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# Use of Flavin Photochemistry to Probe Intraprotein and Interprotein Electron Transfer Mechanisms

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Photoexcitation of flavin analogs generates the lowest triplet state (via intersystem crossing from the first excited singlet state) in the nanosecond time domain and with high quantum efficiency. The triplet, being a strong oxidant, can abstract a hydrogen atom (or an electron) from a reduced donor in a diffusion-controlled reaction. If the donor is a redox protein, the oxidation process can be used to initiate an electron transfer sequence involving either intramolecular or intermolecular reactions. If the donor is an organic compound such as EDTA, the neutral flavin semiquinone will be produced by H atom abstraction; this is a strong reductant and can subsequently transfer a hydrogen atom (or an electron) to an oxidized redox protein, thereby again initiating a sequence of intramolecular or intermolecular processes. If flavin photoexcitation is accomplished using a pulsed laser light source, the initiation of these protein electron transfer reactions can be made to occur in the nanosecond to microsecond time domain, and the sequence of events can be followed by time-resolved spectrophotometry to obtain rate constants and thus mechanistic information. The present paper describes this technology, and selected examples of its use in the investigation of redox protein mechanisms are given.

**KEY WORDS:** Time-resolved spectrophotometry; transient kinetics; redox proteins; flavin triplet state; flavin semiquinone; protein–protein interactions.

## INTRODUCTION

Intraprotein and interprotein electron transfer processes are key components of biological energy conversion systems such as respiration, photosynthesis, nitrogen fixation, etc. Although these reactions can in principle be monitored by time-resolved spectrophotometry, a major requirement for the application of such methodology is the ability to synchronize observation with reaction progress. In the photosynthetic system, which is the paradigm for the use of time-resolved spectroscopy to elucidate electron transfer mechanisms, this is made possible by the fact that light absorption by chlorophyll molecules initiates the injection of electrons into the transport pathway, a process which occurs exceedingly rapidly (picoseconds). Thus, pulsed laser excitation can be used as the triggering event, and the progress of electrons through the various carriers can be observed by a variety of spectroscopic signatures. In our laboratory, we have developed technology to mimic this in nonphotobiological redox systems (Jung and Tollin, 1981; Ahmad *et al.*, 1981; Simondsen and Tollin, 1983; Tollin *et al.*, 1986; Cusanovich *et al.*, 1988; Tollin and Hazzard, 1991; Tollin *et al.*, 1993), utilizing the well-known excited-state chemistry of riboflavin and its analogs as the means of reaction initiation. The following discussion will provide an overview of this methodology, along with some selected examples from our recent work.

## PRIMARY PHOTOCHEMICAL PROCESSES

The lowest triplet state of flavins ($^3$F), which can be populated in a few nanoseconds via intersystem

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crossing (ISC) from the first excited singlet state ($^1F$), is a strong oxidant, capable of removing H atoms or electrons from a variety of molecules. Triplet state formation has a high quantum efficiency; for example, in the case of riboflavin, approximately 70% of the absorbed photons generate the triplet species. The redox properties of the flavin triplet can be utilized either to produce a strong reductant, the flavin semiquinone ($F^{\bullet}$), by oxidizing a sacrificial donor ($AH_2$), or to directly oxidize a reduced redox protein ($P_{red}$), according to the following equations:

$$F + h\nu \rightarrow {}^1F \xrightarrow{ISC} {}^3F \quad (1)$$

$${}^3F + AH_2 \rightarrow FH^{\bullet} + AH^{\bullet} \quad (2)$$

$${}^3F + P_{red} \rightarrow F^{\bullet} + P_{ox} \quad (3)$$

Reactions (2) and (3), written as H atom transfer and electron transfer, respectively, are generally diffusion-controlled, and thus can be made to occur on the microsecond time scale (the exact time course depends upon the magnitude of the second-order rate constants for these reactions, and the concentrations of $AH_2$ or $P_{red}$). If EDTA (or similar donors) are used as the reactant in Eq. (2), the $AH^{\bullet}$ produced is highly unstable and rapidly ($< < 1$ ms) undergoes decarboxylation, reduction of an additional molecule of oxidized flavin to generate another neutral flavin semiquinone $FH^{\bullet}$, and fragmentation to produce stable products (Traber *et al.*, 1982). The flavin anion semiquinone ($F^{\bullet}$) formed in reaction (3) will equilibrate, depending upon the pH and its $pK_a$, with the neutral semiquinone species (for example, the $pK_a$ for riboflavin is 8.4). The flavin semiquinones are both kinetically unstable and strong reductants (the reduction potential for riboflavin semiquinone is $-230$ mV and for deazariboflavin semiquinone is $-650$ mV). Thus, in the absence of any other reducible species, the semiquinone will disproportionate to form oxidized and reduced flavin [Eq. (4)], or, in the presence of an oxidized redox protein, will transfer a reducing equivalent to the protein prosthetic group [Eq. (5)]:

$$2\,FH^{\bullet} \rightarrow F + FH_2 \quad (4)$$

$$FH^{\bullet} + P_{ox} \rightarrow F + P_{red} \quad (5)$$

Reactions (4) and (5) are, of course, in competition with one another; thus, if the concentration of $P_{ox}$ is high enough, reaction (5) will predominate. One must keep this competition in mind, however, in interpreting experimental data. An additional complication can arise due to the fact that $FH_2$ is also a strong reductant [at least in the case of ordinary flavins; with deazaflavins, the $FH_2$ species is much less reactive (Edmondson *et al.*, 1972)]. As a consequence, at low $P_{ox}$ concentrations, a secondary protein reduction can occur via $FH_2$, which contributes to the overall kinetics and must be taken into account. This additional reaction can usually be recognized by two properties: it is slower than the initial reduction, and its amplitude diminishes with increasing protein concentration. A similar complication occurs in the protocol of reaction (3); thus, the initial protein oxidation reaction generates a stoichiometric amount of flavin semiquinone, which can then react further via Eqs. (4) and (5). Again, however, this occurs on a much slower time scale than reaction (3), and can usually be readily deconvoluted.

Figure 1 presents examples of reactions (2)–(5). In panel A is shown a transient obtained under anaerobic conditions (dioxygen is an efficient triplet quencher, and also can act as an electron acceptor) at a monitoring wavelength of 500 nm with 5-deazariboflavin (dRf) in 100 mM phosphate buffer, pH 7.0, in the presence of 1 mM EDTA. The initial rapid rise in absorbance corresponds to the formation of $dRfH^{\bullet}$ via reactions (1) and (2); the subsequent decay is due to the second-order disproportionation reaction of Eq. (4). Panel B corresponds to an experiment in which yeast flavocytochrome $b_2$ (lactate dehydrogenase) is present in the sample cell under these same conditions (Walker and Tollin, 1991). As its name implies, this enzyme contains both flavin (FMN) and heme *b* cofactors; at the monitoring wavelength used (438.5 nm), the principal absorbing species is the FMN center. The observed absorbance decrease subsequent to flash excitation corresponds to the bimolecular reduction of the FMN prosthetic group by $dRfH^{\bullet}$ via reaction (5). Panel C shows a kinetic transient obtained at 557 nm, a wavelength which monitors mainly heme. A biphasic absorbance increase is seen, which reflects the direct reduction of the heme by $dRfH^{\bullet}$ via reaction (5) (rapid phase), and a slower interprotein transfer from a reduced FMN center in one protein molecule to an oxidized heme center in another (see Walker and Tollin, 1991 for details; see below for further discussion of interprotein reactions). The interprotein process can also be observed at 438.5 nm, on a slower time scale than that presented in panel B (data not shown; cf. Walker and Tollin, 1991).

