A remarkable charge transfer (CT) band is described in the bifurcating electron transfer flavoprotein (Bf-ETF) from *Rhodopseudomonas palustris* (*Rpa*ETF). *Rpa*ETF contains two FADs that play contrasting roles in electron bifurcation. The Bf-FAD accepts electrons pairwise from NADH, directs one to a lower-reduction midpoint potential (E') carrier, and the other to the higher-E' electron transfer FAD (ET-FAD). Previous work noted that a CT band at 726 nm formed when ET-FAD was reduced and Bf-FAD was oxidized, suggesting that both flavins participate. However, existing crystal structures place them too far apart to interact directly. We present biochemical experiments addressing this conundrum and elucidating the nature of this CT species. We observed that *Rpa*ETF missing either FAD lacked the 726 nm band. Site-directed mutagenesis near either FAD produced altered yields of the CT species, supporting involvement of both flavins. The residue substitutions did not alter the absorption maximum of the signal, ruling out contributions from residue orbitals. Instead, we propose that the residue identities modulate the population of a protein conformation that brings the ET-flavin and Bf-flavin into direct contact, explaining the 726 nm band based on a CT complex of reduced ET-FAD and oxidized Bf-FAD. This is corroborated by persistence of the 726 nm species during gentle protein denaturation and simple density functional theory calculations of flavin dimers. Although such a CT complex has been demonstrated for free flavins, this is the first observation of such, to our knowledge, in an enzyme. Thus, Bf-ETFs may optimize electron transfer efficiency by enabling direct flavin-flavin contact.

Electron transfer is central to energy metabolism in all domains of life. Of the redox cofactors used, FMN and FAD are among the most versatile (1, 2). The flavin moiety is inherently poised between one-electron and two-electron redox activities by the tunable stability of the flavin’s semiquinone states (3), and the flavin can couple electron transfer to proton transfer (4). At one extreme, dehydrogenases transfer pairs of electrons as hydride between NAD(P)H and closed-shell metabolites (5, 6). The striking example of 2-naphthyl-CoA reductase transfers a strongly reducing hydride (7). In contrast, other flavoproteins carry single electrons between client enzymes, alternating between the flavin’s oxidized state (OX) and its anionic semiquinone (ASQ) in the case of canonical ETFs (8) or between neutral semiquinone (NSQ) and anionic hydroquinone (AHQ) in the case of flavodoxin’s low-reduction midpoint potential (low-E’) couple describing acquisition of an electron (9). The environments produced by the protein are thus crucial in shaping the redox reactivity displayed by the enzyme (9, 10) as well as the flavin’s spectral signatures (11–13).

In bifurcating ETFs (Bf-ETFs) the ET-FAD is analogous to the single FAD of canonical ETFs (14) and undergoes reduction by one-electron (1e⁻) steps from OX to ASQ to fully reduced AHQ (15). The Bf-FAD unique to Bf-ETFs replaces a structural AMP present in canonical ETFs (14, 16). The ET-FAD is bound in the head domain, whereas the Bf-FAD is bound in the base with its flavin in the interface between the S (small) and L (large) subunits (Fig. 1) (14, 16). The presence of two FADs with heavily overlapped optical signals complicates studies of their individual contributions (17–19). However, a combination of biochemical, spectroscopic, thermodynamic, and computational tests established the above roles and reactivities of the individual flavins (16), confirming homology-based models (14) and elegant demonstration of the direction of electron transfer (18).

In contrast to the ET-FAD, the Bf-FAD’s semiquinone state is thermodynamically unstable, enabling this flavin to reduce carriers with E’ values below the flavin two-electron (2e⁻) E’, so long as an oxidant able to “pay for” generation of the transient semiquinone is present (20, 21). This latter role is played by the ET-FAD. Thus, the Bf-FAD accepts pairs of electrons from NADH (E°NADH = −320 mV) and sends one electron and most of the energy to a low-E’ ferredoxin or the NSQ/HQ couple of flavodoxin (E° ≤ −400 mV (22–24)), whereas transfer of the other electron to a high-E’ acceptor (25, 26) renders the overall reaction favorable (21, 26–29). Thus, the system trades quantity for quality, producing low-E’ electrons able to drive energetically demanding reactions such as nitrogen fixation, although NADH cannot (27, 30).

The presence of two FADs may also be responsible for another feature present in *Rpa*ETF but not canonical ETFs. An absorbance band at 726 nm grows as the ET-FAD is reduced but diminishes as the Bf-FAD is reduced, suggesting that it originates from interaction between reduced ET-FAD and oxidized Bf-FAD or between the two FADs in their semiquinone states (19). Signals in this spectral region commonly arise from charge transfer (CT) species involving a flavin and a substrate or product (31–38). However, formation of the 726 nm band was shown to be independent of the identity of the electron donor, making it intrinsic to the ETF (19). The possibility of a flavin triplet seems unlikely, given the lifetime of this signal (39).

Herein we report progress in elucidating the origin and nature of this intriguing species, focusing on the two FADs and...
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Figure 1. Structural models of RpaETF showing the D conformation (A), the B-like conformation (B) and details of the placement of the two flavins relative to one-another in the B-like conformation, with selected nearby amino acid side chains (C). The large (L) subunit, also known as the ETFα or FixB subunit, is shown in cyan, and the small (S) subunit, also known as the ETFβ or FixA subunit, is shown in green. Bf-FAD and ET-FAD are shown in orange and blue, respectively. Conserved residues Tyr-37.S and Cys-174.L are shown in magenta and red, respectively. The electron-donating conformation (D conformation) (45) structure is modeled based on the structure of Acetobacterium woodii caffeoyl-CoA reductase–associated BF-ETF (AwoCarCDE: PDB entry 6FAH (44)), whereas the bifurcating-like (B-like) conformation structure is modeled based on the Acidaminococcus fermentans BF-ETF (AFeETF: PDB entry 4KPU (14)). The large red arrow in A depicts the rotation of the head domain to form the B-like conformation in B, whereas the small red arrow in B shows further head domain rotation required to form the bona fide B conformation in which we propose that the two flavins are brought into van der Waals contact. In panel C the enlarged view of the region circled in panel B provides closest-approach distances between the residues discussed in the text, based on the structural model.

their binding sites (Fig. 1). We found that each of the two FADs is required for formation of the 726 nm band. Similarly, mutations affecting either binding site were found to affect the amplitude with which the 726 nm band is formed. Consistent with studies on free FMN in solution (32, 40), we also present evidence that the proposed complex of two FADs responsible for the 726 nm band (henceforth referred to as the “726 nm species”) possesses inherent stability, as it survives even as protection provided by the protein structure is weakened.

Given the well-documented >80° rotation of the ETF head domain (41–45) and its effect of moving the surface-exposed ET-FAD relative to the Bf-FAD, we suggest that the 726 nm band reflects further rotation of the head domain that places the ET-flavin in contact with the BF-flavin. We note the possible significance to electron transfer of direct flavin-flavin contact, especially in the context of the gating role that has been proposed for head domain rotation (14, 45).

