0.9 \text{ kcal}\cdot\text{mol}^{-1}$). Binding of two hemes to both $[\alpha\text{-l-}\alpha]_2$ and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ shifts the midpoint of the denaturation profile to higher Gdn.HCl concentration, but renders the process less cooperative. No attempt was made to fit the denaturation profile of $[\text{H-}\alpha\text{-l-}\alpha]_2$ or $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$ as the process was not fully reversible because of heme aggregation on unfolding.

Comparison of the flavin and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ fluorescence emission spectra revealed that the intensity is quenched 6-fold on attachment to the peptide (quantum yield Φ_{F} , flavin = 0.30, and $[\text{Fl-}\alpha\text{-l-}\alpha]_2 = 0.05$), which is a general property of sulfur-linked flavinyl peptides (22, 33). Stepwise addition of iodide, a dynamic fluorescence quencher, was used to provide an indication for the degree of shielding that the peptide environment affords the flavin in $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ from bulk solvent. The data were fit to the Stern–Volmer equation to obtain values for dynamic (K_{SV}) and static (V) quenching. The values for K_{SV} and V were $30.4 \pm 1.5 \text{ M}^{-1}$ and $2.3 \pm 0.4 \text{ M}^{-1}$ for flavin and $7.7 \pm 0.3 \text{ M}^{-1}$ and $0.2 \pm 0.1 \text{ M}^{-1}$ for $[\text{Fl-}\alpha\text{-l-}\alpha]_2$, respectively. Thus, the quenching constants are 4-fold (K_{SV}) and 13-fold (V) lower for $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ compared with flavin in solution. The difference in the magnitude of K_{SV} is comparable to values reported in the literature for the degree of shielding conferred by the protein environment in natural flavoproteins (28). This implies that the peptide matrix significantly shields the flavin from quenching agents in bulk solution.

The dissociation constants (K_{D}) of the two hemes (H) bound to $[\alpha\text{-l-}\alpha]_2$ are 0.035 and 0.135 μM ($\text{SD} \pm 20\%$), similar to reported values for related maquettes (3–5). The K_{D} values for H from $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ are 0.13 and 1.5 μM ($\pm 20\%$), approximately one order of magnitude weaker than the corresponding values for $[\alpha\text{-l-}\alpha]_2$, implying that the presence of the flavin adjacent to the heme hinders binding. The K_{D} values of OMH bound to $[\alpha\text{-l-}\alpha]_2$ (0.05 and 0.17 μM) and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ (0.15 and 1.2 μM) are similar to H.

Cofactor Potentiometry and Maquette Topology. The potentiometric properties of the flavin were examined at pH 8.0 for alteration induced by the protein environment. Fig. 3 shows Nernst plots of redox titrations for the two-electron couple of free flavin, Cys-Fl and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$, along with the one-electron heme couples for $[\text{H-}\alpha\text{-l-}\alpha]_2$, $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$, $[\text{OMH-}\alpha\text{-l-}\alpha]_2$, and $[\text{OMH:Fl-}\alpha\text{-l-}\alpha]_2$. Table 2 summarizes these data. The 50-mV rise in midpoint potential for $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ compared with free flavin is not simply due to covalent attachment of the flavin to the peptide because the midpoint potential of the $\text{Fl}_{\text{ox}}/\text{Fl}_{\text{red}}$ couple is unaltered either when attached to cysteine (Cys-Fl) or to a surface exposed cysteine on cytochrome *c* (21). This indicates that the shift in the flavin midpoint potential in $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ is caused by the peptide environment, which stabilizes the fully reduced bound flavin by $1.15 \text{ kcal}\cdot\text{mol}^{-1}$ relative to the oxidized form. In natural flavoproteins, the two-electron $\text{Fl}_{\text{ox}}/\text{Fl}_{\text{red}}$ couple is also typically within 100 mV of that for free flavin. Some natural flavoproteins are engineered to stabilize the single reduced flavosemiquinone (Fl_{sq}) (34, 35); however, it is not clear from Fig. 3A whether the tetra- α -helix protein interior stabilizes Fl_{sq} relative to solution. The best Nernst fit to the redox data has an n value of 1.6 and could reflect either such stabilization or a small amount of heterogeneity in the flavin environment.