Figure 1, panel D, shows a transient obtained with a sample containing dRf and reduced flavocytochrome $b_2$ in the absence of EDTA. The loss of absorbance at

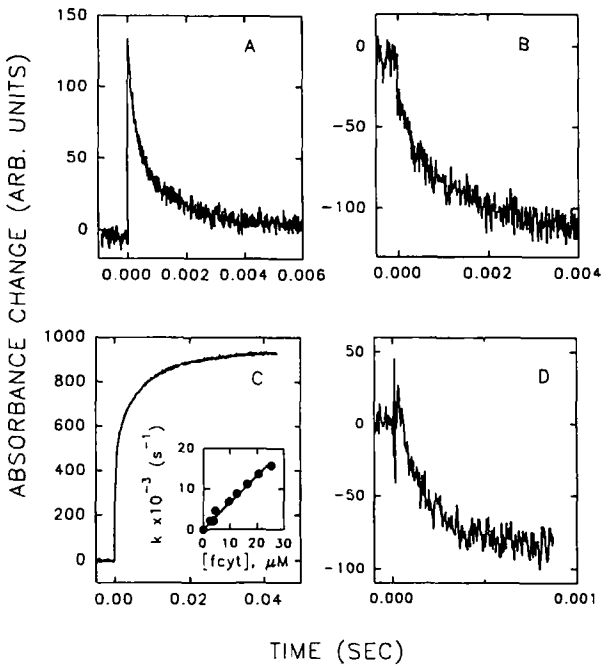


**Fig. 1.** (A) Transient obtained at 500 nm from solution containing 90 μM 5-deazariboflavin and 1 mM EDTA in 100 mM phosphate buffer, pH 7.0, showing formation and disproportionation of deazariboflavin semiquinone. (B) Transient obtained at 438.5 nm from solution in A after addition of 3.6 μM oxidized flavocytochrome $b_2$ (from *Saccharomyces*), showing reduction of FMN cofactor by deazariboflavin semiquinone. (C) Transient obtained at 557 nm from solution in A after addition of 7.2 μM oxidized flavocytochrome $b_2$, showing biphasic reduction of heme cofactor by deazariboflavin semiquinone. Inset shows protein concentration dependence of fast phase of heme reduction. (D) Transient obtained at 557 nm from solution containing 100 μM deazariboflavin and 3.5 μM reduced flavocytochrome $b_2$, showing oxidation of reduced heme cofactor by deazariboflavin triplet state.

557 nm reflects heme oxidation by the dRf triplet state; the observed rate constant obtained from these data is 6600 $s^{-1}$, which corresponds to a second-order rate constant of $1.9 \times 10^9$ $M^{-1}s^{-1}$, consistent with diffusion control.

By monitoring these reactions as a function of protein concentration, accurate values for the second-order rate constants for reactions (3) and (5) can be determined. An example is shown in the inset to Fig. 1, panel C; the second-order rate constant (for the initial rapid phase of heme reduction by $dRfH^{\bullet}$) obtained from these data is $7.1 \times 10^8$ $M^{-1}s^{-1}$; cf. Walker and Tollin, 1991. Although the electron transfer processes represented by Eqs. (3) and (5) are nonphysiological, they can provide useful information. Thus, by varying the structure of the flavin it is possible to probe the steric and electrostatic environment of a protein redox center (Tollin *et al.*, 1986; Cusanovich *et al.*, 1988). This can be used as a method for assessing functional relationships between members of a homologous series of redox proteins (kinetic taxonomy). It is also possible to use these reactions to test the functional integrity of a redox center in a site-specific mutant (Hurley *et al.*, 1993).

Some additional features of the methodology are also worthy of note: (i) the intensity of the pulsed laser which is used to initiate the photochemistry is purposely kept low, so that substoichiometric quantities of flavin triplet and semiquinone are generated relative to the redox protein; this assures that pseudo-first-order conditions apply, and that only a single electron can be removed or added to each protein molecule; (ii) the irradiated volume in the sample cell is kept small (< 1% of the total volume) so that only minor conversion to product occurs, and thus samples can be subjected to multiple flashes for signal averaging and for determination of protein concentration dependences; (iii) because the oxidant and/or reductant is generated exogenously, no chemical modification of the protein is necessary. This last point is of some interest because it also provides one of the limitations of this methodology, i.e., since the initiation process depends upon *bimolecular* chemistry [reactions (2), (3), and (5)], in some cases this becomes the major factor controlling the time resolution of the experiment. Other contributions to this volume (Durham, Gray, Millett) describe unimolecular protocols to achieve rapid reaction initiation, in which the light-activated chromophore is covalently attached to the redox protein. Another limitation of the present methodology derives from the fact that light absorption is used to initiate the reaction; thus, the wavelength range over which observation can be made without perturbing the sample is necessarily proscribed by this fact.

