Flavoproteins can dramatically adjust the thermodynamics and kinetics of electron transfer at their flavin cofactor. A versatile regulatory tool is proton transfer. Here, we demonstrate the significance of proton-coupled electron transfer to redox tuning and semiquinone (sq) stability in photolyases (PLs) and cryptochromes (CRYs). These light-responsive proteins share homologous overall architectures and FAD-binding pockets, yet they have evolved divergent functions that include DNA repair, photomorphogenesis, regulation of circadian rhythm, and magnetoreception. We report the first measurement of both FAD redox potentials for cyclobutane pyrimidine dimer PL (CPD-PL, Anacystis nidulans). These values, $E_1(hq/sq) = -140$ mV and $E_2(sq/ox) = -219$ mV, where hq is FAD hydroquinone and ox is oxidized FAD, establish that the sq is not thermodynamically stabilized ($\Delta E = E_2 - E_1 = -79$ mV). Results with N386D CPD-PL support our earlier hypothesis of a kinetic barrier to sq oxidation associated with proton transfer. Both $E_1$ and $E_2$ are upshifted by $\sim 100$ mV in this mutant; replacing the N5-proximal Asn with Asp decreases the driving force for sq oxidation. However, this Asp alleviates the kinetic barrier, presumably by acting as a proton shuttle, because the sq in N386D CPD-PL oxidizes orders of magnitude more rapidly than wild type. These data clearly reveal, as suggested for plant CRYs, that an N5-proximal Asp can switch on proton flux independently of ET, tuning the properties of their N5-proximal residue to adjust the extent of proton transfer, H-bonding patterns, and changes in protein conformation associated with electron transfer at the flavin.

Electron transfer (ET) within and between proteins is a ubiquitous and essential molecular process in biology. Proteins must tightly control the rates of ET and lifetimes of radical intermediates to use this fundamental chemical reaction for a vast array of cellular functions. A significant control mechanism involves coupling of the ET to a proton transfer (PT) (1–3). The PT ensures heightened discrimination toward subtle changes in protein structure. As compared with ET, it has a much shorter range and is very sensitive to the relative orientation of a few atoms (the proton, donor, and acceptor), and its kinetics and thermodynamics can be adjusted dramatically by tuning pK_a values over a very large range (3). Consequently, proton-coupled ET (PCET) is widespread in nature, notably in biological energy conversion and redox catalysis. Understanding the mechanisms for PCET and identifying its role in protein evolution are central problems in chemical biology.

Flavoproteins are major players in cellular redox reactions (4). The flavin cofactor can exist in three redox states: the two-electron reduced hydroquinone (hq), which is often anionic; the one-electron reduced semiquinone (sq), as a neutral or anionic radical; and the fully oxidized form (ox) (Fig. 1a). Flavoproteins can tune the reduction potentials of sq and ox, $E_1$ and $E_2$, respectively (Fig. 1a), over many hundreds of mV, together with the kinetics of ET (5, 6), as required for function. In addition, because the flavin pK_a values are highly dependent on its redox state, flavoproteins may use PT to adjust the reaction driving force and/or act as a kinetic gate. One such gating mechanism, typified by the flavodoxins (7–9), involves protein dynamics. A PT necessarily changes the pattern of H-bond donors and acceptors presented by the flavin cofactor. This triggers reorientation of local residues and perhaps larger protein conformational changes, which may rate-limit redox reactions. Studies of several classes of flavoproteins, including flavodoxins (9), reductases (10–12), and electron transfer flavoproteins (ETFs) (13, 14), are beginning to reveal that PT and associated protein conformational changes serve not only to regulate the redox potentials of the flavin but also the reaction kinetics, with important functional consequences.

Although most flavoproteins use the ground state of their cofactor in redox chemistry, several light-activated flavoproteins are known (15). These include phototropins, BLUF domains, and the photolyases (PLs) and cryptochromes (CRYs). CRYs are the subject of increasingly intense investigation as they have numerous core functions in nature and human health, and very little is understood regarding their mechanisms of action and evolution (16–19). Their roles include plant photomorphogenesis (17), photoentrainment of circadian rhythm in plants and some animals (e.g. insects) (19, 20), light-independent regulation of the circadian clock in other animals (e.g. mammals) (19, 21, 22), and even magnetorecep-
tion (23–25). Significantly, although CRYs have evolved very different biological roles, they are related to PL both evolutionarily and structurally (Fig. 1, b and c) (16–18, 26, 27). The PL enzymes repair cyclobutane pyrimidine dimers (CPDs) or 6-4 photoproducts in DNA, using photoinduced ET from their flavin-adenine dinucleotide (FAD) cofactor. Recent investigations have focused attention on the importance of redox tuning, particularly of the stability and protonation state of the FAD sq, in the evolution of remarkable functional diversity within the PL/CRY family (28–33). However, little is known regarding redox regulation in these proteins. Although electrochemical potentials have been evaluated for Arabidopsis thaliana CRY1 (34) and Vibrio cholerae CRY-DASH (35), no measurements of $E_1$ in any PL have been reported, and the two values of $E_1$ currently in the literature differ by $\pm 50$ mV (34, 35). Furthermore, it is likely that the mechanism(s) for redox regulation in PLs and CRYs involve more than adjusting the thermodynamics of ET at the FAD cofactor. For instance, the reactivity of the sq radical toward oxidation varies dramatically within these proteins, and the sq is exceptionally resistant in CPD-PL. We recently proposed that sq stability in CPD-PL is rooted in a kinetic barrier imposed by rate-limiting deprotonation (33).

