Equilibrium and ultrafast kinetic studies manipulating electron transfer: A short-lived flavin semiquinone is not sufficient for electron bifurcation

Received for publication, May 1, 2017, and in revised form, June 11, 2017. Published, Papers in Press, June 14, 2017, DOI 10.1074/jbc.M117.794214

John P. Hoben, Carolyn E. Lubner, Michael W. Ratzloff, Gerrit J. Schut, Diep M. N. Nguyen, Karl W. Hempel, Michael W. W. Adams, Paul W. King, and Anne-Frances Miller

From the Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506, the National Renewable Energy Laboratory, Golden, Colorado 80401, and the Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602

Edited by Ruma Banerjee

Equilibrium and ultrafast kinetic studies manipulating electron transfer: A short-lived flavin semiquinone is not sufficient for electron bifurcation

Flavin-based electron transfer bifurcation is emerging as a fundamental and powerful mechanism for conservation and deployment of electrochemical energy in enzymatic systems. In this process, a pair of electrons is acquired at intermediate reduction potential (i.e., intermediate reducing power), and each electron is passed to a different acceptor, one with lower and the other with higher reducing power, leading to “bifurcation.” It is believed that a strongly reducing semiquinone species is essential for this process, and it is expected that this species should be kinetically short-lived. We now demonstrate that the presence of a short-lived anionic flavin semiquinone (ASQ) is not sufficient to infer the existence of bifurcating activity, although such a species may be necessary for the process. We have used transient absorption spectroscopy to compare the rates and mechanisms of decay of ASQ generated photochemically in bifurcating NADH-dependent ferredoxin-NADP⁺ oxidoreductase and the non-bifurcating flavoproteins nitroreductase, NADH oxidase, and flavodoxin. We found that different mechanisms dominate ASQ decay in the different protein environments, producing lifetimes ranging over 2 orders of magnitude. Capacity for electron transfer among redox cofactors versus charge recombination with nearby donors can explain the range of ASQ lifetimes that we observe. Our results support a model wherein efficient electron propagation can explain the short lifetime of the ASQ of bifurcating NADH-dependent ferredoxin-NADP⁺ oxidoreductase I and can be an indication of capacity for electron bifurcation.

Electron transfer bifurcation is emerging as the third fundamental mechanism of energy conservation in biology (1, 2). In this process, a pair of electrons is acquired at intermediate reduction potential (intermediate reducing power), but the electrons are each passed to different acceptors, as implied by the term “bifurcation.” This process accomplishes a critical redistribution of energy between the two electrons. One passes to a high-potential acceptor whose reduction makes the reaction favorable overall despite passage of the other electron to an acceptor with a reduction midpoint potential lower than that of the initial donor. Thus, one strongly reducing carrier, such as reduced ferredoxin, is produced at the expense of two medium-potential electrons from NADH. The ability to produce more powerful reducing equivalents allows organisms to conduct reactions that would otherwise not be possible, such as cleavage of the triple bond of N₂, which removes a critical nutrient limitation and makes more environments habitable (3–6).

In both flavin- and quinone-based bifurcation (7), it is deemed crucial that the bifurcating cofactor be able to acquire electrons in pairs but that the radical semiquinone (SQ)² state involved in one-electron chemistry be thermodynamically unstable (high energy) (2, 8). Such a species would not accumulate in equilibrium titrations, but if formed transiently as part of a reaction, it would be a potent reductant able to transfer an electron to a low-potential acceptor (1, 2, 8). By definition, such a species is difficult to observe and characterize. However, recent transient absorption spectroscopy (TAS) on NADH-dependent ferredoxin-NADP⁺ oxidoreductase I from Pyrococcus furiosus (NfnI) (9) succeeded in doing so by photoexciting active-site flavin and using rapid time-resolved (100 fs to 5.1 ns) dual-beam spectroscopy to observe the resulting species (9). We were thus able to detect anionic flavin SQ and measure its lifetime (absorption decay at 366 nm, τ = 10 ps). Rapid reoxidation of this species could be understood in terms of modest distances to nearby electron carrier cofactors and permitted estimation of the midpoint potential associated with the flavin OX/ASQ redox couple. This is presumed to provide the driving force for this process, a pair of electrons is acquired at intermediate reduction potential (intermediate reducing power), and each electron is passed to a different acceptor, one with lower and the other with higher reducing power, leading to “bifurcation.” This process accomplishes a critical redistribution of energy between the two electrons. One passes to a high-potential acceptor whose reduction makes the reaction favorable overall despite passage of the other electron to an acceptor with a reduction midpoint potential lower than that of the initial donor. Thus, one strongly reducing carrier, such as reduced ferredoxin, is produced at the expense of two medium-potential electrons from NADH. The ability to produce more powerful reducing equivalents allows organisms to conduct reactions that would otherwise not be possible, such as cleavage of the triple bond of N₂, which removes a critical nutrient limitation and makes more environments habitable (3–6).

2 The abbreviations used are: SQ, semiquinone; AB, 2-aminobenzoic acid; Abs, absorbance; ASQ, anionic semiquinone; BA, benzoic acid; CR, charge recombination; CT, charge transfer; D, donor; F*, photoexcited flavin; Fld, flavodoxin from D. vulgaris; NfnI, NADH-dependent ferredoxin-NADP⁺ oxidoreductase I from P. furiosus; NADOX, NADH oxidase from T. thermophila; NR, nitroreductase from E. cloaca; NSQ, neutral semiquinone; OX, oxidized; PAB, 2-(phenylamino)benzoic acid; PADS, phase-associated difference spectrum/spectra; TAS, transient absorption spectroscopy.
Rapid electron transfer distinguishes electron bifurcation

force for electron transfer (ET) into an endergonic pathway. Thus, observation of a short-lived ASQ provided important confirmation of the proposal for flavin-based electron bifurcation in this complex flavin–and iron-sulfur cluster–containing enzyme (1, 8).