## INTRAPROTEIN ELECTRON TRANSFER REACTIONS

If the reduced protein which is present in reaction (3), or the oxidized protein in reaction (5), has more than one redox center (denoted by A and B below, with the assumption that reaction occurs initially at site A), subsequent intraprotein electron transfer processes can occur, as shown in Eq. (6):

$$A_{ox}B_{red} \overset{k_{et}}{\rightleftarrows} A_{red}B_{ox} \quad (6a)$$

$$A_{red}B_{ox} \overset{k_{et}}{\rightleftarrows} A_{ox}B_{red} \quad (6b)$$

This, of course, is subject to both thermodynamic and kinetic constraints, i.e., the initial reaction must occur predominantly (although not necessarily exclusively) at the center having the appropriate redox potential [the higher potential for reaction (6a) and the lower potential for reaction (6b)], and rapidly enough so that the secondary transfer is observable. Examples of reaction (6a) are given in Fig. 2, in which the substrate-reduced form of the multi-copper protein ascorbate oxidase (panel A; Hazzard *et al.*, 1994a), and substrate-reduced flavocytochrome $b_2$ (panel B; Hazzard *et al.*, 1994b), are oxidized by deazariboflavin triplet state. In the case of ascorbate oxidase, the kinetics were monitored at 610 nm, a wavelength which reflects the oxidation state of the blue Type 1 copper center. The biphasic absorbance increase which is observed (cf. panel A), corresponding to oxidation of the Type 1 center, has rate constants of $10^4$ $s^{-1}$ and $1.2 \times 10^3$ $s^{-1}$ which are independent of protein concentration (data not shown; cf. Hazzard *et al.*, 1994a), indicating two parallel intramolecular processes. The interpretation of these results is that the initial oxidation by dRf triplet occurs at the trinuclear Type 2,3 center, which has no appreciable absorption in the wavelength region accessible to this methodology ($\lambda > 400$ nm, because of light absorption by dRf), followed by intraprotein electron transfer from the reduced Type 1 copper to each of these centers, occurring in individual protein molecules. It is of interest to point out that the rate constants for these intramolecular transfers are one to two orders of magnitude larger than is observed for internal electron equilibration following reduction of the Type 1 center in the fully oxidized enzyme (data not shown; $k = 1.6 \times 10^2$ $s^{-1}$, cf. Meyer *et al.*, 1991). Thus, the redox state of the enzyme apparently controls the rates of intramolecular communication between the various copper centers. Although the structural basis for this is presently unknown, it is clearly an important component of the enzymatic reaction process.

In the flavocytochrome $b_2$ case (panel B), the initial rapid absorbance decrease corresponds to heme oxidation by dRf triplet. (cf. also Fig. 1, panel D). This is followed by a protein concentration-independent (data not shown) return of absorbance corresponding to intramolecular electron transfer from reduced FMN to oxidized heme (Hazzard *et al.*, 1994b).