The E_{m8} values of the two hemes in $[\text{H-}\alpha\text{-l-}\alpha]_2$ are -220 and -105 mV , respectively, and are the same within experimental error as previously determined values for heme incorporated into related maquettes (3–5). These midpoint potentials are raised in comparison with free *bis*-imidazole heme: $E_{\text{m8.5}} = -235 \text{ mV}$ (J. Schiffman and P.L.D., unpublished observations), illustrating the relative destabilization of the charged ferric-heme with respect to the neutral ferrous form within the low dielectric protein interior (7). Moreover, the 100-mV splitting of the heme potentials is consistent with negative heme–heme

FIG. 3. Oxidation-reduction potentials of flavin and hemes in the maquettes. Nernst plots illustrating $\text{Fl}_{\text{ox}}/\text{Fl}_{\text{red}}$ ($n = 2$) midpoint potentials of: (A) flavin ($\circ$), Cys-Fl ($\triangle$), and $[\text{Fl-}\alpha\text{-l-}\alpha]_2$ ($\bullet$); (B) $\text{H}_{\text{ox}}/\text{H}_{\text{red}}$ ($n = 1$) midpoint potentials of $[\text{H-}\alpha\text{-l-}\alpha]_2$ ($\diamond$), $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$ ($\blacklozenge$), $[\text{OMH-}\alpha\text{-l-}\alpha]_2$ ($\square$), and $[\text{OMH:Fl-}\alpha\text{-l-}\alpha]_2$ ($\blacksquare$). The midpoint potentials are reported in Table 2.

electrochemical cooperativity brought about by the close proximity of two charged ferric hemes in the protein interior (3). This splitting of the potentials has been used to indicate that the $(\text{H-}\alpha\text{-l-}\alpha)$ subunits in $[\text{H-}\alpha\text{-l-}\alpha]_2$ have adopted a *syn* topology (Fig. 1B) (A. M. Grosset & P.L.D., unpublished observations). However, for the flavin containing *holo* maquette $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$, there is no evidence for splitting of the heme potentials; both are at -153 mV . This effect has been shown to be the result of the subunits adopting an overall *anti* topology, which would place the hemes at diametrically opposite ends of $[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$ (Fig. 1C). The *anti* topology greatly diminishes the electrostatic effects attributed to the splitting of the midpoint potentials and gives rise to the same well defined

Table 1. Midpoint potentials of the flavin and heme redox couples

Redox couple	E_{m8} , mV	n
Flavin, ($\text{Fl}_{\text{ox}}/\text{Fl}_{\text{red}}$)	$-138 \pm 5^*$	2
Cys-Fl, ($\text{Fl}_{\text{ox}}/\text{Fl}_{\text{red}}$)	$-146 \pm 5^*$	2
$[\text{Fl-}\alpha\text{-l-}\alpha]_2$, ($\text{Fl}_{\text{ox}}/\text{Fl}_{\text{red}}$)	-95 ± 10	1.6
$[\text{H-}\alpha\text{-l-}\alpha]_2$, ($\text{H}_{\text{ox}}/\text{H}_{\text{red}}$)	-105 ± 5 (0.68)	1
	-220 ± 10 (0.32)	1
$[\text{H:Fl-}\alpha\text{-l-}\alpha]_2$, ($\text{H}_{\text{ox}}/\text{H}_{\text{red}}$)	-153 ± 10	1
$[\text{OMH-}\alpha\text{-l-}\alpha]_2$, ($\text{OMH}_{\text{ox}}/\text{OMH}_{\text{red}}$)	$+14 \pm 5$ (0.37)	1
	-63 ± 5 (0.63)	1
$[\text{OMH:Fl-}\alpha\text{-l-}\alpha]_2$, ($\text{OMH}_{\text{ox}}/\text{OMH}_{\text{red}}$)	-30 ± 10	1

The numbers in parentheses refer to the relative amplitude of the two $n = 1$ couples contributing to the redox reaction.

*These values were obtained from both equilibrium titration and the xanthine/xanthine oxidase system as described in *Materials and Methods*. In both cases, the values determined were the same within experimental error.

midpoint potential for both hemes. The reason that $[H:Fl-\alpha-l-\alpha]_2$ adopts an *anti* and not a *syn* topology as in $[H:\alpha-l-\alpha]_2$ is probably due to the presence of the flavin sterically driving the assembly equilibrium to this conformation, as observed for a related maquette with incorporated spin label probes (2).

The midpoint potentials of OMH in $[OMH:\alpha-l-l-\alpha]_2$ and $[OMH:Fl-\alpha-l-\alpha]_2$ are ≈ 120 mV more positive than that of the analogous maquettes containing H, due to the electron withdrawing substituents on the OMH porphyrin macrocycle stabilizing the reduced state. The patterns of the redox behavior for OMH and H when bound to $[\alpha-l-\alpha]_2$ or $[Fl-\alpha-l-\alpha]_2$ were identical (Fig. 3B), implying that the adopted maquette topologies are influenced by the same factors.