Although necessary, an unstable SQ may not suffice to produce bifurcating activity. Specifically, we have proposed that conservation of energy requires that the system include a mechanism allowing only one electron to pass down the exergonic path, thus constraining the other electron to pass along the endergonic path from the high-energy SQ (2). We additionally propose that highly efficient ET would be a signature of bifurcating activity (9) because this could effectively propagate the high-energy electron to carriers distant from the low-energy (exergonic) carriers. Conformational change has also been invoked as a mechanism that could interrupt/disconnect a path for ET once one electron has taken that path, thus preventing a second one from following (6, 10). We note that in NfnI, this appears to be built into the natures of the cofactors present. Thus, the flavin proposed as the site of bifurcation is flanked on two sides by iron-sulfur clusters. An Fe₅S₄ cluster believed to have a high potential can readily accept one electron but not a second because of the very large energetic separation between successive oxidation states of such clusters (9). However, the other cluster is only 7.5 Å away based on the crystal structures (9, 10) and provides a highly competitive second path for electron escape from the flavin. Although the midpoint potential of this Fe₅S₄ cluster is very low, this is offset regarding ET efficiency by the short distance between it and the flavin. Moreover on-going electron propagation is enhanced by a subsequent short and favorable ET step to another nearby Fe₅S₄ cluster that increases the irreversibility of ET. Thus, existing data suggest that an unstable SQ state is essential in conjunction with efficient ET along two physically separate paths.

To elucidate the significance of efficient ET in conferring bifurcating activity on a flavin, we have compared the bifurcating flavoenzyme NfnI with two non-bifurcating flavoenzymes that nonetheless also thermodynamically suppress the SQ state. Nitroreductase from Enterobacter cloacae (NR) (11, 12) and NADH oxidase from Thermus thermophilus (NADOX) (13, 14) are structural homologues that are known or shown herein to lack a stable SQ state (15). Their comparison with NfnI thus holds constant the suppression of SQ, so we can focus on other features that may contribute to the ability of NfnI to bifurcate. As a control system with a very stable SQ, we have also included flavodoxin from Desulfovibrio vulgaris (Fld) (16) in our comparisons.

A critical concept is the distinction between thermodynamic and kinetic stability. Thermodynamic instability (suppression at equilibrium) is not necessarily accompanied by rapid reaction, although a correlation is common. A thermodynamically unstable species can persist if no efficient mechanism is available for its reaction. Thus, bifurcation is expected to require not only driving force based on favorable thermodynamics, but also mechanistic features that allow it to kinetically outcompete other potential fates for the high-energy species proposed.

To address this, we extended the TAS approach that was successful in NfnI to detect ASQ in each of our systems and discovered ASQ lifetimes ranging over 2 orders of magnitude, demonstrating that short lifetime alone does not necessarily imply thermodynamic instability of the flavin ASQ per se. Moreover, the lifetime of NADOX ASQ was comparable with that of NfnI ASQ, showing that a short-lived ASQ is not a guarantee of bifurcating activity, although we maintain that it is necessary. We are able to understand the different ASQ lifetimes represented in our set of proteins in terms of the presence of ET relays able to mediate multiple ET steps and thereby separate the photogenerated hole and electron from one another, versus the dominance of simple electron donors that result in the photogenerated hole remaining close to the ASQ, where it can rapidly recombine with the ASQ.

Results

TAS as a function of time after a short pulse of irradiation at 400 nm was used to probe for possible formation of ASQ (Fig. 1). Photochemical formation of ASQ is understood to be the result of photoexcited flavin (F*) abstracting an electron from a nearby residue, such as Trp or Tyr (17, 18), producing the radical form of the donor residue (Trp⁺ or Tyr⁺; see Fig. 3) (18–20). In flavoproteins where the ASQ is thermodynamically unstable, it is expected to react rapidly, reducing a nearby electron carrier or recombining with the donor radical to return to the oxidized (OX) state. Thus, the time courses of ASQ absorbance (365–375 nm) and OX absorbance (454–467 nm) are expected to reveal ASQ formation and loss of OX upon illumination, which both eventually return to pre-illumination levels.

We compared four different flavin sites using TAS. As reported previously (9), photoexcitation of NfnI produced increased absorbance at 365 nm compared with reference intensities (positive ΔA₃65 upon subtraction of the spectrum without prior illumination). NR and NADOX also yielded
increased $A_{375}$ or $A_{370}$, respectively, and spectral analysis described below confirms their attribution to ASQ. This is the first observation of SQ in NR and NADOX, wherein the SQ state is thermodynamically unstable (supplemental Fig. S1) (15). Absorbance at 370 nm was also produced in Fld but was too short-lived to provide quality spectra and definitive assignment of the species present (supplemental Figs. S2 and S3).

Our set of flavin environments produced flavin ASQ lifetimes spanning $>2$ orders of magnitude, indicating that decay kinetics are exquisitely responsive to the context of the flavin and potentially informative on the processes contributing to ASQ decay (Fig. 1). In each case, absorbance diagnostic of OX recovered on a similar or slightly longer time scale, indicating recovery of the pre-illumination state of the system (19, 21). NR had the longest-lived ASQ, consistent with naive expectations for non-bifurcating systems where the ASQ need not be very strongly suppressed. However, the comparable lifetimes of ASQ in NfnI and NADOX suggest that the capacity to bifurcate is not an automatic consequence of a short-lived unstable ASQ and, thus, that short-lived ASQ cannot be interpreted alone as diagnostic of bifurcation activity. The most rapid ASQ decay in our set is that of Fld, which is only fleetingly observed at our time resolution, consistent with the studies of He et al. (22).