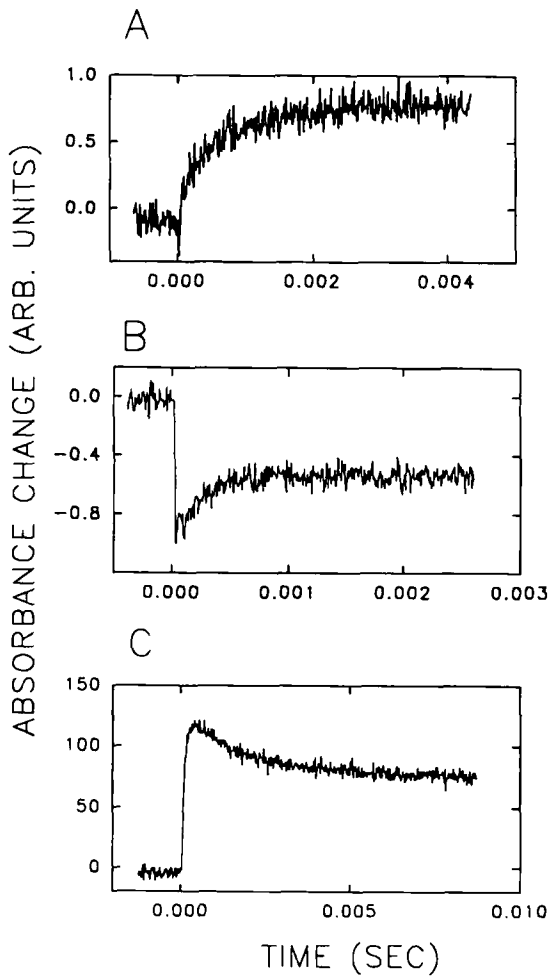


**Fig. 2.** (A) Transient obtained at 610 nm from solution containing 100 μM deazariboflavin and 40 μM reduced ascorbate oxidase (from zucchini) in 100 mM phosphate buffer, pH 7.0, showing biphasic oxidation of reduced Type 1 copper center via intramolecular electron transfer to Type 2,3 center. (B) Transient obtained at 557 nm from solution containing 100 μM deazariboflavin and 15 μM reduced flavocytochrome $b_2$ in 100 mM phosphate buffer, pH 7.0, showing oxidation of reduced heme cofactor by deazaflavin triplet state and intramolecular re-reduction via electron transfer from reduced FMN cofactor. (C) Transient obtained at 557 nm from solution containing 90 μM deazariboflavin, 1 mM EDTA, 5 mM pyruvate, and 5.1 μM oxidized flavocytochrome $b_2$ in 100 mM phosphate buffer, pH 7.0, showing reduction of heme cofactor and intramolecular reoxidation via electron transfer to FMN cofactor (compare with Fig. 1C).

An example of reaction (6b) is given in Fig. 2, panel C (Walker and Tollin, 1991), in which oxidized flavocytochrome $b_2$ is reduced by dRfH$^{\bullet}$ in the presence of pyruvate, which is the enzymatic product of lactate

dehydrogenation. The rapid initial rise in absorbance corresponds to heme reduction via reaction (5). The subsequent absorbance decrease, which is protein concentration-independent (data not shown), corresponds to the reoxidation of the heme via intramolecular transfer to the FMN cofactor [reaction (6b)]. This trace should be compared to that shown in Fig. 1, panel C, which shows a comparable experiment carried out in the absence of pyruvate. In this latter situation, intramolecular electron transfer is not observed. This result has been interpreted (Walker and Tollin, 1991) as an example of ligand-gated intraprotein electron transfer, a process which is of considerable interest in terms of control mechanisms in redox enzymes. It is also important to note that, for the fully reduced flavocytochrome $b_2$, pyruvate binding turns off the intramolecular electron transfer process shown in Fig. 2, panel B (data not shown; cf. Hazzard *et al.*, 1994b). Thus, as with ascorbate oxidase, the intramolecular communication between redox centers changes with the state of reduction of the cofactors. Again, the structural basis for this is presently unknown.

## INTERPROTEIN ELECTRON TRANSFER REACTIONS

If a second redox protein is present under the conditions represented by Eq. (3) or (5), secondary protein–protein electron transfer reactions can occur, again subject to thermodynamic and kinetic constraints (see above). In this situation, however, the constraints can be modulated by having the higher-potential (under oxidizing conditions) or lower-potential (under reducing conditions) protein present in stoichiometric excess, thus favoring removal of electrons from (or entry of electrons into) the appropriate protein in the initial reaction with exogenous flavin. An example of this for the reductive protocol is given in Fig. 3. In panel A, a transient is shown which was obtained (Walker *et al.*, 1991) at 507 nm in a dRf/EDTA solution (phosphate buffer, pH 7.0, 310 mM ionic strength) containing 35 μM ferredoxin and 10.3 μM ferredoxin:NADP$^+$ reductase (both proteins from the cyanobacterium *Anabaena* PCC 7119). This wavelength corresponds to an isosbestic point for the FAD cofactor of the reductase, and thus the absorbance change monitors the [2Fe-2S] cluster of the ferredoxin (and also the dRfH$^\bullet$ species). As is evident, immediately after the laser flash there is a rapid rise in absorbance due to dRfH$^\bullet$ formation. This is followed