Here, we report electrochemical potentials for the two FAD redox couples in CPD-PL. These results establish that the sq is thermodynamically destabilized relative to both hq and ox ($\Delta E = E_2 - E_3 = -79$ mV) and confirm the significant role of kinetic mechanisms in redox regulation. We further demonstrate that the N5-proximal residue, conserved as an Asn in all PLs and as an Asn/Asp/Cys in CRYs, plays a critical role. In N386D CPD-PL kinetic stabilization of the sq is lost; the Asp residue in this protein serves as a proton shuttle to alleviate the kinetic barrier. The N5-proximal residue also has a dramatic impact on the thermodynamics of ET. The midpoint potential of N386D CPD-PL is $\pm 100$ mV more positive than wild type. Unexpectedly, both $E_1$ and $E_2$ are more positive in wild type.
the mutant, such that the $\Delta E$ value is largely unchanged, and the sq remains thermodynamically destabilized. These results emphasize the importance of PT to redox tuning and evolution within the PL/Cry family and highlight common regulatory themes among flavoproteins generally.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Characterization**—Anacystis nidulans CPD-PL was prepared as described previously (33), and analogous procedures were used for the N386D mutant; the coding DNA for N386D CPD-PL was generated by site-directed mutagenesis and sequenced. Briefly, the CPD-PLs were overexpressed in Escherichia coli BL21 (DE3)pLysS cells grown in 8-liter batches at 37 °C. Expression was induced by the addition of 600 μM isopropyl 1-thio-β-D-galactopyranoside at an $A_{600}$ of ~0.9 (after ~3–4 h), and cell growth was continued for 3 h at 37 °C. Cells were harvested by centrifugation (5000 rpm) for 4 °C for 10 min, and the resulting pellet was washed and resuspended in 3 × 20 ml of column buffer (10 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 100 mM NaCl, pH 7.0), flash-frozen, and allowed to thaw overnight on ice. The cell-free extract was prepared by sonication followed by centrifugation (16,000 rpm) for 1 h at 4 °C. The filtered cell-free extract was loaded onto a 5-ml heparin column, washed with a salt gradient (80–200 mM NaCl) and assayed for purity by SDS-PAGE and UV-visible spectroscopy. Elutions containing CPD-PL were pooled, exchanged into column buffer, reapplied to a heparin column, and eluted as described above. The CPD-PL elutions were again pooled and exchanged into DEAE-Sepharose column buffer (10 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 40 mM NaCl, 5 mM β-mercaptoethanol, pH 7.0) and then applied to a DEAE-Sepharose column. CPD-PL was eluted with a salt gradient (80–200 mM NaCl) and assayed for purity by SDS-PAGE. Pure fractions were combined, exchanged into storage buffer (10 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 450 mM NaCl, 10% glycerol and 450 mM NaCl). Samples for experiments at pH 5.4 were prepared in TRIS-HCl buffer with 10% glycerol and 450 mM NaCl. Samples for experiments at pH 5.4 were prepared in phosphate buffer (10 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 450 mM NaCl, pH 7) containing 10% glycerol and 10 mM EDTA (for photoreduction). Samples for experiments at pH 9.0 were prepared in TRIS-HCl buffer with 10% glycerol and 450 mM NaCl. Samples for experiments at pH 9.0 were prepared in phosphate buffer. The cuvette for electrochemical measurements was fitted with a platinum wire working electrode (0.5-mm diameter with platinum mesh) and a calomel reference electrode calibrated prior to the measurements (+260 mV at 10 °C, pH 7). The cuvettes were made anaerobic by 4–6 cycles of evacuation/flushing with ultrapure argon; the cell was then sealed. The solution in the side arm was photoreduced (deep blue) with white light prior to each experiment. The indicator solution serves to scavenge extraneous oxygen and provides a sensitive measure of its presence in the cell.