To understand the significance of ASQ lifetime, it is necessary to examine the mechanism by which ASQ decays, because comparison of rates is not meaningful if they correspond to different events. Thus, for the example of NR, the time courses of absorbance at several diagnostic wavelengths were compared, and these revealed intervals dominated by distinct processes (Fig. 2). Phase 1 was chosen as the time in which negative intensity at 455 nm had already been established but in which negative intensity at 565 nm was still growing, as was positive intensity at 375 nm. At later times (phase 2), intensity at 375, 455, and 565 nm had all stabilized, but weaker intensity at 515 nm displayed continued growth. In phase 3, negative intensity at 565 nm had begun to return to zero as had positive inten-

---

**Figure 2.** TAS of NR reveals long lifetimes for multiple dynamic components and permits observation of events that are difficult to observe in the other more rapidly evolving systems studied. **a**, select phases (boxes) and transients (traces) used in the identification of species and dominant dynamics, respectively. Time 0 was adjusted by $+1.99$ ps. $\Delta A_{abs}$, difference absorbance. **b**, transient difference spectra (photoexcited minus not photoexcited) at times corresponding to the start and end of each phase. **c**, sample equilibrium spectra for the four flavin redox states: OX, hydroquinone (HQ), ASQ, and NSQ. **d**, PADS corresponding to non-equilibrium ET events following photoexcitation. The colors identifying the different phases (a) have been applied to the PADS associated with them (d) as well as the events depicted in Fig. 3a.
Rapid electron transfer distinguishes electron bifurcation

sity at 375 nm, whereas at the longest times (phase 4), we observed concerted decay at all wavelengths. To discern the identities of the species growing and decaying in each interval, difference spectra were generated by subtracting the spectrum at the beginning from the spectrum at the end for each phase. In these difference spectra, the non-illuminated background is expected to cancel out, and we can interpret the result as representing changes that occurred during that phase. The four phase-associated difference spectra (PADS) representing intervals 1–4 are shown in Fig. 2d, accompanied by the individual spectra (photoexcited minus not photoexcited) collected at the beginning and end of each phase in Fig. 2b. The approach was verified in part by observation of the expected neutral semiquinone (NSQ) signal in the Fld control (supplemental Fig. S3d). The analogous analyses of NADOX and NfnI are provided in supplemental Figs. S2 and S3.

For NR in phase 1, the PADS in Fig. 2d displays a well-formed signature of ASQ with a maximum at 377 nm accompanied by intensity from 480 to 520 nm that is also consistent with ASQ (cf. Fig. 2c and Ref. 23). Positive intensity between 510 and 530 nm has been interpreted as absorption by photoexcited flavin (F*) (19, 24) as well as Trp.

Figure 3. a, simplified scheme for ET processes resulting from photoexcitation (hv) and varying depending on the flavin environment and context in each system. Processes (arrows) and resulting species are colored according to the phases in which they are most prominent (Fig. 2a) and the PADS in which their spectral signatures appear (dark orange, ET; green, change in the flavin environment; sea foam gray, CR). H+ indicates proton transfer, b, overlay of the flavin sites of NADOX (magenta), flavodoxin (green), NfnI (tan), and NR with BA bound (blue). b was generated using Chimera (53) and the Protein Data Bank coordinate files 1NOX.pdb (13), 1KQC.pdb (34), 5JFC.pdb (9), and 1J8Q.pdb (39) for NADOX, NR, NfnI, and Fld, respectively. Distances between cofactors in NfnI are provided in Å. The active-site flavins of the four proteins were overlaid, using the L-FAD of NfnI because it is the one believed to bifurcate.

that of NR (Fig. 1). This is consistent with the lower fluorescence emission of NADOX than NR and possible quenching of F* by a Trp residue near the flavin that is replaced by Thr in the structure of NR (Fig. 3b and supplemental Fig. S4) (13). Thus, we hypothesized that the Trp distinguishing NADOX from NR serves as an electron donor in the production of ASQ, but its proximity to the flavin permits relatively rapid charge recombination (CR) that rapidly returns the populations of Trp+ and ASQ to zero (Fig. 3a).

An extreme form of the same situation is found in the flavin-binding site of Fld, which sandwiches the flavin between a Trp and a Tyr side chain (Fig. 3b). As anticipated, flavin fluorescence is strongly quenched in Fld, and this is consistent with onset of absorbance at 370 nm within the time resolution of our instrument (supplemental Fig. S2d), presumably due to electron abstraction from the nearby Trp to form ASQ (25). The Fld protein stabilizes SQ as the neutral form, and the proton transfer required to generate NSQ from ASQ can be inferred from rapid formation of difference absorption from 510 to 660 nm consistent with the spectrum of NSQ shown in Fig. 2c (supplemental Fig. S3d). Growth of this species occurs during only a short interval, consistent with the rapid disappearance of the
ASQ from which NSQ is formed. The short lifetime of the ASQ is expected due to CR with the nearby oxidized Trp$^+$ formed by electron abstraction, in processes that have been described in detail (22). Even the NSQ is short-lived in this system, despite its well-known thermodynamic stability (16). This can be understood to be a consequence of the reactivity of the Trp$^+$ radical that is present along with photogenerated flavin NSQ but absent from the Fld NSQ formed in normal biochemical contexts.

In the case of NfnI, phase 1 displays formation of ASQ at both 365 and 480–510 nm, which decays in phase 2, accompanied by recovery of OX at $\lambda_{max} = 455$ nm (supplemental Fig. S3c). However, there is no Trp or Tyr stacked against the active-site flavin (Fig. 3b), arguing against the mechanism of CR demonstrated ever, there is no Trp or Tyr stacked against the active-site flavin (9). Table 1 summarizes the species and their lifetimes obtained by fitting biphasic exponential processes yielded $K_d$ values. We speculate that this could reflect an increase in the $K_d$ upon flavin reduction, as has been demonstrated for benzoic acid (15). We note also that the use of a donor with a larger $K_d$ results in attenuation of the effect even at the same donor concentration (benzoic acid (BA); Fig. 4), as does use of a lower concentration of donor (supplemental Fig. S3).

The donors also span a range of reduction midpoint potentials ($E^\circ$; Table 2). The higher $E^\circ$ of BA would be expected to make it a less favorable source of an electron for the photoactive electron carrier cofactors that can propagate the electron further, which has been demonstrated in the case of NfnI (9). Thus, although we attribute the decay of ASQ to reoxidation in all four cases, we propose that it represents CR in NR, NADOX, and Fld, as opposed to the ET into a chain of electron carrier cofactors that can propagate the electron further, as has been demonstrated for benzoic acid (15). We note also that the use of a donor with a larger $K_d$ results in attenuation of the effect even at the same donor concentration (benzoic acid (BA); Fig. 4), as does use of a lower concentration of donor (supplemental Fig. S3).