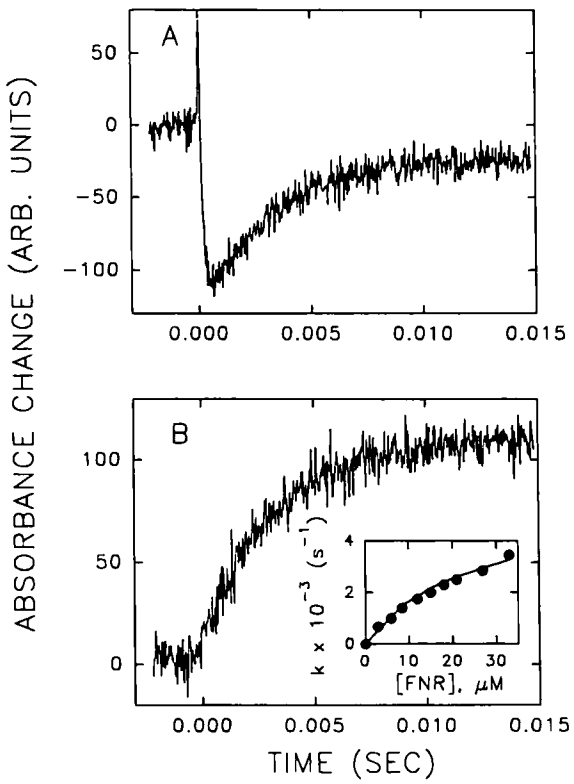


**Fig. 3.** (A) Transient obtained at 507 nm from solution containing 100 μM deazariboflavin, 0.5 mM EDTA, 35 μM ferredoxin (from *Anabaena* 7119), and 10.3 μM ferredoxin:NADP$^+$ reductase (from *Anabaena* 7119) in 4 mM phosphate buffer, pH 7.0, ionic strength 310 mM (adjusted with NaCl), showing ferredoxin reduction and reoxidation. (B) Transient obtained from solution in A at 600 nm showing reduction of FAD cofactor of ferredoxin:NADP$^+$ reductase. *Inset:* Ferredoxin:NADP$^+$ reductase concentration dependence of rate constant for FAD reduction by reduced ferredoxin (ionic strength 10 mM). Solid curve corresponds to theoretical fit based on minimal two-step mechanism (see text).

by a sharp absorbance decrease corresponding to ferredoxin reduction and dRfH$^\bullet$ oxidation. The subsequent slow increase in absorption is a consequence of ferredoxin reoxidation due to electron transfer to the reductase. The latter is confirmed by measurement at 610 nm (panel B), a wavelength which monitors FAD semiquinone formation; the rate constant obtained from the 610 nm rise is the same as that obtained from the slow increase at 507 nm, consistent with this interpretation. By varying the concentration of the higher potential protein (in this case the ferredoxin reductase), a second-order rate constant for the interprotein reaction can be obtained. In a situation in which the reaction proceeds via an intermediate complex, as shown by the (minimal) mechanism in Eq. (7), a nonlinear con-

centration dependence may be observed (an example of this is shown in the inset to Fig. 3, panel B):

$$P^1_{red} + P^2_{ox} \overset{K_d}{\rightleftarrows} [P^1_{red} - P^2_{ox}] \overset{k_{et}}{\rightarrow} P^1_{ox} + P^2_{red} \quad (7)$$

Such data can be fit using a nonlinear least squares procedure based upon the exact solution to the differential equations describing this mechanism (Simondsen *et al.*, 1982; Simondsen and Tollin, 1983), to obtain values for $K_d$ and the limiting first-order rate constant $k_{et}$ (a minimum value for the second-order rate constant for complex formation can also be obtained from this analysis). Note that $K_d$ refers to the interaction between reduced $P^1$ and oxidized $P^2$, a situation which is *only* observable by kinetic methodology. For the case shown in the inset to Fig. 3, the values obtained are $K_d = 8$ μM and $k_{et} = 3800\ s^{-1}$ (the minimum value for the second-order rate constant is $3 \times 10^8\ M^{-1}s^{-1}$; Walker *et al.*, 1991).