Results
Formation and reversibility
When WT RpaETF was reduced stepwise using dithionite or the xanthine and xanthine oxidase system (46) the 726 nm band grew to maximum amplitude halfway through the titration and then decreased again (Fig. 2A). Qualitatively similar behavior was observed with NADH as the reductant, although maximization and decay of the signal occurred at somewhat higher NADH concentrations, suggesting that stoichiometric additions of NADH do not fall in the tight-binding regime (see below). However, the 726 nm species itself does not appear to be a transient intermediate as it was stable over days under inert atmosphere. The band is seen to extend to long wavelengths as for other flavin CT bands, but it displays apparent structure (Fig. 2B). Whereas vibrational structure is evident in absorption bands of individual flavins, it is generally not observable on the broader supramolecular CT transitions (47). We speculate that the protein environment may suppress movement of one partner relative to the other that might otherwise be associated with the transition.

The 726 nm band could also be formed in oxidative titrations (Fig. 2C). When NADH was used to oxidize RpaETF that had been fully reduced by prior treatment with dithionite, the CT band at 726 nm grew initially, concurrent with the appearance of spectral features of OX flavin (Fig. 2C). Upon the addition of excess NADH, it was even possible to decrease the amount of 726 nm species present as more flavin was converted to OX (below). Therefore, the 726 nm species represents a state function rather than an intermediate of a particular path.

Sensitivity to pH
The 726 nm band appeared at 726 ± 2 nm at pH 8 and pH 9; however, the amplitude of the 726 nm band was 4.5-fold larger at pH 9, in titrations with NADH. Thus, it does not appear to reflect the conjugate acid of an acid/base equilibrium, and we rule out the NSQ state of a flavin, although that of 8-formyl flavin can absorb in this range (48) (but see Ref. 49). Thus, although the use of pH 9 incurs the risk of increased 8-formyl flavin formation (48), we used pH 9 to optimize our ability to detect and quantify the 726 nm signal to elucidate the nature of the species responsible. Other work under way is testing a possible relationship between formation of the 726 nm species and accumulation of modified flavin, because the latter is also favored by higher pH and correlated with a particular conformation of the ETF (48) (Fig. S1). The higher 726 nm species yield at high pH suggests that the 726 nm species is favored by deprotonation of an amino acid side chain nearby or equivalently that another state in equilibrium with the 726 nm species...
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is disfavored by the amino acid’s deprotonation. To identify candidate amino acids, one must know where the 726 nm species resides.

Absence of either ET-FAD or Bf-FAD abolishes the 726 nm band

In our previous studies, we showed that the 726 nm species correlates with the oxidation states of both ET-FAD and Bf-FAD (19). We therefore proposed a delocalized CT involving both flavins (50). Consistent with this, a RpaETF variant retaining the ET-FAD but lacking Bf-FAD was found to not form the 726 nm band (16), even at pH 9. In that variant, the ET-FAD retained WT-like behavior, including the same succession of two 1e− reductions via an ASQ intermediate, as well as E° values and spectra similar to those of WT RpaETF. Because the reactivity of the ET-FAD was not greatly altered, perturbation of the ET-flavin could not explain the absence of the 726 nm band from this variant. Instead, we propose that loss of the 726 nm band was due to the absence of Bf-FAD.

To test for a similar requirement for ET-FAD, we exploited the facts that 1) the ET-FAD binds with a lower affinity than does the Bf-FAD of Bf-ETFs and 2) binding is predominantly via the ADP moiety rather than the FMN component of FAD (14, 17, 51, 52). Therefore, we used ADP to displace RpaETF’s ET-FAD, to produce RpaETF containing only the Bf-flavin.

RpaETF was titrated with concentrated ADP solution while the absorbance was monitored at 400 nm (17), (Fig. 3A). Once the shape of the absorption spectrum ceased to change, we inferred that the remaining FAD was more resistant to displacement and therefore mostly in the Bf site. This RpaETF retained ET-FAD in 24% of sites based on the amplitude of ASQ that could be formed. Upon titration with dithionite, ET-
FAD–depleted \textit{RpaETF} formed much less of the 726 nm species than did \textit{WT RpaETF} \textbf{(compare Fig. 3B with Fig. 2A)}. The small amount produced was attributable to the fraction of ET sites retaining FAD even after treatment.

Interestingly, the resting state enzyme prior to treatment with ADP contained a discernable amount of the 726 nm species \textbf{(Fig. 3A, red curve)}. This seemed more resistant to ADP competition than the population with both flavins OX, possibly reflecting a conformational difference. However, this residual 726 nm signal decreased as the sample was reduced, and additional 726 nm intensity was not formed from the OX FAD present, in contrast with the untreated case \textbf{(Fig. 2A)}. Thus, the absence of ET-FAD prevents formation of the 726 nm band.

At the end point of reduction \textbf{(in excess dithionite)} ET-FAD–depleted \textit{RpaETF} displayed a species resistant to further reduction \textbf{(magenta spectrum in Fig. 3B)}. Because free FAD has a higher reduction midpoint potential than that of Bf-FAD \textbf{(19)}, we expect it to be fully reduced and propose instead that the re-calctrat species corresponds to bound FAD. To test the possibility that a two-electron donor is required, we employed NADH under the same conditions, with the same result.

In case ET-FAD is involved in forming the 726 nm species, but is not a component of the species, we first generated a maximal amount of 726 nm species via titration with dithionite and then displaced ET-FAD using ADP \textbf{(Fig. 4A)}. Most of the FAD that remained bound occupied the Bf site, based on the previously assigned CD signatures of Bf-FAD and ET-FAD \textbf{(16, 19)} \textbf{(Fig. 4B)}, and deconvolution of the observed CD spectrum indicated that only 26% of ET sites retained FAD.

Displacement of ET-FAD from half-reduced \textit{RpaETF} was accompanied by drastic diminution of the 726 nm band to only 26% of its previous intensity, demonstrating a requirement for the ET-FAD even for the retention of preformed 726 nm band \textbf{(Fig. 4A)}. The surviving small population retaining the 726 nm band underwent 2e\textsuperscript{−} reduction to HQ when additional dithionite was added, confirming the presence of Bf-FAD \textbf{(Fig. 4C)}. Increased signal from OX as well as HQ FAD was observed as ADP was added \textbf{(456 and 320 nm; Fig. 4A).} Thus, we propose that disruption of the 726 nm species and attendant supramolecular orbitals restores population and transition moment amplitude to the electronic transitions that characterize monomeric flavins. Indeed, this replicates in reverse the diminution of band intensity between 300 and 500 nm observed in conjunction with CT band formation when old yellow enzyme was titrated with p-chlorophenol \textbf{(31)}, strengthening our assignment of the 726 nm band to a CT complex.

Thus, regardless of whether ET-FAD is displaced before or after formation of the 726 nm species, its displacement eliminates the 726 nm species. We conclude that the ET-flavin and the Bf-flavin are both indispensable for the 726 nm species.