The use of small-molecule electron donors instead of mutagenesis enabled us to vary both the reduction potential and the dissociation constant ($K_d$) of the donor (Table 2). We anticipated that if a long flavin-to-donor distance is responsible for the long lifetime of the NSQ ASQ, then provision of exogenous donors that bind close to the flavin should shorten this lifetime.

The addition of 2-(phenylamino)benzoic acid (PAB) or 2-aminobenzoic acid (AB) results in rapid decay of ASQ and restoration of OX that occurs on a time scale comparable with formation of ASQ and thus greatly diminishes the extent to which ASQ accumulates (Fig. 4). In both cases, a small fraction of the sites appear to retain a comparatively long-lived ASQ despite the large concentration of donors used compared with their $K_d$ values. We speculate that this could reflect an increase in the $K_d$ upon flavin reduction, as has been demonstrated for benzoic acid (15). We note also that the use of a donor with a larger $K_d$ results in attenuation of the effect even at the same donor concentration (benzoic acid (BA); Fig. 4), as does use of a lower concentration of donor (supplemental Fig. S3).

To test our hypothesis that the NfnI-like lifetime of the NADOX ASQ can represent CR and that the comparative absence of this behavior in NR reflects an absence of the active-site Trp seen in NADOX, we employed aromatic substrate analogs in a “chemical rescue” strategy to provide a Trp surrogate.

### Table 1

**Processes and lifetimes in proteins**

| Processes | NR | NADOX | Fld |
|-----------|----|-------|-----|
| **Lifetimes (ps)** | | | |
| ASQ (decay) | 426 ± 27 | 29 ± 3 | 14 ± 1 |
| Mechanism | CR | CR | ET |
| OX (recovery) | 513 ± 34 | 60 ± 6 | 17 ± 2 |
| F$^*$ Abs$^a$ | 443 ± 15 | 40 ± 2 | 2.3 ± 0.1 (72%$^a$) |
| F$^*$ Emission | 337 ± 17 | 16.6 ± 0.4 | 8 ± 1 |

$^a$ *Lifetime fit to a biphasic exponential process.*

### Table 2

**Effects of exogenous donors on NR ASQ decay and OX recovery**

| Donor$^a$ | $E^\circ$$^b$ | $K_d$$^c$ | Donor concentration | t (375 nm) | t (455 nm) |
|-----------|----|-----|----------------|------------|------------|
| None | 743 | 47 ± 5 | 0 | 618 ± 54 | 679 ± 48 |
| PAB | 977 | 32 ± 2 | 25 | 482 ± 21 | 636 ± 40 |
| AB | >1405 | 1405 | 2500 | 1082 ± 200 (17%) | 585 ± 74 (18%) |

$^a$ *Structures of the donors are provided in supplemental Fig. S6.*

$^b$ *All values are ±10 mV; for literature values, see Refs. 49–52.*

$^c$ *Determined via fluorescence-monitored titrations performed at 10°C in 50 mM phosphate and 50 mM KCl with 5% ethanol at pH 7.0.*

$^d$ *Fits to biphasic exponential processes yielded t (375 nm) = 0.6 ± 0.7 ps (97%) and 143 ± 98 ps (3%) and t (455 nm) = 1.5 ± 0.1 ps (85%) and 776 ± 110 ps (15%).*

$^e$ *Lifetime fit to a biphasic exponential process; monophasic fits failed to converge in these cases.*
flavin, yet it appears to have the highest yield of ASQ. Hence, it appears that the photoactive flavin is a sufficiently strong oxidant that it can draw on any of the three donors. This is consistent with the fact that none of the donors is more difficult to oxidize than Trp and Tyr, which are known sources of an electron for photoactivated flavin (17, 18). CR of ASQ with oxidized donor is expected to be more favorable for the higher $E^\circ$ donor BA, yet this is the donor with the least impact on ASQ lifetime. Therefore, it appears that driving force of ET is not the principal determinant of the variations of ASQ recombination rate in our time regime.

Efficient electron transfer is expected to reflect an effective mechanism as well as a short distance between partners and favorable thermodynamics. Consistent with this, we find that PAB, which shortens the ASQ lifetime by 10 orders of magnitude, also efficiently quenches fluorescence (confirming rapid ET; Fig. 5b) and displays a charge transfer band upon binding to NR (evidence for favorable orbital overlap and short distance (30–32); Fig. 5d). Indeed, a charge transfer (CT) band is also observed in the rapid-ET Y98W mutant of Fld (22, 33). In contrast, Fig. 5 (a and c) shows that the least effective donor (BA) does not produce a CT band and is a much poorer quencher of the flavin fluorescence. Nonetheless, titration of NR with either donor demonstrates saturation and thus binding in a discrete site, which has been shown by crystallography to be directly over the re face of the flavin for BA (34).