**Oxidation Experiments and Kinetic Analysis with N386D CPD-PL**—Absorption spectra were recorded at 10 ± 0.5 °C on a Varian Cary 100 spectrophotometer equipped with a temperature-controlled multicell holder. Spectra were scanned from 800 to 200 nm with a 1-nm step size, 1-s integration time, and 1-nm spectral bandwidth. Anaerobic protein samples were photoreduced by irradiation with white light (300 watts; ~10 cm) for 20–40 min in an ice water bath until fully reduced. Absorption spectra were recorded during the reduction to monitor the changes in FAD redox state. Fully reduced, anaerobic samples were made aerobic by releasing the seal of the sample cell and allowing them to react with O$_2$ at 10 °C for ~1–3 days. Time-dependent absorption spectra (~130) were recorded, using the Varian software scanning kinetics program. Because little sq is detected during oxidation of the N386D mutant, the change in absorbance at 450 nm was fit to a single exponential growth to yield the rate constant, $k$ for formation of oxidized FAD (ox).

**Spectroelectrochemistry**—Redox titrations of CPD-PL and its N386D mutant were carried out according to the method of Dutton (36), in the presence of the following mediators: benzyl viologen (1 mM), 2-hydroxy-1,4-naphthoquinone.
(15 μM), 2,5-dihydroxy-1,4-benzoquinone (15 μM), 5,8-dihydroxy-1,4-naphthoquinone (15 μM), duroquinone (15 μM), phenazine ethosulfate (1 μM), phenazine methylsulphate (1 μM), 1,2-naphthoquinone (15 μM), and anthraquinone-2-sulfonic acid (1 μM). The redox potential was adjusted stepwise (5–20 mV/step) by the addition of small volumes (~2.5 μl) of sodium dithionite as reductant or potassium ferricyanide as oxidant. The solution was mixed and stirred for 5 min. The potential range scanned varied from ~350 mV to +350 mV (versus NHE, normal hydrogen electrode), depending on the protein and the experiment. After each step, the solution was allowed to equilibrate until the open circuit potential stabilized, ΔE < 1 mV/15 min, and two or more identical UV-visible spectra were recorded. Typical equilibration times were 25–60 min.

The spectroelectrochemical data were analyzed using the Nernst equation, modified according to the Beer-Lambert law to express the total change in absorbance (or extinction coefficient) in terms of the absorbance contributions of each redox state of FAD (10, 11). Because essentially no sq was formed during equilibrium redox titrations of CPD-PL or its N386D mutant, absorbance changes were analyzed at 445 nm, which is near a maximum for the oxidized FAD. Plots of ε_{445} versus potential were first fit to the Nernst equation for a two-electron transfer, Equation 1

$$\varepsilon_{445} = \frac{\varepsilon_{ox} 10^{(E_{ox}/RT) + 0.028} + \varepsilon_{sq} 10^{(E_{sq}/RT) - 0.028} + 1}{2}$$  \hspace{1cm} (Eq. 1)

where ε_{ox} and ε_{sq} are the extinction coefficients of the oxidized FAD and its fully reduced hydroquinone, respectively, at 445 nm. The factor, 0.028 V in Equation 1, is the value of RT/nF for a two-electron transfer (n = 2) at the experimental temperature of 10 °C. Because this equation contains only three parameters, two of which we have determined in independent experiments (at 445 nm: ε_{ox} = 11 000 M⁻¹ cm⁻¹, ε_{sq} = 1750 M⁻¹ cm⁻¹), accurate E_M values are obtained from this treatment. This is confirmed by the observation that the calculated extinction coefficients are very close to the known values, and fixing them in the analysis does not change the output E_M value. In addition, the E_M values derived from 5–7 independent experiments with both wild type and the N386D mutant were very reproducible (S.E. ± 2 mV). To extract the (hq/sq) potential E_1 and the (ox/sq) potential E_2, the change in absorbance/extinction coefficient at 445 nm was fit to the modified two-step Nernst equation, Equation 2

$$\varepsilon_{445} = \frac{\varepsilon_{ox} 10^{(E_{1}/RT) + 0.056} + \varepsilon_{sq} 10^{(E_{2}/RT) - 0.056} + 1}{\varepsilon_{ox} 10^{(E_{1}/RT) - 0.056} + \varepsilon_{sq} 10^{(E_{2}/RT) + 0.056}}$$  \hspace{1cm} (Eq. 2)

where ε_{sq} is the extinction coefficient of the FAD sq at 445 nm. The factor, 0.056 V in Equation 2, represents the value of RT/nF for a single electron transfer (n = 1) at the experimental temperature of 10 °C. This equation now has five parameters, and although such single wavelength analysis has been reported to extract the two redox couples of flavoproteins (12, 34), we constrained the fit using our E_M values (i.e. E_1 = E_M * 2 – E_2). We could further reduce the number of parameters to three by using our measured value for ε_{sq} = 3670 M⁻¹ cm⁻¹ at 445 nm. Fixing this extinction coefficient is reasonable because its value does not alter the calculated potential. We found this analysis to be robust and to yield reproducible mid-point potentials for both redox couples. Although the standard errors of the 5–7 trials (±7 mV) are expectedly larger than those for E_M, they are well within the range of those reported in the literature.