\textbf{Identities of the flavin oxidation states}

Difference spectra were calculated to reveal spectral changes associated with formation and then disappearance of the 726 nm band in a dithionite titration of \textit{WT RpaETF} \textbf{(Fig. 5)}. In difference spectra depicting spectral changes \textit{versus} the starting spectrum of \textit{RpaETF} containing OX ET-FAD and OX Bf-FAD (\textit{ETOX/BfOX}), maximal 726 nm amplitude correlated less well with initial conversion of OX flavin to ASQ \textbf{(gained amplitude near 374 nm with little net change near 456 nm; Fig. 5, B and C)} but correlated well with subsequent consumption of ASQ to form HQ \textbf{(lost amplitude near 374 nm, and near 510 nm which is almost isosbestic for conversion of OX to ASQ but loses intensity as ASQ is converted to HQ; Fig. 5C)}. Difference spectra for the second half of the titration, in which the 726 nm species undergoes reduction, showed loss of that species to be accompanied by reduction of an OX flavin to HQ \textbf{(456 and 374 nm, the Bf-FAD; Fig. 5D)}. Thus, optical spectra indicate that ET-

\textbf{Figure 4. Displacement of FAD by ADP in the half-reduced state WT \textit{RpaETF}.} \textbf{A}, UV-visible spectra of half-reduced WT \textit{RpaETF} (68.7 \textmu M, \textit{black trace}) and effect of titration to 0, 1.59, 3.19, 4.78, 6.37, 7.97, 9.56, 11.16, 12.75, 14.34, 15.94, 23.91, 39.84, 63.75, 95.62, and 159.37 m\textsuperscript{M} ADP \textbf{(black to pink)}. \textbf{B}, identification of bound FAD by CD after FAD displacement in half-reduced protein \textbf{(black trace)}. For comparison, the CD spectrum of Bf-FAD seen in half-reduced \textit{RpaETF} is shown \textbf{(red trace)} along with that of ET-FAD \textbf{(blue trace)} obtained by subtraction of the Bf-FAD CD spectrum from the native protein CD spectrum made up of both Bf-FAD and ET-FAD. \textbf{C}, resumed reductive titration of the half-reduced WT \textit{RpaETF} at pH 9.0. All samples were in 20 mM bis-Tris propane, pH 9.0, 200 mM KCl, 10% (w/v) glycerol.
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Figure 5. Flavin oxidation state changes associated with formation of the 726 nm species. A. reductive titration of WT RpaETF (68.7 μM) by 3.42 mM sodium dithionite at pH 9.0 showing fully oxidized state (red trace), ASQ maximum (dashed black trace), half-reduced state (solid black trace), and fully reduced state (purple trace). B, amplitudes of the 726 nm species, sum of ASQ and OX (as amplitude at 374 nm), and OX species (as amplitude at 456 nm) versus electron equivalents provided, based on A. C, difference spectra (observed spectrum – fully oxidized state spectrum) showing spectral changes that accompany formation of the 726 nm species. Only spectra collected in the course of 726 nm species formation are included. The difference spectrum with the maximum amount of 726 nm species is shown in black. Formation of the 726 nm species is associated with loss of ASQ based on negative absorbance changes at 520 nm. D, difference spectra (observed spectrum – fully oxidized state spectrum) showing spectral changes that accompany consumption of the 726 nm species. The difference spectrum resulting from the final spectrum is shown in black. Loss of 726 nm species is associated with loss of an OX flavin based on changes at 460 and 370 nm.

FADHQ and Bf-FADOX are present when the 726 nm band has maximum amplitude in WT RpaETF. We therefore propose that the 726 nm species could be a flavinOX-flavinHQ CT complex, specifically ETHQ/BfOX.

At maximum, ~65% of sites formed the 726 nm species, based on a plot of its amplitude versus absorbance at 378 nm (Fig. S2). This value is similar to the prediction of 80% obtained based on the E° values associated with the redox equilibria by which the ETHQ/BfOX state is formed and consumed.

Replacements of Tyr-37.S: Delocalized charge transfer between ET-FAD and Bf-FAD or conformational effect?

Situated about 4 Å away from the ET-FAD in the B-like conformation, and 15 Å from the Bf-FAD (14), the conserved Tyr at position 37 is an attractive candidate to facilitate electron hopping between the flavins (53, 54) or delocalized CT (50), because the flavins are 18 Å apart in the B-like conformation of Bf-ETF has been captured crystallographically (Fig. 1B) (45). Because long-range electron transfer has been observed via Tyr and Trp but is considered negligible via Phe (53), we tested for a requirement for Tyr or Trp at position 37 for formation of the 726 nm species.

The Y37F.S variant formed little if any of the 726 nm band during reduction by NADH (Fig. 6A). Instead, we observed a broad and weak CT band extending from 620 nm to beyond 800 nm (Fig. 6A). At least two such bands were detected in the Bf-ETF from Pyrobaculum aerophilum upon reduction using NADH or upon the addition of NAD⁺ to reduced material (15). Because NADH and presumably NAD⁺ bind at the Bf-FAD site (14, 16, 18), these broad CT bands likely reflect Bf-FADHQ-NAD⁺ complexes in more than one conformation (15). Indeed, no such band was formed when the reductant was dithionite (Fig. S3). The finding that Y37F.S RpaETF forms hardly any 726 nm species also argues against the signal being due to an impurity co-purifying or bound to the ETF, as it is all but eliminated by this one amino acid substitution.

Y37W.S RpaETF accumulated a low yield of 726 nm band that can be largely explained by low FAD content (see Fig. 6B and figure legend). After accounting for a flavin stoichiometry of only 1.2 per ETF dimer (Table S1), the 726 nm band was not stronger than that of WT, although the side-chain E° of Trp is considered to be equally or better able to mediate hole hopping as compared with Tyr (53). This argues against efficient hole hopping via residue 37. If the side chain of Tyr-37.S participates in long-range CT, the band might also be expected to occur at a different wavelength in the Y37W variant (e.g. see Ref. 55). Instead, Y37W.S RpaETF yielded a signal at 726 ± 2 nm. Therefore, the diminished intensity likely stems from other causes. The impaired FAD binding to the Y37W.S variant suggests that the added bulk of the Trp side chain perturbs the flavin-binding site(s) and could similarly affect the stability of the conformation corresponding to the proposed 726 nm species.

Y37F.S RpaETF’s lack of 726 nm band can be understood on the same basis. In contrast to Y37W.S, Y37F.S was notably more stable than WT, in the ETOX/BfOX state. Thus, the effects of amino acid substitution at position 37 appear to extend beyond the ET-FADHQ/Bf-FADOX state in which CT could occur, and the absence of the 726 nm band from Y37F.S could
result of conformational effects. We favor the interpretation that altered bulk at position 37 modulates the stability of conformations that compete against the one responsible for the 726 nm band, thus changing its population without changing its nature.

**Substitution of Cys-174.L makes the 726 nm complex resistant to reaction with NADH/NAD^+**

The Bf site is devoid of aromatic amino acids, but electron-rich Cys and Met are in principle also able to act as CT donors to oxidized flavins (53, 56), and Cys-174.L is situated adjacent to the flavin C6 of Bf-flavin (Fig. 1). It is conserved among Bf-ETFs from diazotrophic bacteria, such as *R. palustris*, *Azotobacter vinelandii*, and *Rhodospirillum rubrum* (57), but is replaced by Ala in most nondiazotrophic Bf-ETFs, implying that it is not essential for electron bifurcation but could play a role related to diazotrophy.

The greater strength of the 726 nm band at pH 9 than at pH 8 raised the possibility that ionization of Cys-174.L is involved in the 726 nm band. We tested this by replacing Cys-174.L with alanine. C174A.L RpaETF retained full flavin occupancy comparable with WT (Table S1) and likewise produced maximal amplitude of the 726 nm band when the protein was half-reduced by dithionite, but with nearly 3-fold lower maximum yield (Fig. 7A versus Fig. 2A).