Rapid electron transfer distinguishes electron bifurcation

Figure 5. Fluorescence quenching (a and b) and formation of a charge transfer band near 550 nm (c and d) upon binding of the exogenous donor BA (a and c) or PAB (b and d) to NR. Panel b shows that flavin fluorescence is substantially quenched by the substrate analog PAB with saturation behavior diagnostic of specific binding (orange curve in inset of panel b) as opposed to Stern–Volmer type quenching (blue line in inset). Panel a shows that quenching of fluorescence upon the addition of BA is also best described by specific binding but with a 10-fold smaller electron transfer rate (same color convention in inset of panel a). Temperatures of 10 °C (fluorescence) and 25 °C (absorbance) are compared. For fluorescence quenching by BA, the fit to the Stern–Volmer equation (Equation 1) neglecting the data at the four highest donor concentrations yielded $y = 1.016 + 0.00046x$, and the fit to specific binding (Equation 2) yielded an intercept of $1.004 \pm 0.007$, $k_{ET}T_0 = 0.34 \pm 0.02$, and $K_d = 420 \pm 60 \mu M$. For quenching of fluorescence by PAB, the Stern–Volmer fit neglecting the data at the four highest donor concentrations yielded $y = 1.15 + 0.059x$, and the fit to specific binding yielded an intercept of $1.06 \pm 0.07$, $k_{ET}T_0 = 4.4 \pm 0.2$ and $K_d = 47 \pm 5 \mu M$. For both models, the data adhere well to the intercept of 1.00 predicted by theory. In absorbance-monitored titrations, panel c shows that BA yielded insignificant increase in absorbance near 550 nm (orange arrow in panel c and inset), but panel d shows that the addition of PAB produced a CT band near 550 nm that displayed saturation behavior consistent with specific binding (orange arrow in panel d and inset), albeit not in excellent conformity to the simplest model for independent binding at two identical sites: $A = \Delta A_{bs} \times$ [donor]/([donor] + $K_d$) + $b$, where $b$ is the background, $\Delta A_{bs}$ is the maximal absorbance change upon binding, and $K_d$ is the dissociation constant describing binding. Fit to the data yielded $K_d = 27 \pm 2 \mu M$, $\Delta A = 0.027 \pm 0.0007$, and $b = 0.002 \pm 0.0006$. The slightly lower $K_d$ observed at 25 °C is consistent with slightly greater fluorescence quenching and static quenching, albeit incomplete.
Data adhered well to a model wherein binding of the donor produces partial quenching via introduction of additional ET that augments preexisting non-radiative decay and radiative decay (fluorescence) (see supplemental materials) (35). This analysis of fluorescence quenching yields a dissociation constant that is consistent with the one that emerges from analysis of the CT band amplitude versus [PAB], supporting the model. Thus, the fluorescence quenching achieved upon saturating NR with PAB indicates that radiative decay accounts for only 1% of photoexcited flavin, versus 4% for NR in the absence of exogenous donor, based on 25% for FMN in solution (36). In contrast, BA has only a very minor effect on the fluorescence quantum yield, which remains almost 4% even at saturating BA. This is consistent with the failure of BA to produce a CT band, indicating the absence of good orbital overlap with the flavin (Fig. 5c). Thus, we conclude that the rate of ET from BA to F* is slow compared with the rate of radiative decay and infer that the rate of CR from flavin ASQ to BA** is similarly slower than from ASQ to PAB**. This provides an explanation for the discernible increase in ASQ yield observed in the presence of BA versus PAB (Fig. 4). We note that signatures of electronic coupling between cofactors were also observed in Nfnl, consistent with the rapid rates of ET between them (9).

Thus, our experiments with exogenous donors demonstrate that increased CR can change the kinetics and ASQ amplitudes displayed by NR to behaviors more closely resembling those of NADOX and even Fld (Figs. 1 and 4 and supplemental Fig. S2). Therefore, the short lifetime of NADOX can be explained by CR. This is in contrast to Nfnl, where the short lifetimes have been explained in terms of ET (9).

Discussion

Electron bifurcation was first elucidated in the guise of the quinone-mediated Q cycle in respiratory ET (7) and has more recently been recognized at the heart of a variety of enzyme complexes executing more reducing chemistry (1, 2). In the latter, it was proposed that the bifurcating step is mediated by a flavin (1), consistent with flavins’ lower potentials and inherent tendency to undergo two-electron chemistry with only weak population of the SQ species corresponding to one-electron reduction. However, there are numerous flavoenzymes that execute two-electron chemistry but not electron bifurcation. Given bifurcation’s recent recognition as a third fundamental mechanism for biological energy conservation, alongside substrate-level phosphorylation and chemiosmotic potential-based phosphorylation (37), it is important to identify factors required for a flavin site to execute bifurcation. Thermodynamic suppression of the SQ is deemed essential because this species would constitute a strongly reactive electron donor able to reduce low-potential acceptors, such as ferredoxin and semireduced flavodoxin. Generation of such an SQ is thought to be the result of oxidation of doubly reduced flavin by a high-potential electron acceptor, and this step is understood to provide the driving force for formation of the strongly reducing SQ. In the current studies, we have dispensed with a high-potential one-electron acceptor by providing the driving force for this step via photoexcitation.

A strongly reducing SQ state does not suffice to explain bifurcation, because there must be a mechanism requiring the SQ to transfer its electron to the low-potential acceptor rather than the high-potential acceptor. In Nfnl, a combination of the distances between electron carriers, and their $E^0$ values was proposed to produce rates of ET that favor rapid separation of the two electrons derived from the bifurcating flavin along two different ET paths (9). Thus the highly reactive reducing equivalent is transferred quickly to Fe$_4$S$_4$ clusters distant from the high-potential acceptor that oxidizes the initial two-electron reduced flavin, and we have directly observed a short-lived ASQ state for the bifurcating flavin (9).

Whereas the necessity of a high-energy short-lived SQ is not in doubt, the current work addresses its sufficiency. A short lifetime for a high-energy flavin SQ can reflect a variety of processes and mechanisms (Figs. 3a and 6). When oxidized flavin is photoexcited, it rapidly acquires an electron at the expense of residues nearby in a reaction that is driven by the high energy of the photoexcited state (F*). However F* can also return to the ground OX state by radiative decay in which excess energy is released as an emitted photon in fluorescence. Non-radiative decay can occur in competition, mediated by local motions that dissipate the excess energy. Additional pathways, such as inter-system crossing, have been documented (27) but are believed to be slower and thus less germane to bifurcation. Our TAS experiment is able to observe radiative decay via the emissive signal of F* as well as ET via the population of ASQ. Moreover, we can also observe the sum of all restoring processes via the recovery of OX, which (like emission) appears as a negative signal. The fate of ASQ can be addressed via formation of the optical signature of NSQ in response to proton acquisition, or simple concurrent disappearance of ASQ and restoration of OX in the case of flavin reoxidation. In this connection, several seminal studies have succeeded in observing amino acid radicals that result from initial ET to the flavin and documented ET back from ASQ to the oxidized donor D$^+$ as a mechanism for ASQ decay by CR (Fig. 6) (20, 22). Whereas we detected optical signatures with properties consistent with amino acid radicals (see supplemental Fig. S3 for signals at 515 nm (NR), 518 nm (NADOX), 522 nm (Nfnl), and 521 nm (Fld)), further studies, such as time-resolved infrared spectroscopy, are needed for a definitive assignment (38).