**RESULTS**

It has long been known that the sq in CPD-PL is stable toward oxidation (37). We have reported extremely sluggish kinetics for this reaction and have shown, through kinetic isotope effects, that oxidation is rate-limited by PT (33). Recent electrochemical studies, aimed at measuring the one-electron redox potentials of CPD-PL, reported values for E_1 ranging from −48 to 0 mV, the same within experimental error for *E. coli* and *A. nidulans* CPD-PL (34, 35). In these experiments, the hq-sq redox couple was titrated, with quantitative production of the sq whereas the sq-ox couple proved inaccessible at potentials as high as ~400 mV. In similar electrochemical titrations of the hq-sq couple of *A. nidulans* CPD-PL, we measured a comparable value of E_1 = −50 mV (33). However, we also found that over extended equilibration time (e.g. >30 min to several hours), the absorption spectra and electrochemical potentials did not stabilize (33). We suspected that the sq observed during these experiments may be a kinetic product, with the slow kinetics of PT contributing to the accumulation of sq and inhibiting further oxidation and equilibration of the system. Consistent with this conclusion is the severe hysteresis observed between oxidative and reductive titrations over both redox couples (supplemental Fig. S1). No sq is detected in reductive titrations starting with the fully oxidized protein (Fig. 2a). Although full oxidative titrations are kinetically unfeasible (>15–20 h for equilibration at some steps), we are confident that equilibration is achieved in the reductive direction (15 min to 2 h) and that accurate potentials can be derived from these data. An analogous kinetic barrier, associated with rate-limiting PT, is observed in titrations of the anionic sq-hq couple of *Methylophilus methylotrophus* ETF (13, 14). Due to prohibitively long equilibration times for oxidation of the hq, redox potentials for this protein have likewise been obtained by titrations in the reductive direction only (13). For CPD-PL, the variation in absorbance at 450 nm with potential (Fig. 2b), when fit to a modified Nernst equation (see "Experimental Procedures"), yields E_M = −182 ± 2 mV, E_1 = −140 ± 7 mV, and E_2 = −219 ± 7 mV. Its sq is clearly destabilized relative to both hq and ox. With ΔE = E_2 – E_1 = −79 mV, the maximum yield of sq under thermodynamic equilibrium would be <10%. The buildup of sq during non-equilibrium oxidation of the hq by O_2 (33) and in titrations of the hq-sq couple only (33–35) is likely a kinetic phenomenon and may be responsible for the significantly more positive E_1 values previously reported. The very negative E_1 is consistent with predictions made based on the relative binding affinities of CPD-PL for FAD sq and ox (−191 > E_2 > −242) (33). This value confirms that the resistance of the sq toward oxidation is due to a kinetic barrier rather than thermodynamic stabilization. Because CPD-PL uses the hq-sq
The identity of the N5-proximal residue plays a central role in redox regulation by flavoproteins. In flavodoxins, this residue is a Gly that orients its backbone carbonyl to provide an H-bond acceptor to the protonated flavin N5 of the hq and neutral sq radical (7–9). Complete oxidation requires deprotonation, and the Gly flips to offer its backbone NH as an H-bond donor to N5. This pattern of H-bonding thermodynamically stabilizes the neutral sq and presents a kinetic barrier to its oxidation. Similar mechanisms coupling PT, changes in H-bonding, and protein dynamics are operative in other flavoproteins (10–12). In Type I animal CRY, where the N5-proximal residue is a Cys, the sq exists as an anion radical that neutral form (28, 32, 38) and may be further reduced to the hq (32). The Asp may act as the proton donor/acceptor (32, 38).

In CPD-PL, the N5-proximal Asn is not likely to act as a proton shuttle given that in solution it is very difficult to protonate (pKa ≈ 17). Instead, it has been proposed to stabilize the neutral sq radical by providing its side chain carbonyl as an H-bond acceptor to the protonated flavin N5 (39). As in flavodoxins, full oxidation of CPD-PL requires sq deprotonation and loss of this H-bond, unless the Asn flips orientation to present its side chain amino groups as an H-bond donor to the N5. The intrinsic rate of deprotonation and/or conformational change may serve to kinetically gate the oxidation.

To test this hypothesis, we investigated N386D CPD-PL (A. nidulans, supplemental Fig. S2). The absorption spectra recorded during after purification of this protein under aerobic conditions clearly reveal loss of the sq stabilization provided by wild type. As compared with blue CPD-PL, which possesses near quantitative sq, the yellow mutant is completely oxidized (Fig. 2c). It is not surprising that mutation of
the N5-proximal residue should have a large affect on the FAD redox properties; indeed, mutation of this Asn to Ser in E. coli PL also severely destabilized the sq (39). However, the results with the Ser mutant do not establish how the conserved Asn stabilizes the neutral sq in wild type CPD-PL. No redox potentials or oxidation kinetics were reported for this mutant. Moreover, like Asn, an N5-proximal Ser can provide an H-bond acceptor to stabilize the neutral sq and is unlikely to act in PT. By replacing the N5-proximal Asn with Asp, we aimed to specifically address the role of PT in sq stability within these proteins.