The lower yield of 726 nm complex in C174A.L could result from elevation of the Bf-FAD’s $E^\text{°}_{\text{OX/HQ}}$ because more favorable reduction of the Bf-flavin would diminish the extent to which Bf-FADOX co-exists with ET-FADHQ. Redox titrations revealed that the $E^\text{°}_{\text{OX/HQ}}$ of the Bf-FAD is $-216 \pm 5$ mV ($n = 3$) (versus $-223$ mV for WT; both calculated based on values measured at pH 9 (19); Fig. S4). This small of an effect is surprising, given that at pH 9, Cys-174.L is predicted to be anionic, and flavin reduction is predicted to yield AHQ, so we would anticipate relief from an electrostatic impediment to reduction, in C174A.L. Further tests are required to determine whether the Bf-FAD (together with Cys-174.L) takes up two protons upon 2e⁻ reduction, making the reduction electrostatically neutral. However, we note that the 726 nm band was stronger at pH 9 than at pH 8 even for C174A.L RpaETF, demonstrating that ionization of Cys-174.L is not the basis for the pH effect.

Although less likely to respond to replacement of Cys-174.L, the $E^\text{°}_{\text{ASQ/HQ}}$ of the ET-FAD could also affect yield of the 726 nm species. The results of two independent titrations yielded $E^\text{°} = -90$ mV for the ASQ/HQ couple of the ET-flavin in C174A.L versus a value of $-83$ mV for WT (19) (Fig. S5). The two small shifts in $E^\text{°}$ conspire to predict a 7% decrease in yield of the 726 nm species in C174A.L, assuming that the 726 nm species involves the HQ state of ET-flavin and the OX state of Bf-flavin (Fig. S6B). This fails to account for the 3-fold diminution observed, indicating that factors additional to the flavin $E^\text{°}$ values contribute to the lower 726 nm species yield in C174A.L versus WT RpaETF.

When the physiological electron donor NADH was employed to reduce C174A.L RpaETF, the 726 nm band did not diminish toward the end of the titration in the usual NADH
concentration range (Fig. 7 B versus A) and Fig. 6C). C174A.
L’s recalcitrance could reflect lower reactivity with NADH, as the C174A.L RpaETF also accumulated less ASQ early in the tita-
nation, similar to what has also been observed among canonical ETFs (58). When electrons were supplied using dithionite or xanthine/xanthine oxidase via a mediator, more ASQ formed, and the 726 nm band diminished as the system became fully reduced (Fig. S4). Thus, C174A.L RpaETF’s impeded reaction with NADH appears to be particular to this reductant.

To test whether C174A.L’s 726 nm species was resistant to reduction in particular, we tested its formation and decay in oxidative titrations. Fig. 8 (A and B) shows that the 726 nm species has the status of an intermediate that formed and then declined in the course of oxidative titrations of WT RpaETF with NAD$^+$, as in reductive titrations. C174A.L that had been fully reduced by dithionite also formed the 726 nm species upon oxidation with NAD$^+$ (Fig. 8C). As for WT, the signature of OX flavin was seen to grow in tandem with that of the 726 nm species, consistent with participation of Bf-FAD$_{OX}$. However, a much higher concentration of NAD$^+$ was required to form the 726 nm species starting from fully reduced C174A.L than for WT (Fig. 8D, note different horizontal axes). Similarly, prominent accumulation of NADH was observed in the presence of reduced C174A.L (Fig. 8C, near 340 nm). This could reflect excess dithionite present as required to fully reduce the C174A.L RpaETF to start with and then ETF-catalyzed reduction of added NAD$^+$ by the excess dithionite. Given that both reduction of C174A.L RpaETF by NADH and its oxidation by NAD$^+$ required higher concentrations of the nicotinamide, and given that the flavin $E^*$ in effect was not very different from that of WT, we conclude that C174A.L RpaETF has a diminished affinity for NADH/NAD$^+$.

As the physiological substrate, NADH may be more than simply an electron donor, as its binding, or release of NAD$^+$, could be coupled to reorientation of ETF’s head domain (14). Demmer et al. (45) found that the NADH-binding site is obstructed in the D conformation of Clostridium difficile ETF (45). Thus, it may be that substitution of Ala for Cys-174.L favors the D conformation especially when ET-FAD is reduced. If so, the lowered maximal yields of 726 nm species in C174A.L suggest that the 726 nm species corresponds to a conformation other than D. Regardless, the finding that the 726 nm species of C174A.L RpaETF resists further reduction with NADH provides a convenient means of accumulating it for detailed study.

**The 726 nm complex persists even as protein tertiary structure dissolves**

Studies of FMN in aqueous solution document formation of change-transfer dimers of FMNH$_{HQ}$-FMNO$_{OX}$ and demonstrate that the association between an FMNH$_{HQ}$ and an FMNO$_{OX}$ is inherently favorable (40) (Fig. S7). Therefore, if the 726 nm complex corresponds to a flavin$_{HQ}$-flavin$_{OX}$ association, it should persist (temporarily) if the protein structure around it is compromised.

To dissolve protein structure without forming precipitate, we adopted the method of Gross et al. (59) wherein cetyltrimethylammonium bromide (CTAB, a cationic surfactant) presumably loosens the protein tertiary structure but does not produce protein precipitation. Indeed, even after heating to 100°C, RpaETF displayed no aggregation in the presence of 10 mM CTAB. Near-UV CD reflecting Trp side chains in anisotropic ordered environments (60) decayed with a half-time of only 3 min upon the addition of CTAB (Fig. 9B). However, absorbance at 726 nm diminished with a half-time of ~37 min (Fig. 9A).

Even before the addition of CTAB, the flavin fluorescence of ET$_{HQ}$/B$_{OX}$ RpaETF was already greatly quenched compared with that of fully oxidized (ET$_{OX}$/B$_{OX}$) RpaETF, consistent with CT character in the 726 nm state (61, 62) (black versus gray curves in Fig. 9C). However, in the course of a 1-h incubation in CTAB, flavin fluorescence intensity dropped further, suggesting increased quenching, presumably by the attached adenine upon FAD dissociation (63, 64). Because heating in air produced little additional effect, it appears that the effect was almost complete after 1 h in CTAB.

Trp fluorescence was almost fully unmasked by a 1-h treatment with CTAB (Fig. 9D). For the flavins as well as the Trps, fluorescence emission maxima shifted to longer wavelengths consistent with greater exposure to polar solvent (65). Thus, global signatures of protein tertiary structure indicated that the core of RpaETF was no longer packed in a unique long-lived structure but became less ordered and extensively hydrated in the course of a 1-h incubation with CTAB. (Also see Fig. S8.) Despite the almost complete loss of protein structural integrity, the 726 nm band retained almost half of its amplitude over the same time interval. The 726 nm species nonetheless represents...
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**Discussion**

We report a work in progress, as this story appears to involve additional variables such as pH, possible correlation with flavin modification, and factors that may modulate the orientation of
the head domain relative to the base. However, we present evidence that the 726 nm band involves participation by both of the flavins of Rpa ETF, apparently as ET-FADHQ and BF-FADOX. The Y37F.S, Y37W.S, and C174A.L substitutions that affect 726 nm yield can each act via multiple mechanisms, but a unifying hypothesis is that they alter the relative stability of an orientation of the head domain that places the two flavins in van der Waals contact with one another. The resolved features of the 726 nm band (compared with conventional CT bands) suggest that the proposed flavinHQ to flavinOX transition is associated with very little reorganization within the complex (69) (see, for example, Ref. 70). This is consistent with the proposed 726 nm CT complex being coupled to the much larger inertias of protein domains.