The several paths by which a high-energy ASQ can react provide ample justification for our finding that a short-lived ASQ does not suffice to predict or explain bifurcation activity. Not only does our non-bifurcating NADOX system display an ASQ lifetime comparable with that of bifurcating Nfnl, but Fld has the shortest ASQ lifetime of all of our systems, and it is known to stabilize a NSQ. Thus, we find that short-lived ASQ is not causative but instead is a consequence of more fundamental properties necessary for bifurcation.

Table 1 compares the ASQ lifetimes in the several systems that we have compared and summarizes the different mechanisms that appear principally responsible for ASQ decay after photochemical production. The long ASQ lifetime in NR can be explained by the relatively long distances to the amino acid side chains that most likely served as donors in the photogeneration of ASQ. ET to the NR flavin is supported by the observed
Rapid electron transfer distinguishes electron bifurcation

We cannot build ET pathways into NR to test the significance of this proposed mechanism, but we were able to manipulate the balance between charge propagation and CR by accelerating the latter via provision of exogenous donors. Indeed, upon the addition of high concentrations of donors, CR appears to occur on a time scale competitive with ASQ formation and thereby prevent its accumulation. Thus, we propose that the relatively rapid decay of NADOX ASQ represents CR with a donor radical, in this case the Trp residue near the flavin (Fig. 6). In this model, we contend that the similar ASQ lifetimes of NADOX and NfnI stem from different dominant mechanisms of ASQ decay (Table 1). We propose that the bifurcating activity specific to NfnI stems from its efficient ET chains, one of which can rereduce the donor oxidized by the photoexcited flavin, and the other of which can accept an electron from ASQ, mediating its decay. Together, these two paths can separate the photogenerated hole from the electron, forestalling rapid CR and mediating bifurcation in vivo.

In conclusion, our comparison of a set of complementary flavoproteins shows that whereas a short-lived ASQ may be an expected consequence of the high-energy SQ held to be necessary for bifurcation, it is not sufficient. By comparing and manipulating different mechanisms of ASQ reoxidation, we show that rapid CR can shorten the lifetime of a photochemically generated ASQ as much as, and even more than, the efficient ET documented previously for NfnI. The necessity for ET along two separate paths is not in doubt, but our work cautions that neither thermodynamic suppression of an SQ state nor a short-lived SQ state once formed suffices to diagnose a system as bifurcating. TAS is demonstrated to be an invaluable method of elucidating the steps by which a photochemical system evolves, and electrons may move within a system.

Experimental procedures

Reagents

The exogenous electron donors/NR substrate analogs benzoic acid (≥99.5%; catalogue no. 242381), 2-aminobenzoic acid (≥98%; catalogue no. A89855), and 2-(phenylamino)benzoic acid (≥95%; catalogue no. 78153) were obtained and used directly from Sigma. The plasmid (pKK223-3) containing the WT-NADOX gene (TTHA0425) from T. thermophilus HB8 (ATCC 27634) was a gift from Professor Erdmann (Laboratorium für Biochemie, Universität Bayreuth, Germany). Primers were ordered from Integrated DNA Technologies (Coralville, IA; Rapid HPLC Oligo service).

Production of flavodoxin

His-tagged flavodoxin was produced from the Desulfovibrio vulgaris gene cloned into pET32a+ (EMD Biosciences, Darmstadt, Germany) to add a hexahistidine tag to the N terminus (40). The protein was purified from the BL21 (DE3) strain (Novagen, Madison, WI) by IMAC according to the vendor’s instructions Qiagen, Hilden, Germany). The concentration of the protein was determined via absorbance at 272 and 445 nm (ε_{272} = 47,600 M^{-1} cm^{-1}, ε_{445} = 10,200 M^{-1} cm^{-1}) (41).
Nitroreductase production

Wild-type NR from *E. cloacae* was expressed and purified as described previously (12, 42). The concentration of holo-NR was determined based on the extinction coefficient of the bound flavin, $\epsilon_{454} = 14.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Production of wild-type NADOX

The gene for *T. thermophilus* NADOX was cloned into an overexpression vector as follows. The forward primer was designed to introduce an EcoRI restriction site, a ribosome binding site, and a silent mutation to convert a rare Ala codon to a more common Ala codon (Ala-3, GCG) better able to support overexpression in *Escherichia coli*: 5'-TTT GAA TTC GAA GGA GAT ATA CAT ATG GAA GCG ACC CTT CCC GT-3'. The reverse primer was designed to introduce a HindIII restriction site and a silent mutation to introduce a stop codon that is more common for *E. coli* (TAA): 5'-TTT TTT TAA GCT TTT AGC GCC AGA GGA CCA CC-3'. High-fidelity DNA polymerase (Phusion M0530, New England Biolabs) was used to amplify the gene for NADOX from pKK223-3 isolated from *E. coli* JM109 host cells, by PCR. Difficulties with the ligation reaction were overcome by not exposing the DNA to UV light, and the use of "touch down" PCR to obtain clean PCR products (43). The PCR product was gel-purified and ligated into digested vector pET-21a (+) (catalog no. 69740-3, EMD Biosciences). The gene sequence was confirmed by DNA sequencing.

NADOX was overexpressed from transformed *E. coli* lysY/Pl grown in Terrific Broth (12 g/liter tryptone, 24 g/liter yeast extract, 4 ml/liter glycerol, pH 7.5, supplemented with 100 $\mu$g ml$^{-1}$ ampicillin). Induction of expression and protein purification were carried out as for NR (12). Protein concentration was determined using the Bradford assay (catalog no. B6916, Sigma-Aldrich) and the flavin content was determined using $\epsilon_{450} = 17.61 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (44).

Production of NfnI

NfnI from *P. furiosus* was expressed and purified as detailed by Lubner et al. (9). Flavin content of purified proteins was determined aerobically using a standard fluorescence method (45). Briefly, flavin from the protein was released by denaturing in 0.02% SDS at 95–100°C for 30 min and subjected to phosphodiesterase cleavage (catalogue no. P5263, Abnova, Taipei City, Taiwan) prior to measuring fluorescence on a Lumina fluorescence spectrometer (Thermo Fisher Scientific).