To investigate whether the N386D mutation impacts the kinetic barrier to sq oxidation, we examined the reaction of the fully reduced protein with O2. Our previous investigations of CPD-PL, CRY-DASH, and several other mutants, established that this oxidation occurs within the proteins (33), and reactivity toward O2 can be used as a quantitative gauge of sq kinetic stability (5, 6). The sq is obtained by photoreduction of fully oxidized N386D CPD-PL with white light in the presence of EDTA. At neutral pH, a small amount of sq is observed during photoreduction (supplemental Fig. S3). Oxidation of the mutant differs markedly from wild type CPD-PL. First, essentially no sq (<10%) is detected during conversion of the hq to ox in the N386D mutant, whereas sq forms quantitatively in wild type CPD-PL (Fig. 2d). Second, the overall oxidation is near completion in ~3 h, whereas it requires weeks in wild type CPD-PL (Fig. 2e). In CPD-PL, oxidation of the hq to sq, with rate constant \( k_1 = 12 \times 10^{-5} \text{s}^{-1} \) is much faster than the oxidation of the sq, with rate constant \( k_2 \ll 0.4 \times 10^{-5} \text{s}^{-1} \) \((k_2 \gg k_1, \text{Fig. 1a) (33)}\). In N386D CPD-PL, little sq is observed, it is likely that \( k_2 \gg k_1 \). It is possible that oxidation occurs via direct transfer of two electrons to the hq (with rate constant \( k_1 \), Fig. 1a). However, \( O_2 \) generally acts as a single electron oxidant, and in analogous oxidation experiments with the homologous CRY-DASH, where little sq accumulates, our kinetic analysis indicates that oxidation proceeds by two single-ET steps with \( k_2 \gg k_1 \) (33). The overall rate constant for oxidation of N386D CPD-PL, derived from the formation of ox at 450 nm (Fig. 2e), is \( k = 18 \times 10^{-5} \text{s}^{-1} \). Thus, complete oxidation of the mutant is more rapid than sq formation in wild type CPD-PL (\(45\)-fold rate enhancement for ox formation). In fact, the sq in N386D CPD-PL is even more reactive than in CRY-DASH (Fig. 2e). Kinetic stabilization of the sq in CPD-PL is clearly lost by conversion of the N5-proximal residue from Asn to Asp. Evidence that this mutation facilitates oxidation specifically by impacting PT comes from kinetic isotope effects. The large kinetic isotope effect of ~4 for sq oxidation in wild type CPD-PL (33) is reduced to ~2 in the mutant (supplemental Fig. S4).

The simplest explanation for the accelerated rate of oxidation and more facile PT in N386D CPD-PL is that the N5-proximal Asp acts as a proton shuttle, increasing the rate of PCET. By replacing the N5-proximal Asn with Asp, we aimed to specifically address the role of PT in sq stability within these proteins.

The simplest explanation for the accelerated rate of oxidation and more facile PT in N386D CPD-PL is that the N5-proximal Asp acts as a proton shuttle, increasing the rate of PCET. By replacing the N5-proximal Asn with Asp, we aimed to specifically address the role of PT in sq stability within these proteins.
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an H-bond donor without conformational change. Preorganization by Asp of the proton acceptor and H-bond donor may lower the barrier to deprotonation and accelerate the rate of sq oxidation relative to wild type. An analogous model has been proposed for plant CRYs whereby the conserved N5-proximal Asp, protonated when the protein is fully oxidized, donates its proton to the flavin to generate the neutral sq, and ultimately the anionic hq, during photoreduction (32, 38). The N5-proximal Asn in wild type CPD-PL is not expected to function in such a proton exchange. The pKₐ of its conjugate acid is likely less than that of the oxidized N5–H(+) such that it does not accept that proton during oxidation. Similarly, the high pKₐ of the amide protons may prevent Asn from providing a proton during protein reduction. Oxidation/reduction in wild type CPD-PL requires PT from another source, such as a bound/mobile water (29), and conformational rearrangement of the N5-proximal Asn to re-establish H-bonding.

Additional experimental evidence in support of Asp-386 acting as a proton shuttle comes from the pH dependence of photoreduction. During photoreduction of N386D CPD-PL at pH 7 and 5.4, a small amount of sq accumulates on route to the hydroquinone, as evidenced by its characteristic absorption between 550 and 700 nm (supplemental Fig. S3). The spectral changes recorded during photoreduction at pH 9, however, are notably different. In particular, the long wavelength absorption due to the neutral sq is nearly absent, and absorption bands distinctive of the sq anion radical are evident. These include a sharp peak at 400 nm, increased absorption at 360 nm, and a peak at 480 nm with trailing absorption between 500 and 550 nm (Fig. 2f). In contrast, photoreduction of wild type CPD-PL does not produce the anionic sq (supplemental Fig. S3). These observations are consistent with a model in which Asp-386 is protonated in the oxidized protein at pH 7; it can donate this proton to FAD, allowing photoreduction through the neutral sq, and accept the proton to facilitate oxidation. At pH 9, Asp-386 is more likely to be deprotonated in the oxidized protein, and photoreduction produces the sq anion radical.