Figure 9. Persistence of the 726 nm species in Rpa ETF treated with CTAB. A, time course of anaerobic treatment of WT RpaETF (9.96 μM) bearing the 726 nm species (black trace) in 10 mM CTAB, showing spectra after 5 min (red), 10 min (orange), 20 min (lime), 30 min (green), 40 min (cyan), 50 min (blue), 60 min (purple), and then final treatment with heat aerobically (magenta). The inset shows amplitude of the 726 nm band as a function of CTAB treatment time. Amplitudes were fit to a single-exponential decay in time. A shoulder around 520 nm persisted in the absorbance spectrum collected after heat denaturation, and the short-wavelength maximum was shifted to 360 nm versus 375 nm for authentic FAD consistent with possible modification of some of the flavin (48, 113). B, near-UV CD spectra of the same sample as described in A with the inset showing time course of tertiary structural change based on molar ellipticity at 280 nm. Negative amplitudes were fit to a single-exponential decay in time. C, fluorescence emission spectra of FAD in the absence of the 726 nm species (ETOX/BFOX gray), bearing the 726 nm species before treatment with CTAB (black, presumed ETHQ/BFOX) versus after 60-min treatment with CTAB (purple) and after final aerobic heat treatment (magenta). Excitation was at 466 nm. D, fluorescence emission spectra of tryptophan excited at 295 nm for Rpa ETF bearing the 726 nm species before treatment with CTAB (black), after a 60-min treatment with CTAB (purple) and after further heat treatment (magenta).

Figure 10. Computed spectra of HQ lumiflavin (LF) (light blue), OX LF (green), sum of HQ LF and OX LF (teal), and LFHQ·LFOX dimer (dark blue) compared with the experimental spectrum of FMN in water (dashed black line). The left vertical axis applies to the experimental spectrum of FMN, and the right vertical axis applies to the computed spectra of lumiflavin models. The ball-and-stick structure shows the geometry-optimized structure of the LFHQ·LFOX dimer, where LFHQ is in light blue and LFOX is in green. The starting structure was based on the stacked flavin dimer of T. thermophilus dodecin; geometry optimization and TD-DFT calculations employed the cam-B3LYP functional with the cc-pVDZ basis (see “Experimental procedures”). The electron density displacement map corresponding to the long-wavelength transition exclusive to the LFHQ·LFOX dimer was generated with an isovalue of 0.0004 electron density lost (green) and gained (blue) and is displayed on the same structure. The clear net transfer of electron density classifies the transition as a CT transition. Computed absorption maxima incorporate a 0.55-eV red shift to optimize agreement with the 446 nm band of FMN.
A corollary of our proposal of van der Waals contact between the two flavins is that the “B-like” conformation captured crystallographically does not represent the full extent to which the head domain can rotate. The latter has already been proposed by the original authors and was the basis for their “B-like” terminology rather than simply “B”, because they postulated a greater amplitude of rotation in solution, resulting in a flavin-flavin distance of 14 Å rather than the 18 Å captured in the crystal structure (14). Likewise, we note that relatively understanding side-chain and loop movements suffice to allow the head domain orientation to be further rotated, bringing the flavins closer together. Building on the earlier work, we present the first evidence to our knowledge that the two flavins may come in contact.

Our proposal of a CT complex between the flavins themselves is consistent with the lack of significant wavelength shift or linewidth change upon substituting nearby Cys, Tyr, or Trp that are known to be able to engage in CT with flavins (62, 71, 72). CT bands between flavins and stacked aromatic groups generally apply to OX flavin and manifest as absorbance extending to longer wavelengths than the 500 nm cutoff typifying OX flavin alone (73, 74). They are understood to result from orbital overlap producing hybrid orbitals with supramolecular character (36, 75). Participation of Tyr-37.5 in the 726 nm complex is unlikely because Tyr (or Trp) is expected to act as a CT donor to an OX flavin, whereas our titrations in Figure 5 demonstrate that the flavin near Tyr-37.5 adopts the HQ state in the 726 nm species (the ET-flavin; Figure 1). Meanwhile, the Bf-flavin is not mobile relative to residue 37 and is 12 Å away from the nearest aromatic side chain based on our model and available structures (14). Cys is competent to form a CT complex with OX Bf-flavin, especially upon ionization (76, 77). However, Cys-174.L was proven not to be required for 726 nm species formation by our C174A.L variant. These findings corroborate the lack of wavelength change, arguing against inclusion of Cys-174.L or Tyr-37.5 in the CT species. Thus, the simplest proposal consistent with the data in hand at present is that the 726 nm species is comprised of E\textsubscript{H}Q B\textsubscript{OX}.

Although our proposal is novel in the context of ETFs, ample structural precedent for π-stacked flavin dimers is provided by the dodecins. Bacterial dodecin from Thermus thermophilus displays pairs of si-si stacked flavins, whereas re-re stacked pairs are observed in Halobacterium salinarum dodecin (66, 67). Interestingly, dodecins extend the π-stack by contributing Trp side chains that flank the flavin dimer on either side to generate a 4-layer stack. Absorbance extends to wavelengths longer than 500 nm (66, 67) as in the Tyr/flavin/Trp stack of flavodoxin (71, 78) and riboflavin-binding protein (79). In an elegant study wherein Trp analogs varying with respect to electron-donating ability were installed in dodecin in place of the stacked Trp, analogs with lower tendency to donate electron density showed less long-wavelength absorbance, whereas the presence of the better electron donor 4-NH\textsubscript{2}-Trp resulted in a resolved CT band centered near 670 nm, and accelerated electron transfer to photoexcited flavin (55).

Dodecins are believed to prevent photodamage catalyzed by flavins by passivating the flavin’s intrinsic photoreactivity (80). The binding mode allows retention of the OX flavin’s absorbance spectrum but essentially abolishes fluorescence and triplet formation due to very rapid electron transfer from the stacked Trp that outcompetes other events (55, 62, 67). Similarly rapid electron transfer between two flavins would be expected if one were present as the HQ, as we propose here.

Thermodynamic and optical spectroscopic data document binding of flavin dimers in flavodoxin (81), and one of the most celebrated structures in molecular biology, that of B-form DNA, reveals that π-stacking among DNA bases provides the structural basis for the stability of the hydrophobic core of DNA (82). These examples demonstrate the plausibility of stacking among flavins.

The inherent favorability of such an interaction is demonstrated by the fact that riboflavin and FMN dimerize with a K\textsubscript{d} of ~10 mM for OX/OX dimers or HQ/HQ dimers that can be substantially attributed to the hydrophobic effect because it is unique to aqueous solutions (40) and dimerization is suppressed above room temperature (83). This is consistent with model studies and computation indicating that π-stacked OX/OX flavin dimers are stabilized by ~2 kJ/mol (84, 85), reflecting dispersion interaction between the participating π systems (68, 86) and hydrogen bonding (68, 83). OX/HQ π-stacked dimers display a diagnostic shoulder at 485 nm and long-wavelength absorption extending beyond 550 nm (87, 88).

When one of the two flavins is reduced to the HQ state, additional dipolar stabilization produces a K\textsubscript{d} of 0.5 mM (40). Such OX-HQ dimers are presumably at equilibrium with flavin SQs, monomers or dimers (89). However, EPR and UV-visible spectral signatures argue that in half-reduced solutions with flavin concentrations above 2 mM, the dominant species is the OX-HQ dimer (32, 40, 90). This is consistent with the E\textsuperscript{+} values of free flavin that favor disproportionation of SQ, which is populated to a maximum of only 1% in dilute solution at half-reduction (3, 91). The flavin HQ-OX complex CT absorption maximum varies in the range of 770–1100, depending on the pH and presence of additives (40, 89). Considering that Bf-ETFs constrain two flavins to be within 41 Å of one another (D conformation), which constitutes an effective concentration of 6 mM, we should not be surprised if the flavins were to dimerize in competition with other equally favorable interactions.