Light-Activated Electron Transfer from Flavin to Heme. Favorable intramolecular electron transfer from Fl_{sq} to heme requires a Fl_{ox}/Fl_{sq} couple E_{m8} more negative than the heme E_{m8} . In solution, Fl_{ox}/Fl_{sq} is ≈ 30 mV lower than the Fl_{ox}/Fl_{red} midpoint. Thus, if Fl_{sq} is not stabilized by the protein relative to solution, electron transfer from Fl_{sq} to H_{ox} would be unfavorable by ≈ 30 mV. However, electron transfer from Fl_{sq} to OMH_{ox} would be favorable by ≈ 100 mV. Only if Fl_{sq} is stabilized by the protein would Fl_{sq} to H_{ox} electron transfer become favorable.

Fig. 4 demonstrates that under continuous illumination, $[H:Fl-\alpha-l-\alpha]_2$ and $[OMH:Fl-\alpha-l-\alpha]_2$ can support photoreduction. The inset to Fig. 4 shows that reduction of OMH is essentially complete after 5 min of irradiation, whereas H reduction takes ≈ 60 min. Also apparent is a significant 5-min lag phase before the onset of H reduction in $[H:Fl-\alpha-l-\alpha]_2$, presumably caused by a requirement for accumulation of reduced flavin before initiation of thermodynamically unfavorable heme reduction. No such lag phase is evident for $[OMH:Fl-\alpha-l-\alpha]_2$. This demonstrates that the difference in free energy, ΔG° , for intramolecular electron transfer in both systems affects the rate of accumulation of reduced heme. Both H and OMH reduction occur slowly because the reaction is light-limited and the quantum yield of flavin fluorescence in the *holo* maquettes is low ($\Phi_f \approx 0.01$). When the light was switched off, the sample remained fully reduced, illustrating that the reduced state of the system is stable under anaerobic conditions. On aeration of the samples, reoxidation rapidly occurred. In control experiments (not shown), where $[H:\alpha-l-\alpha]_2$ and $[OMH:\alpha-l-\alpha]_2$ (unflavinated) were irradiated with light, no photoreduction occurred, illustrating the absolute

FIG. 4. Photoreduction of the flavo-heme maquettes on continuous light illumination. UV-visible reduced minus oxidized difference spectra of $[H:Fl-\alpha-l-\alpha]_2$ and $[OMH:Fl-\alpha-l-\alpha]_2$, illustrating the generation of fully reduced maquettes. The sample concentrations were $10 \mu M$. (Inset) Time course of the reduction process (●) $[H:Fl-\alpha-l-\alpha]_2$ reduction and (○) $[OMH:Fl-\alpha-l-\alpha]_2$ reduction.

FIG. 5. Laser-induced rapid accumulation of reduced heme. (A) Laser flash photolysis absorbance difference spectrum of $10 \mu M$ $[OMH:Fl-\alpha-l-\alpha]_2$ with the diode array detection gated $10 \mu s$ after the laser flash. The spectrum is the mean of 500 acquisitions. Superimposed on this is the steady-state acquired reduced minus oxidized difference spectrum of the same sample, to illustrate that the absorbance difference is due to OMH reduction. (B) Time course for OMH reduction, obtained from a series of transient absorption spectra gated at set intervals after the laser flash (0.03 -, 0.05 -, 0.07 -, 0.1 -, and 1.0 - μs delay). The absorption difference at 430 – 460 nm was used to monitor this process (arrows in A). The data are fit to a single exponential, with the indicated time constant (τ_c) for reduction.

requirement of flavin for mediating heme reduction in this system.

The microscopic time course for accumulation of reduced species in $[OMH:Fl-\alpha-l-\alpha]_2$ was probed by pulsed laser excitation, with subsequent detection of absorbance difference transients at set time intervals after the laser flash. Fig. 5A shows the difference spectrum for $[OMH:Fl-\alpha-l-\alpha]_2$ obtained $10 \mu s$ after the laser flash. The spectrum is consistent with the reduced minus oxidized difference spectrum shown in Fig. 4 for $[OMH:Fl-\alpha-l-\alpha]_2$, implying that reduced OMH is generated rapidly in the maquette. It was not possible to observe H reduction in $[H:Fl-\alpha-l-\alpha]_2$ for the laser-activated experiments, consistent with the findings from the continuous light illumination experiments that the ΔG° for Fl_{sq} to H_{ox} electron transfer is unfavorable. Fig. 5B depicts the time course for the OMH reduction process, where the data points were obtained for a series of transient absorption spectra gated at set time intervals after the laser flash (spectra not shown). The rate of accumulation of reduced OMH is well described by a single exponential process, with a time constant (τ_c) of ≈ 100 ns. Whether τ_c actually reflects the intramolecular electron transfer from Fl_{sq} to OMH_{ox} , depends on a number of factors. The scheme below illustrates the photochemical processes and electron transfer events that can occur on light irradiation in