It is worth noting that the pH-dependent photoreduction profiles are not inconsistent with the largely pH-independent oxidation kinetics because the mechanistic paths followed during these reactions differ, as do the relevant pKₐ values. When photoreduction is initiated, FAD is fully oxidized and lacks a proton at N5. If the proximal Asp is deprotonated (e.g. pH = 9), photoreduction may generate the sq anion radical. To complete reduction to the hq, this anion would have to extract a proton from another source. If the Asp is protonated (e.g. pH = 7), it may transfer its proton to the sq radical anion provided that its pKₐ is less than that of the neutral sq. In contrast, when oxidation is initiated, the flavin N5 is protonated and presumably H-bonded to Asp-386 (deprotonated at pH 7). In this case, transfer of the proton from FAD to Asp-386 to complete oxidation will depend on the relative pKₐ values of the Asp and the flavin N5–H(+).

Consistent with the observation that the N386D mutation alleviates the kinetic barrier to sq oxidation in CPD-PL, we find fully reversible reductive and oxidative titrations over both redox couples in N386D CPD-PL. In sharp contrast to wild type, where equilibration could only be achieved in the reductive direction, equilibration is achieved in standard times (~0.5–2 h), and no hysteresis is observed (supplemental Fig. S1). Like wild type, and as expected from oxidation kinetics of the N386D mutant, no sq is detected during interconversion of hq and ox under equilibrium conditions (Fig. 2a, inset). Significantly, the Eₘ of −90 ± 2 mV obtained for N386D CPD-PL is nearly 100 mV more positive than wild type (Fig. 2b). Moreover, both E₁ = −56 ± 7 and E₂ = −124 ± 7 are upshifted for the mutant and by a similar amount, 84 and 95 mV, respectively (Fig. 3b, supplemental Table S1). Thus, although reduction of ox and sq is made more thermodynamically favorable by the mutation, ΔE = E₂ − E₁ = −68 mV is effectively the same as wild type, and the sq remains thermodynamically prone to disproportionation. The increases in E₁ and E₂ indicate that both the hq and the sq are thermodynamically stabilized by the mutation (Fig. 3b). Based on electrostatic arguments alone, one might have predicted that replacing a neutral Asn with the (likely) negatively charged Asp would lower the driving force for reduction of the sq and decrease E₂. One explanation for the observed increase in both redox potentials, consistent with the model proposed to rationalize the oxidation kinetics, is that the deprotonated Asp serves as a stronger H-bond acceptor to the hq and sq than does the neutral Asn.

DISCUSSION

The significance of the N5-proximal residue to evolution within the PL/CRY family, and specifically to tuning the reactivity of the sq intermediate, has received considerable attention over the past few years (29–32, 38–40). There is general agreement that the identity of this residue adjusts the protonation state of the sq. However, two models for the photocycle and mechanism of the action of CRY, differing in the role of this residue, have been presented. Investigations of plant CRYs have led to a model where the ground/dark state is fully oxidized (28, 38). Light absorption induces photoreduction through a strictly conserved Trp triad to produce a one-electron reduced signaling state. PT following this reduction has been proposed as a key feature that distinguishes plant CRYs from animal CRYs, as well as PLs. Specifically, in plant CRYs, which possess an N5-proximal Asp, PT takes place to generate the neutral sq radical, whereas PT does not occur in animal CRYs and PLs, which lack the Asp. On the other hand, based on studies of Type 1 animal CRYs and CPD-PL, a common, anionic ground state, either the sq anion radical or the fully reduced hq, has been proposed for the entire family (29, 30). Light absorption by either of these dark states yields a potent one-electron reductant that serves as the signaling/catalytically active state. In this model, the identity of the N5-proximal residue governs whether the hq or sq serves as the dark state, and correspondingly, whether the E₁ or E₂ redox couple is used for photoinduced ET. If the N5-proximal residue is a Cys, as it is in Type 1 animal CRYs, PT and further reduction to the hq does not occur; the sq anion radical serves as the ground state. If the N5-proximal residue is an Asp or Asn, as it is in the other CRYs or PLs,
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PT from the Asp or a water molecule generates the hq ground state.