The natural proclivity of flavins to form dimers could also cause otherwise short-lived or infrequent states to be captured when concentrations are high. π-Stacked flavin dimers have been directly observed in structures resulting from crystals soaked in 23 mM FMN (92), 1 mM FMN (93) or grown from 0.5 mM enzyme in a 0.5 mM solution of FMN (94). In these latter cases, the mechanisms of the enzymes in question allow that the flavin dimers observed may reflect bona fide catalytic intermediates.

The EmoB crystal structure shows a si-si stack of two flavins, with a Tyr side chain below the tightly bound one (93). EmoB is a flavin reductase proposed to accept hydride from NADH in a reductive half-reaction and then pass it to FMN substrate in an oxidative half-reaction that would involve a flavin-to-flavin hydride transfer facilitated by stacking of the two flavins (93). In dihydromethanopterin reductase (DmrB), a weakly bound FMN is resolved stacked against the tightly bound FMN to form a re-re π-stacked dimer. (94). Here too, the enzyme is
proposed to employ tightly bound FMN to reduce a substrate FMN that binds via a transient stacked conformation (94).

In EmoB and DmrB, it appears that both flavins were OX, and long wavelength spectra were not shown in either case. However, given that the tightly bound FMNs are both proposed to alternate between OX and HQ states, and the substrate FMN is proposed to bind as OX when the tightly bound FMN is HQ, we expect that the O-S and E-P intermediates should represent π-stacked OX/HQ, as we propose for RpaETF. Based on our work, we predict that if the oxidative reactions of EmoB or DmrB can be observed at long wavelengths with sufficiently short dead times, they will display transient flavin_HQ-flavin_OX CT bands.

In our case, we do not think that we form a π-stacked dimer in a single one of the two binding sites. The flavin stoichiometry of our preparations is close to 2 per ETF heterodimer, and the flavins have distinct E° values different from those of free flavin, indicating that the two flavins occupy different binding sites. Moreover, amino acid substitutions in either binding site decreased the yield of the 726 nm species, indicating that both protein sites contribute to its population. Thus, we hypothesize that the two sites are allowed come together by head domain movement and that favorable flavin-flavin interaction stabilizes this conformation (40).

Events that modulate the head domain orientation would acquire control over whether the proposed CT transition state could form. Candidates are binding/dissociation of partner proteins (45) or binding/dissociation of NADH/NAD⁺ (14). Whereas such a species may be fleeting in turnover, our use of high pH and the absence of natural partner proteins may enable it to accumulate, for lack of normal electron acceptors or a proton coupled to electron transfer. Thus, our proposed conformationally enabled π-stacked diflavin CT complex could coordinate several requirements for energy-efficient electron bifurcation in the heart of Bf-ETFs.

Experimental procedures

Site-directed mutagenesis, overexpression, and purification of R. palustris ETF and its variants

Generation of a construct for the expression of WT RpaETF was described elsewhere (19). The vectors pMCSG28 and pMCSG21 were acquired from the DNASU Plasmid Repository (Tempe, AZ, USA) and augmented with the genes for Etfs (also known as FixA) and Etfl (also known as FixB), respectively. The plasmids of Etfs variants Y37F.S and Y37W.S and Etfl variant C174A.L were prepared using mutagenic primers (5'-CAATCCTTTGATCTGGCGCCTCG-3' (Y37F.S coding), 5'-ATGATCGTGGACACGCGGTGC-3' (Y37W.S non-coding), 5'-CAATCCTTTGATCTGGCGCCTCG-3' (Y37F.S non-coding), 5'-ATGATCGTGGACACGCGGTGC-3' (Y37W.S non-coding), 5'-GCTGCTGGGCAGATCTCTACACGC-3' (C174A.L coding), and 5'-GAGCCCCGGGAA-GTGGCGACGC-3' (C174A.L non-coding)) according to the Q5® Site-Directed Mutagenesis Kit procedure (New England Biolabs, Ipswich, MA, USA). After DNA sequencing verification (Eurofins Genomics, Louisville, KY, USA), the Escherichia coli strain NiCo21(DE3) (New England Biolabs) was transformed with both plasmids for expression of desired RpaETF variants along with pGro7 (Takara Bio, Mountain View, CA, USA) for co-expression of molecular chaperones GroES/EL. Cells were grown in 500 ml of Terrific Broth supplemented with 20 mg/liter riboflavin and 2 mM MgSO₄ along with carbenicillin (100 μg/ml), spectinomycin (100 μg/ml), and chloramphenicol (50 μg/ml) at 37°C, shaking at 250 rpm, to an A₆₀₀ of ~2. After fully cooling the culture to 18°C, groES/EL gene expression was induced with 1 mg/ml l-arabinose. After 30 min of growth, ETF gene expression was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Cultures were then grown for an additional 16 h at 18°C. Cells were harvested by centrifugation at 11,899 × g, 4°C for 6 min, and the pellet was stored at −80°C after washing once in PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4).

Frozen cell pellet was thawed and resuspended in 25 ml of BugBuster (EMD Millipore, Burlington, MA, USA) containing 1 mM 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride, 1 mM FAD (Chem-Impex International, Wood Dale, IL, USA), 2 μl of Benzonase® Nuclease HC, and 2 μl of rLysosome™ Solution (EMD Millipore) and further incubated at 4°C for 2 h with stirring. After centrifugation at 20,000 × g for 30 min at 4°C, the supernatant was filtered through a 0.22-μm syringe
filter. The resulting protein solution was mixed with 2 ml of pre-equilibrated nickel-nitritotriacetic acid resin (EMD Millipore) and incubated at 4 °C with stirring for 1 h. Then the mixture was transferred to a column at 4 °C. After collecting the flow-through, the column was washed with 20 column volumes of 20 mM Tris, pH 7.8, 500 mM KCl containing 20 mM imidazole. Finally, the column was developed with 2 column volumes of 20 mM Tris, pH 7.8, 500 mM KCl containing 200 mM imidazole, and the eluate was collected in different fractions. After SDS-PAGE analysis, imidazole was removed from the pooled pure fractions by passage over a 10DG column (Bio-Rad) equilibrated with 20 mM bis-Tris propane, pH 9.0, 200 mM KCl, 10% (w/v) glycerol. Any apoproteins were then reconstituted by overnight incubation of the protein in 1 mM FAD at 4 °C in darkness. Excess flavin was then removed by gel filtration on a 10DG column (above) prior to prompt use or flash-freezing in liquid nitrogen and storage at −80 °C. Prior work had included tris(2-carboxyethyl)phosphine in buffers as a precaution (19), but it was found to diminish yields of the 726 nm species, so it was not used in the current work unless stated.

**Flavin content quantification**

Protein concentration was determined using the Pierce 660 nm protein assay (Thermo Fisher Scientific). No correction was found to be necessary for the use of BSA as the standard, based on parallel protein concentration determination based on the absorbance at 280 nm of TCA-denatured and urea-reddissolved protein (96). To quantify the flavin content, the protein sample was denatured by heating at 100 °C in the dark for 10 min. After cooling to 4 °C, the sample was centrifuged at 15,000 × g for 2 min to remove denatured proteins. The concentration of FAD in the supernatant was then quantified spectrophotometrically using the extinction coefficient ε_{280} = 11.3 mm M −1 cm −1 (97).