An example of interprotein electron transfer for the oxidative protocol is given in Fig. 4. In this experiment (Hazzard and Tollin, unpublished data), lactate-reduced *Saccharomyces* flavocytochrome $b_2$ and reduced horse heart cytochrome *c* were flashed in the presence of dRf. The upper panel shows a transient obtained at 547 nm (an isosbestic point for the heme of flavocytochrome $b_2$); the initial loss in absorbance corresponds to cytochrome *c* oxidation by the flavin triplet and the slower rise represents heme *c* re-reduction. The latter process has a $k \simeq 300\ s^{-1}$, a value which is protein concentration independent (not shown). In the lower panel, a transient obtained at 557 nm (an isosbestic point for cytochrome *c*) is presented. Again, the initial absorbance decrease corresponds to heme *b* oxidation by the flavin triplet (occurring in parallel with heme *c* oxidation). The slower absorbance decrease, which reflects a secondary heme oxidation, has the same protein concentration-independent rate constant ($300\ s^{-1}$) as does the heme *c* reduction process shown in the upper panel. We interpret these data in terms of intracomplex electron transfer from the flavocytochrome heme to the heme prosthetic group of cytochrome *c*.

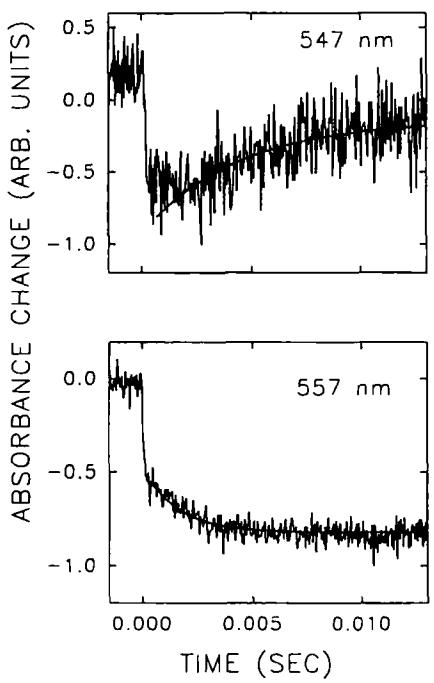


**Fig. 4.** Transients obtained at 547 and 557 nm from solution of 100 μM deazariboflavin, 20 μM *Saccharomyces* flavocytochrome $b_2$ and 20 μM horse heart cytochrome *c* in 10 mM phosphate buffer, pH 7.0, containing 80 mM NaCl. Proteins reduced prior to laser photolysis by addition of 50 μM L-lactate. Solid curves through data correspond to single exponential fits.

## CONCLUSION

As the above discussion has illustrated, the photochemical generation of strong oxidants and reductants, coupled with time-resolved spectrophotometry, allows the direct observation of the kinetics of intraprotein and interprotein electron transfer processes in a wide variety of redox proteins. The major limitations of this technology lie in the use of bimolecular chemistry to initiate the reactions, which places an upper limit on the time resolution, and the necessity of restricting observation to wavelengths which are not absorbed by the photoactive species. Although the first of these limitations can be obviated by covalent attachment of the photoinitiator to a redox protein, this approach presents additional problems associated with the possibility of introducing structural perturbations into the system caused by the chemical modification. The second limitation, although intrinsic to all photochemical methodologies, is not a problem when high-energy radiation is used as the means of generating the initial electron donor or acceptor. Again, however, such pulse radiolysis procedures have their own unique difficulties, primarily associated with the exceedingly high reactivity (and therefore low specificity) of the free radical species formed under the conditions of the experiment. As is always true, one must accept tradeoffs in using any of these procedures, and therefore

each of them has a place in the armamentarium of the redox enzymologist.

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