The redox potentials for *A. thaliana* CRY1, *E* 1 = −161 mV and *E* 2 = −153 mV (34), may provide evidence that the ground state of FAD in plant CRYs is fully oxidized. For instance, using these values and a typical plant cell potential of −100 mV, Brettel and co-workers (34) estimated that ~90% of plant CRYs should be oxidized *in vivo*. These authors also measured an *E* 1 of −39 mV for CPD-PL and observed that potentials as positive as 400 mV could not oxidize the sq (34). Consequently, a downshift in both redox potentials for plant CRYs, leading to a stabilization of the oxidized redox state, was proposed as an essential step in the evolution of CRY from CPD-PL. Notably, however, predictions of the *in vivo* oxidation state depend strongly on estimates of the plant cell resting potential. Values as low as −300 mV have been reported (41, 42). Given these potentials, the ground state of *A. thaliana* CRY1 at thermodynamic equilibrium would be the fully reduced hq.

The values of both redox couples for CPD-PL, which we now report, provide important new insight and may be used to assess models for redox regulation and the role of the N5-proximal residue in PLs and CRYs. First, we find that the downshift in the sq/hq redox couple is relatively small because the *E* 1 value we measure for CPD-PL is only ~20 mV more positive than *A. thaliana* CRY1. More significant is the large negative *E* 2 of CPD-PL, which is almost 70 mV lower than plant CRY. These redox potentials suggest that the sq/ox couple is actually upshifted significantly in plant CRYs, that the driving force for complete oxidation is higher in CPD-PL, and that CPD-PL may be mostly oxidized if at thermodynamic equilibrium in the cell. However, it is well established that the catalytically active form of CPD-PL is the hq and that the dark state *in vivo* is the hq/sq (18). A consideration of the thermodynamics of ET may be insufficient to evaluate the functionally relevant redox states. We suggest that CPD-PL uses a kinetic mechanism involving PCET to prevent complete oxidation of its FAD cofactor and ensure efficient use of the *E* 1 redox couple in catalysis (Fig. 3). Thermodynamic destabilization of the sq reduces the energy required to maintain the hq ground state and for re-reduction of the sq, formed transiently during catalysis, to complete the catalytic cycle with high quantum efficiency.

Our results with N386D CPD-PL are consistent with this proposal. As with plant CRYs (34), its sq/ox redox potential, *E* 2, is significantly upshifted relative to wild type. The driving force for oxidation is actually less when the N5-proximal Asn is replaced by Asp. Although the Asn in CPD-PL likely cannot accept a proton from FAD to complete oxidation, this does not present a thermodynamic obstacle to oxidation. The stability of the neutral sq in CPD-PL is rooted in a kinetic barrier to oxidation. This is evident from the significantly enhanced rates of reaction with O2 in the N386D mutant. Although the driving force for oxidation of this mutant is less than wild type, it reacts at a much faster rate, presumably because the N5-proximal Asp can act as a proton shuttle to reduce the barrier (Fig. 3). The Asp also appears to alleviate the kinetic bottleneck in plant CRYs. Here too, the driving force for oxidation is actually less when the N5-proximal Asn can act as a proton shuttle to reduce the barrier (Fig. 3). The Asp also appears to alleviate the kinetic bottleneck in plant CRYs. Here too, the driving force for oxidation is less than CPD-PL, but the rate constants for reaction with O2 are ~10−3 s−1 (28, 43), several orders of magnitude faster than CPD-PL. That the kinetics of PT to (and from) the oxidized flavin are tuned by the N5-proximal residue is also borne out by photoreduction data. During photoreduction, protonation of the anionic sq is not observed in Type 1 animal CRYs (29, 30), requires longer than ms to s in CPD-PL (30, 44), as well as in Type 1 animal CRYs with the Cys-Asn mutation (29), and occurs on the μs timescale in plant CRYs (32).

Although the kinetics of PT may be an important contributor to mechanistic and functional divergence among CRYs and PLs, these kinetics are not governed by the N5-proximal residue alone. In the context of CPD-PL, replacing the N5-proximal Asn with Asp dramatically accelerates the rate of oxidation; this mutation shifts the sq reactivity closer to that in plant CRY and suggests that the N5-proximal Asp in both proteins plays a defining role in PT. Conversely, in Type 1 animal CRYs, replacing the N5-proximal Cys with Asp has no apparent effect on PT because these mutants behave like wild type, generating only anionic sq upon photoreduction (29, 30). Other residues clearly tune the reactivity of the sq toward protonation and oxidation/reduction. Perhaps, within the environment of Type 1 animal CRYs, the relative *pK* a values of the N5-proximal Asp and the N5 of FAD ox/sq are altered such that PT is shut down. Differences in the flavin binding environment are expected because 6 of the 12 residues in direct contact with the isosoolxazine in CPD-PL differ in Type 1 animal CRYs, as do most of the amino acids interacting with the ribitol chain and diphosphate of FAD. Closer homology among FAD-binding residues is found between CPD-PL and plant CRYs (Fig. 1).