Redox titration of NADOX

The procedures and modified cuvette described by Massey were used with only slight changes (46). An anaerobic cuvette (3 ml, 1-cm path length) was equilibrated in a glove box overnight ([O$_2$] $\leq$ 10 ppm). Solutions of 18 $\mu$M NADOX, 2 $\mu$M benzyl viologen, 400 $\mu$M xanthine in 50 mM potassium phosphate, pH 7.5 (2.982 ml) and 6 ng of xanthine oxidase (18 $\mu$l) were rendered anaerobic by 5–10 pump-purge cycles of ~30 s followed by equilibrium in the glove box for 1 h. The solution containing NADOX was placed in the cuvette along with a small magnetic stir bar. Xanthine oxidase was placed in a side arm of the cuvette, separated from the rest of the reaction mixture. After removing the sealed cuvette from the glove-box, the solution was allowed to equilibrate at 25°C in the spectrometer for 15 min. Initial scans were stable and showed the expected trace for fully reduced NADOX. The reaction was initiated by tipping the cuvette to mix the xanthine solution from the side arm into the NADOX solution in the cuvette.

Transient absorption spectroscopy

The ultrafast (100 fs to 5.1 ns) TAS spectrometer employed in this study uses an amplified 4W Ti:sapphire laser (Libra, Coherent, 800 nm, 1 kHz, 100-fs pulse width) to deliver excitatory pulses of light and a Helios spectrometer (Ultrastart Systems, LLC) to probe the sample afterward. A fraction of the 800-nm Libra output was frequency-doubled in $\beta$-barium borate to produce the desired pump wavelength (400 nm in the experiments described here) for sample excitation, which was then directed into the Helios. The pump pulses were passed through a depolarizer and chopped by a synchronized chopper to 500 Hz before reaching the sample. The pump pulse energy was 1.1 $\mu$J/pulse at the sample. Another fraction of the Libra output was guided directly into the Helios for generation of the probe. Within the spectrometer, a white-light continuum of wavelengths, including 340–800 nm, was generated using a 2-mm-thick CaF$_2$ crystal. This beam was split into a probe and a reference beam. The probe beam was focused into the sample, where it was overlapped with the pump beam. The transmitted probe and reference beams were then focused into optical fibers coupled to multichannel spectrometers with CMOS sensors with 1-kHz detection rates. The reference signal was used to correct the probe signal for pulse-to-pulse fluctuations in the white-light continuum.

For all transient absorption measurements, samples were transferred into 50 mm potassium phosphate, pH 7.5, 50 mm KCl by diafiltration (10 kDa; catalogue no. UFC501024, EMD Millipore, Billerica, MA) to remove possible traces of free flavin, immediately prior to collection of spectra. NADOX samples were at pH 7.0. Manipulations were performed in an Mbraun glove box (N$_2$ atmosphere). Each sample was sealed in a 2-mm quartz cuvette and stirred continuously to prevent photodegradation. Protein concentrations were ~250 $\mu$M for NR, NADOX, and Fld, whereas NfnI was run at 200 $\mu$M. Samples with exogenous electron donors were prepared in 250 $\mu$M NR with the exception that the sample containing 25 $\mu$M PAB was 229 $\mu$M in NR. All experiments were conducted at room temperature.

Photoexcitation of flavin at 400 nm was used to initiate formation of ASQ by generating the excited state (P$^*$), which then abstracts an electron from a nearby donor. ASQ then relaxes by various pathways to return to the oxidized ground state. The change in absorbance signal ($\Delta$Abs) was calculated from the absorbance measured by alternating probe pulses with and without prior pump pulse excitation. The time delay between the pump and probe pulses was controlled by a motorized delay stage. The data collection (350 pump shots/time point) was carried out four consecutive times and then averaged. The experiment was repeated 2–3 times for each protein to ensure similar results across different days and preparations. Data were corrected for spectral chrip using SurfaceXplorer (Ultra-
Rapid electron transfer distinguishes electron bifurcation

fast Systems). Additional time 0 ($t_0$) correction was performed by adding 1.99 ps for NR and subtracting 0.30 ps for Fld. Additional baseline correction was performed by subtracting the average AAbs from 790 to 800 for NfnI and from 780 to 800 for NADOX. Fitting for the determination of dynamic lifetimes was performed with slight adjustment to the instrument response function and $t_0$, using Surface Xplorer (Ultrafast Systems). Similar results for lifetimes were obtained using Origin Pro version 9 (Northampton, MA).

Reduction potentials of exogenous donors

Reduction potentials were determined by cyclic voltammetry of 1 mM solutions of PAB, AB, and BA using a glassy carbon working electrode, platinum wire counter electrode, and Ag/AgCl reference electrode (catalog nos. CH1104, CH1115, and CH1111 from CH Instruments Inc., Austin, TX) in 5 ml of 500 mM potassium phosphate, 100 mM KCl, pH 7.0 (47). Saturated CHI111 from CH Instruments Inc., Austin, TX) in 5 ml of 500 mM potassium phosphate, 100 mM KCl, pH 7.0 (47). Saturated CHI111 from CH Instruments Inc., Austin, TX) in 5 ml of 500 

Steady-state absorbance and fluorescence

Measurements were carried out in 1 ml of 50 mM potassium phosphate, 50 mM KCl, 5% ethanol, pH 7.0. Absorption spectra were recorded with a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer, and samples were held at 25 °C in a water-jacketed cell holder. Fluorescence quenching of 5 μM NR was monitored at 10 °C using 450-nm excitation with 5-nm slit widths in a Lumina spectrofluorometer (Thermo, Waltham, MA) using a Peltier temperature controller. Fresh stocks of PAB, AB, and BA were made for titrations using 10-μl aliquots. The mechanism of quenching was probed by fitting the data in Kaleidagraph (Synergy Software) to consider Stern–Volmer versus specific binding–type behavior,

$$F_0/F = c + K_{sv}[Donor]$$

(Eq. 1)

$$F_0/F = c + K/(1 + K_d/[Donor])$$

(Eq. 2)

where $F_0/F$ is the fluorescence in the absence of electron donor divided by the fluorescence at a given concentration of donor, $K_{sv}$ is the Stern–Volmer constant for nonspecific quenching, $K = k_{ET}τ_0$ is the electron transfer rate constant ($k_{ET}$) times the natural lifetime of the emissive state ($τ_0$), and $K_d$ is the dissociation constant for specific binding (35).