**Displacement of flavin by ADP in WT RpaETF and bound flavin identification by CD**

The FAD in RpaETF with a lower binding affinity to the protein was displaced at two different oxidation states using the method of Sato et al. (51). Anaerobic titrations were performed on a HP 8452A spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an Olis controller housed in a Belle Technology glovebox (<1.8 ppm of oxygen) using a 1-cm path length self-masking quartz cuvette with an air-tight closed screw cap (Starna Cells) in 20 mM bis-Tris, pH 9.0, 200 mM KCl, 10% (w/v) glycerol.

In the first case, 221.38 mM ADP (ε_{260} = 15.4 mm M −1 cm −1) (98) was used to titrate 400 µM of 22.2 µM WT RpaETF until the amplitude at 400 nm ceased to change, suggesting that the majority of ET-FADOX had dissociated. Then a reductive titration of the resulting protein sample was performed using 3.32 mM sodium dithionite (ε_{315} = 7.05 mm M −1 cm −1) (99).

In the second case, 400 µM of 68.7 µM WT RpaETF was reduced halfway by 3.42 mM sodium dithionite. Then the resulting protein sample was titrated with 637.48 mM ADP until the absorbance at 726 nm no longer changed. To determine which binding site retained FAD, CD spectra were recorded from 600 to 300 nm at room temperature, with the following parameters: bandwidth, 2.00 nm; scanning speed, 100 nm/min; accumulations, 1. The molar ellipticity [θ] can be calculated using the equation, [θ] = θ/(c × l), where θ is the ellipticity in millidegrees, c is the concentration in millimolar, and l is the cell path length in cm. After the CD measurement, the reductive titration was resumed with sodium dithionite.

**Anaerobic titration of WT, Y37F.S, Y37W.S, and C174A.L RpaETF by NADH, NAD+, and/or sodium dithionite**

Anaerobic titrations were all conducted on an HP 8452A spectrophotometer (Agilent Technologies) equipped with an Olis controller housed in a Belle Technology glovebox (<1.8 ppm of oxygen) using a 1-cm path length self-masking quartz cuvette with an air-tight closed screw cap (Starna Cells) in 20 mM bis-Tris, pH 9.0, 200 mM KCl, 10% (w/v) glycerol.

WT RpaETF was titrated using 400 µl of 21.3 µM protein with 1.22 mM NADH (ε_{340} = 6.22 mm M −1 cm −1) (100). Y37F.S RpaETF was titrated using 400 µl of 29.9 µM protein with 1.50 mM NADH. Y37W.S RpaETF was titrated using 400 µl of 15.9 µM protein with 0.65 mM NADH. C174A.L RpaETF was titrated using 400 µl of 27.4 µM protein with 2.74 mM NADH. WT RpaETF was also titrated using 400 µl of 68.7 µM protein with 3.42 mm sodium dithionite (ε_{315} = 7.05 mm M −1 cm −1) (99). C174A.L RpaETF was also titrated using 400 µl of 20.0 µM protein with 2.00 mm sodium dithionite.

Oxidative titrations using NAD+ were performed similar to the reductive titrations under inert atmosphere. It was not possible to reoxidize with ferricyanide, due to protein precipitation, and reaction with O2 was not employed due to its several complicating side reactions. Fully reduced (via dithionite) WT RpaETF (13.9 µM) was titrated by 2.78 mM NAD+ first to reform the 726 nm species until maximum population was achieved. Then the resulting protein was further titrated by 139.00 mM NAD+ until the 726 nm band was gone. Fully reduced (via dithionite) C174A.L variant (20.0 µM) was titrated by 3 mM NAD+ and then 150 mM NAD+ until the 726 nm band no longer changed. All titrations were done in 20 mM bis-Tris propane, pH 9.0, 200 mM KCl, 10% (w/v) glycerol. The following equation was used to determine the dissociation constant for NAD+ binding:

\[
y = \frac{y_{\text{max}}c}{K_d + c}
\]

(Eq. 1)

where \(y_{\text{max}}\) is the maximum binding shown as ε_{260}, c is NAD+ concentration, and \(K_d\) is the dissociation constant.

**CD and fluorescence analysis of CTAB-treated WT RpaETF 726 nm species**

To denature RpaETF without causing precipitation, 44 µl of 115.5 mM CTAB was mixed thoroughly with 466 µl of 9.96 µM WT RpaETF displaying the 726 nm signal. Rather than maintain a sample poised halfway through a redox titration, this experiment employed 726 nm species that had formed slowly over 20 h in anaerobic storage at 4 °C in 20 mM bis-Tris propane, pH 9.0, 200 mM KCl, 10% (w/v) glycerol inside a Belle Technology glovebox (<1.8 ppm of oxygen). All other
726 nm CT band suggests direct flavin-flavin contact

experiments used material immediately after preparation or flash-frozen immediately and thawed only at the time of use.

The absorption spectra were monitored on a HP 8453 spectrophotometer (Agilent Technologies) using a 1-cm path length self-masking quartz cuvette with an air-tight closed screw cap (Starna Cells) at 0, 5, 10, 20, 30, 40, 50, and 60 min time points after thorough mixing anaerobically on ice. Eventually, the sample was heated at 100 °C in the dark for 10 min. The absorbance change at 726 nm over time was fit into a single-phase dissociation model, \( y = y_0 + (y_{\text{max}} - y_0) \left( 1 - e^{-kt} \right) \) (Eq. 2), where \( y_0 \) is the minimum, \( y_{\text{max}} \) is the plateau, \( t \) is time, and \( k \) is the rate constant.

Near-UV CD spectra were also recorded at 4 °C from 350 to 250 nm to probe protein tertiary structure with the following parameters: bandwidth, 2.00 nm; scanning speed, 100 nm/min; accumulations, 4. The molar ellipticity \( [\theta] \) can be calculated using the equation, 
\[
[\theta] = \theta / (c \times l)
\]
where \( \theta \) is the ellipticity in millidegrees, \( c \) is the concentration (mM), and \( l \) is the cell path length in cm. The molar ellipticity change at 280 nm over time was fit into a one-phase association model,
\[
y = y_0 + (y_{\text{max}} - y_0) (1 - e^{-kt})
\]
(Eq. 2)
where \( y_0 \) is the \([\theta]_{280} \) minimum, \( y_{\text{max}} \) is the \([\theta]_{280} \) plateau, \( t \) is time, and \( k \) is the rate constant.

Similarly, far-UV CD spectra were also recorded using 3.67 μM protein at 4 °C from 250 to 200 nm to probe protein secondary structure with the following parameters: bandwidth, 2.00 nm; scanning speed, 100 nm/min; accumulations, 4. The mean residue ellipticity \( [\theta] \) can be calculated using the equation,
\[
[\theta] = \left( \theta \times 10^6 \right) / (c \times l \times n)
\]
where \( \theta \) is the ellipticity in millidegrees, \( c \) is the concentration (mM), \( l \) is the cell path length in nm, and \( n \) is the number of peptide bonds in the protein. The mean residue ellipticity change at 222 nm over time was fit into a one-phase dissociation model,
\[
y = y_{\text{min}} + (y_0 - y_{\text{min}}) e^{-kt}
\]
(Eq. 3)
where \( y_0 \) is the \([\theta]_{222} \) maximum, \( y_{\text{min}} \) is the \([\theta]_{222} \) plateau, \( t \) is time, and \( k \) is the rate constant.