The functional relevance of regulating the reactivity of the FAD sq in PLs and CRYs has yet to be established. However, several lines of evidence hint at a correlation. For instance, the N5-proximal Asn-to-Ser mutant of CPD-PL was found to be fully oxidized following isolation and purification and to be catalytically inactive *in vitro* and in cells (39). In Type 1 animal CRYs, mutation of the N5-proximal Cys to Asp increases both the stability of the sq radical anion (*i.e.* resistance to O2) and the photoreceptor response in degradation assays (29). It was proposed that the intrinsic stability of the sq anion radical may regulate the photosignaling quantum efficiency in Type 1 animal CRYs. In Type 2 animal CRYs from mice (mCRY1), the D387N and R358K mutations were shown to compromise the function of these proteins as transcriptional repressors *in vivo* (31). This Asp-Arg pair is strictly conserved throughout the PL and CRY family and is seen to form a salt bridge on the *re* face of the isosoolxazine in all high resolution structures solved to date. Although the impact of disrupting this salt bridge on cofactor binding and protein structure is unknown, the authors suggest that the intact bridge serves to position the guanidinium group of Arg-352 over the flavin C4a and to stabilize the sq radical (31). More generally, it was proposed that regulation of sq stability may be central to the diverse functions of CRYs and PLs.

The evolution of reaction mechanisms and function through tuning of sq *pK* a values and reactivity has been described in several other classes of flavoproteins and is likely
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general. For instance, many ETFs function as one-electron carriers that cycle between ox and sq anion radical, although most can be fully reduced to the hq, with the exception of *M. methylotrophus* ETF (13, 14). Here, Arg-237, which is positioned over the si face of the isoalloxazine, is thought to strongly stabilize the anionic sq radical, and this residue is found to induce large thermodynamic and kinetic barriers to hq formation (14). This is analogous to the bottleneck to complete reduction in Type 1 animal CRYS and to complete oxidation in CPD-PLs. Also thematic is the wide variation in redox potentials and sq stabilities in ETFs despite high conservation of residues (including strictly conserved Arg-237) in the flavin-binding pocket. Thus, although a single residue may play the dominant role in redox regulation in one protein (e.g. Arg-237 in *M. methylotrophus* ETF), the same residue in a homologous protein (e.g. Arg-249 in human ETF) often exerts a different effect (14). Undoubtedly the local environment modulates the impact of the N5-proximal residue in PLs and CRYS.

A second example is provided by the FMN-binding/reductase domains of the cytochromes P450 and nitric oxide synthases (NOS). Sharing homology with flavodoxins, the FMN domains of most of these proteins are characterized by a large, positive $\Delta E (E_2 > E_1)$, indicative of thermodynamic stabilization of the sq, and the sq/hq couple is used in ET (11, 12). A proton-coupled sq/ox transition provides significant kinetic stabilization that traps the neutral sq and inhibits oxidation, much like CPD-PL. As in flavodoxins, the kinetic bottleneck is related to a conformational change within a flavin-binding loop (‘50’s loop’ in flavodoxins); this reorients the backbone of a key Gly residue to accommodate the change in h-bonding pattern at the flavin N5 during interconversion between ox and neutral sq. In contrast, in the FMN-binding domain of P450 from *Bacillus megaterium* (P450-BM3), it is the sq anion radical that forms, and much more transiently as its $\Delta E$ is negative ($E_2 < E_1$), and the sq/ox couple is used in ET (10). The thermodynamically destabilized sq anion radical can be kinetically trapped because the proton-coupled transition to the hq introduces a barrier to complete reduction. In the case of P450-BM3, distinct sq reactivity and redox potentials evolved through the loss of the Gly from the binding loop. Presumably this prevents the required conformational change and/or H-bond stabilization of the neutral sq radical. Validation of this model comes from elegant experiments in which the relative values of $E_1$ and $E_2$ are reversed, either by deleting the loop Gly in NOS (45) or by inserting the loop Gly in P450-BM3 (46).

CONCLUDING REMARKS

Although common threads of redox regulation exist among flavoproteins generally, PLs and CRYS form part of a small group of flavin-binding proteins whose functions depend on light. These photoresponsive flavoproteins, the PLs/CRYs, BLUF domains, and phototropins, may utilize similar underlying mechanisms to capture light for catalysis and signaling. Investigations of BLUF domains also suggest that these mechanisms are intimately linked to the sq intermediate (47). In particular, switching of H-bonding patterns, due to the formation/elimination of the sq through PCET, triggers conformational changes that produce a signaling state. It is likely that the many functions of CRYS rely on photoinduced ET and analogous mechanisms for achieving light sensitivity. Our investigations of CPD-PL and its N386D mutant, together with existing data, clearly reveal that the properties of the N5-proximal residue in PLs and CRYS are tuned to regulate both the driving force and the rates of PCET and consequently the reactivity of the sq. Correlation of this reactivity with the evolution of divergent function in these PLs and CRYS remains an important future challenge. Our results further underscore the significance of PCET, as well as kinetic mechanisms of redox regulation, to the action of PLs and CRYS and flavoproteins, generally.

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