Author contributions—J. P. H. prepared all samples prior to analysis, conducted equilibrium experiments, performed detailed analyses, produced NR and NADOX proteins, designed and produced figures, and contributed to editing the paper. C. E. L. and M. W. R. designed and performed the transient absorption spectroscopy experiments; C. E. L. contributed to analysis of the resulting data. G. J. S. and D. M. N. N. provided NADH-dependent ferredoxin-NADP+ oxidoreductase samples and helped to guide interpretation of results in the context of electron bifurcation. K. W. H. produced NR and NADOX proteins and helped with equilibrium experiments; M. W. W. A. and P. W. K. provided feedback on data interpretation and presentation. A.-F. M. conceived experiments, designed and produced figures, engaged in interpretation, and wrote the paper.

References

1. Buckel, W., and Thauer, R. K. (2013) Energy conservation via electron bifurcating ferredoxin reduction and proton/Na+ translocating ferredoxin oxidation. Biochim. Biophys. Acta 1827, 94–113
2. Peters, J. W., Miller, A. F., Jones, A. K., King, P. W., and Adams, M. W. (2016) Electron bifurcation. Curr. Opin. Chem. Biol. 31, 146–152
3. Scott, J. D., and Ludwig, R. A. (2004) Azorhizobium caulinodans electron-transferring flavoprotein N electrochemically couples pyruvate dehydrogenase complex activity to Nf, fixation. Microbiology 150, 117–126
4. Huang, J. I., Heiniger, E. K., McKinlay, J. B., and Harwood, C. S. (2010) Production of hydrogen gas from light and the inorganic electron donor thiosulfate by Rhodopseudomonas palustris. Appl. Environ. Microbiol. 76, 7717–7722
5. Edgren, T., and Nordlund, S. (2004) The fixABCX genes in Rhodospirillum rubrum encode a putative membrane complex participating in electron transfer to nitorgenase. J. Bacteriol. 186, 2052–2060
6. Chowdhury, N. P., Mowafy, A. M., Demmer, J. K., Upadhyay, V., Koelzer, S., Jayamani, E., Kahnt, J., Hornung, M., Demmer, U., Ermiller, U., and Buckel, W. (2014) Studies of the mechanism of electron bifurcation catalyzed by electron transferring flavoprotein (Etf) and butyryl-CoA dehydrogenase (Bedc) of Acidaminococcus fermentans. J. Biol. Chem. 289, 5145–5157
7. Mitchell, P. (1975) The protonmotive Q cycle: a general formulation. FEBS Lett. 59, 137–139
8. Nitschke, W., and Russell, M. J. (2012) Redox bifurcations: mechanisms and importance to life now, and at its origin. Bioessays 34, 106–109
9. Lubner, C. E., Jennings, D. P., Mulder, D. W., Schur, G. J., Zavadnyy, O. A., Hoben, J. P., Tokmina–Lukaszewska, M., Berry, L., Nguyen, D. M., Lipscornb, G. L., Bothner, B., Jones, A. K., Miller, A. F., King, P. W., Adams, M. W. W., and Peters, J. W. (2017) Mechanistic insights into energy conservation by flavin-based electron bifurcation. Nat. Chem. Biol. 13, 655–659
10. Demmer, J. K., Huang, H., Wang, S., Demmer, U., Thauer, R. K., and Ermiller, U. (2015) Insights into flavin-based electron bifurcation via the NADH-dependent reduced ferredoxin-NADP oxidoreductase structure. J. Biol. Chem. 290, 21985–21995
11. Koder, R. L., Jr., and Miller, A.-F. (1998) Steady state kinetic mechanism, stereospecificity, substrate and inhibitor specificity of Enterobacter cloacae nitroreductase. Biochim. Biophys. Acta 1387, 394–405
12. Pitsawong, W., Hoben, J. P., and Miller, A.-F. (2014) Understanding the broad substrate repertoire of nitroreductase based on its simple mechanism. J. Biol. Chem. 289, 15203–15214
13. Hecht, H.-J., Erdmann, H., Park, H. J., Sprinzl, M., and Schmid, R. D. (1995) Crystal structure of NADH oxidase from Thermus thermophilus. Nat. Struct. Biol. 2, 1109–1114
14. Park, H.-J., Reiser, C. O. A., Kondurweit, S., Erdmann, H., Schmid, R. D., and Sprinzl, M. (1992) Purification and characterization of a NADH oxidase from the thermophile Thermus thermophilus HB8. Eur. J. Biochem. 205, 881–885
15. Koder, R. L., Haynes, C. A., Rodgers, M. E., Rodgers, D. W., and Miller, A.-F. (2002) Flavin thermodynamics explain the oxygen insensitivity of enteric nitroreductases. Biochemistry 41, 14197–14205
16. Curley, G. P., Carr, M. C., Mayhew, S. G., and Voordouw, G. (1991) Redox and flavin-binding properties of recombinant flavodoxin from Desulfovibrio vulgaris (Hildenborough). Eur. J. Biochem. 202, 1091–1100
17. Liu, Z., Tan, C., Guo, X., Li, J., Wang, L., Sanar, A., and Zhong, D. (2013) Determining complete electron flow in the cofactor photooxidation of oxidized photolyase. Proc. Natl. Acad. Sci. U.S.A. 110, 12966–12971
18. Mathes, T., van Stokkum, I. H. M., Stierl, M., and Kennis, J. T. M. (2012) Redox modulation of flavin and tyrosine determines photoinduced proton-coupled electron transfer and photoactivation of BLUF photoreceptors. J. Biol. Chem. 287, 31725–31738
19. Hardman, S. J. O., Pudney, C. R. H., and Scrutton, N. S. (2013) Excited state dynamics can be used to probe donor-acceptor distances for H-tunneling reactions catalyzed by flavoproteins. Biophys. J. 105, 2549–2558
20. Gauden, M., Grinstead, J. S., Laan, W., van Stokkum, I. H. M., Avila-Perez, M., Toh, K. C., Boelens, R., Kaptein, R., van Grondelle, R., Hellingwerf,