In a similar experiment to produce samples for fluorescence analysis, 40 μl of 115.5 mM CTAB was mixed thoroughly with 422 μl of 12.1 mM WT RpaETF. Fluorescence emission spectra of the above native WT RpaETF protein, CTAB-treated protein (after 60 min), and further heat-denatured protein were recorded using a 2-mm path length fluorescence quartz cuvette with an air-tight closed screw cap (Starna Cells) on a Thermo Scientific Lumina Fluorescence Spectrometer (Waltham, MA, USA) with the following parameters: for tryptophan fluorescence, excitation wavelength 295 nm, emission wavelength 310–450 nm, excitation slit 2.5 nm, emission slit 1 nm, integration time 20 ms, response time 0.1 s, average number 3; for flavin fluorescence, excitation wavelength 466 nm, emission wavelength 480–720 nm, excitation slit 5 nm, emission slit 2.5 nm, integration time 20 ms, response time 0.1 s, average number 3. Note that upon flavin dissociation, flavin fluorescence becomes strongly quenched by the attached adenine (63, 64).

Reduction midpoint potential determination of Bf-FAD of C174A.L RpaETF

The \( E^* \) (Bf-FAD\(_{Ox/HQ} \)) of C174A.L variant was determined using the xanthine/xanthine oxidase method as described previously (19), using phenosafranin as the mediator-cum-redox buffer and sensor. Linear regression analysis of \( \ln([\text{FAD}_{\text{OX}}]/[\text{FAD}_{\text{RED}}]) \) versus \( \ln([\text{DyF}_{\text{OX}}]/[\text{DyF}_{\text{RED}}]) \) was performed using GraphPad Prism version 8.2.1 for Mac OS X (GraphPad Software, La Jolla, CA, USA) and fit into the following equation,
\[
\ln \left( \frac{[\text{FOX}]}{[\text{FRED}]} \right) = b + n_F \ln \left( \frac{[\text{Dox}]}{[\text{DRED}]} \right)
\]
(Eq. 4)
where \([\text{FOX}]\) is the concentration of the reacting flavin in the oxidized state, \([\text{FRED}]\) is the concentration in the state formed upon reduction, \([\text{Dox}]\) and \([\text{DRED}]\) have the same meanings for the dye, and \( b \) is the intercept produced by the fit. The value obtained for \( b \) was then used with the midpoint potential\(^2\) of the dye, \( E_{D}^{\circ} \), to calculate that of the flavin, \( E_{F}^{\circ} \), using the following equation,
\[
b = \frac{n_F}{25.7 \text{ mV}} \left( E_D^{\circ} - E_F^{\circ} \right)
\]
(Eq. 5)
where 25.7 mV replaces \( R T / F \) (ideal gas constant times absolute temperature divided by Faraday’s constant). For phenosafranin, \( n = 2 \) and \( E_D^{\circ} = -252 \text{ mV at pH 7} \), which was adjusted to \(-312 \text{ mV at pH 9}\) based on phenosafranin’s uptake of one proton per 2 electrons between pH 7 and 9. For Nile blue, \( n = 2 \) and \( E_D^{\circ} = -116 \text{ mV at pH 7} \), which was adjusted to \(-176 \text{ mV at pH 9}\) based on Nile blue’s uptake of one proton per 2 electrons between pH 7 and 9.

Computations

Density functional theory (DFT) calculations were performed using Gaussian 16 (101). Starting geometries for flavin dimers were drawn from the crystal structures of dodecins. Coordinates for a re face to re face dimer were extracted from \( \text{Halobacterium salinarum} \) dodecin (PDB entry 2CCC (66)), and a si-si dimer was extracted from the coordinates of \( \text{Thermus thermophilus} \) dodecin (PDB entry 2V18 (67)). Ribityl chains were truncated to methyl groups, yielding lumiflavin dimers. Hydrogen atoms were added to N5 and N1 of one of the two lumiflavins to create a model for the HQ state, whereas the other lumiflavin was left as OX.

To test the sufficiency of the flavins themselves for formation of CT dimers, the systems were kept as simple as possible, using the polarizable continuum model for water (102). To control for possible effects of the methodology, we compared two functionals: the long-range corrected hybrid exchange-correlation functional cam-B3LYP (103) and another range-separated functional that was found to perform well in TD-DFT calculations on flavins (104): oB97X-D (105). We also varied the basis set used in order to test whether results were general. The correlation-consistent double-\( \zeta \) basis cc-pVDZ (106) was used with the cam-B3LYP functional as in Ref. 68, whereas the 6-
31+G(d, p) basis (107) was used with the ωB97X-D functional as in Ref. 86.

After geometry optimization and checking for imaginary vibrations, time-dependent DFT calculations were performed to identify singlet-singlet transitions with significant strength. The computed excitation energies and oscillator strengths were used to calculate optical spectra for comparison with experimental data. TD-DFT–derived vertical transition energies were too high by ∼0.55 eV, consistent with other work (16, 108). However, this does not interfere with our objective of comparing the features produced by individual flavins with those produced by CT dimers. The TD-DFT method is not ideal for transitions with a large amount of CT character (109), but its failure to treat the electrostatic attraction between CT partners is rectified substantially by use of the cam-B3LYP or ωB97X-D functionals (104), so the current qualitative comparisons can be made with a single industry-standard approach that has an extensive track record for flavins (16, 68, 104).

Transition energies and amplitudes from the computations were used to calculate absorption spectra with Gaussian lines 0.5 eV wide at half-height for transitions with energies up to $v$ = 5 eV ($\lambda$ ≥ 250 nm). Individual bands were calculated using the equation,

$$\epsilon_i(\sigma) = \frac{e^2 N \sqrt{\pi}}{10^3 \ln(10) c^2 m_e^2 \Delta \sigma} e^{-\frac{(\sigma - \epsilon_i)}{2\epsilon_i}}$$

(Eq. 6)

where $\sigma_i$ is the energy of the ith transition in eV; $f_i$ is the oscillator strength of the ith transition; $N$, $c$, and $m_e$ have their usual meanings; and $\epsilon_i(\sigma)$ is the obtained extinction coefficient due to the ith transition as a function of photon energy $\sigma$ (https://gaussian.com/uvvisplot/). Plots of total $\epsilon$ versus wavelength were obtained by summing the contributions from all transitions with oscillator strengths ≥1% of the maximum value obtained for that calculation and converting transition energies to wavelengths using $\lambda = h c / \sigma$, where $h$ is Planck’s constant.

Molecular graphics were produced in PyMOL (110) or Chimera (111), and electron density displacement maps were generated using Gausview 06 at an isovalue of 0.0004 (112).

**Data availability**

Data are contained in the article as well as the supporting information. The authors welcome collegial communications regarding any and all content in this article.

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Abbreviations—The abbreviations used are: OX, oxidized state; ASQ, anionic semiquinone; ETF, electron transfer flavoprotein; NSQ, neutral semiquinone; AHQ, anionic hydroquinone; Bf, bifurcating; $E^*$, reduction midpoint potential; CT, charge transfer; CTAB, cetyltrimethylammonium bromide; bis-Tris, cetyltrimethylammonium bromide bis-Tris propane; DFT, density functional theory; TD-DFT, time-dependent DFT; PDB, Protein Data Bank; LF, lumiflavin.

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**Additional information**

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