Intramolecular Electron Transfer in Yeast Flavocytochrome b_2 upon One-Electron Photooxidation of the Fully Reduced Enzyme: Evidence for Redox State Control of Heme−Flavin Communication†

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ABSTRACT: Flavocytochrome b_2, which has been fully reduced using L-lactate, can be rapidly oxidized by 1 equiv using the laser-generated triplet state of 5-deazariboflavin. Parallel photoinduced oxidation occurs at the reduced heme and at the fully reduced FMN ($FMNH_2$) prosthetic groups of different enzyme monomers, producing the anion semiquinone of FMN and a ferric heme. Following the initial oxidation reaction, rapid intramolecular reduction of the ferric heme occurs with concomitant oxidation of $FMNH_2$, generating the neutral FMN semiquinone. The observed rate constant for this intramolecular electron transfer is 2200 s^{-1}, which is 1 order of magnitude larger than the turnover number under these conditions. A slower reduction of the heme prosthetic group also occurs with an observed rate constant of approximately 10 s^{-1}, perhaps due to intersubunit electron transfer from reduced FMN to heme. The rapid intramolecular electron transfer between the $FMNH_2$ and ferric heme is eliminated upon addition of excess pyruvate (K_i = 3.8 mM). This latter result indicates that pyruvate inhibition of catalytic turnover apparently can occur at the $FMNH_2 \rightarrow$ heme electron transfer step. These results markedly differ from those previously obtained (Walker, M. C., & Tollin, G. (1991) *Biochemistry 30,* 5546−5555) and confirmed here for electron transfer within the one-electron reduced enzyme and for the effect of pyruvate binding, suggesting that intramolecular communication between the heme and flavin prosthetic groups can be controlled by the redox state of the enzyme and by ligand binding to the active site.

Flavocytochrome b_2 from *Saccharomyces cerevisiae* (L-lactate dehydrogenase, EC 1.1.2.3) is a mitochondrial enzyme which catalyzes the two-electron oxidation of L-lactate and the subsequent reduction of two cytochrome *c* molecules (Appleby & Morton, 1954; Labeyrie et al., 1978; Capeillère-Blandin et al., 1980; Labeyrie, 1982), providing an alternative respiratory pathway in yeast (Pajot & Claisse, 1974). The structural, catalytic, biophysical, and molecular biological properties of the enzyme have been recently reviewed by Chapman et al. (1991). Flavocytochrome b_2 has been the subject of much investigation in order to elucidate the three primary reactions carried out by the enzyme: oxidation of substrate, intramolecular electron transfer, and reduction of a second substrate, mitochondrial cytochrome *c*. The three-dimensional structure of the *Saccharomyces* flavocytochrome has been solved to 2.4 Å resolution (Xia et al., 1987; Xia & Mathews, 1990), and this has provided an impetus for the design of a variety of site-specific mutants in order to more fully understand the biochemistry of the enzyme (reviewed in Chapman et al., 1991; cf. also Kay & Lippay, 1992; Miles et al., 1992, 1993; White et al., 1993; Silvestrini et al., 1993; Balme & Lederer, 1994; Rouvière-Fourmy et al., 1994).

The protein exists as a tetrameric ensemble of monomers in solution, each monomer having a molecular weight of 57 000 (Pajot & Claise, 1974). The determination of the three-dimensional crystal structure confirmed what had been suggested by limited proteolytic digestion of the *S. cerevisiae* enzyme (Labeyrie et al., 1966), i.e., that the two prosthetic groups, an FMN and a noncovalently bound protoheme, are located within two distinct domains of the monomeric subunit (Xia et al., 1987; Xia & Mathews, 1990). The larger flavin domain is comprised of 411 residues of the total 511 amino acids (Lederer et al., 1985) and is the site of L-lactate dehydrogenation, via α-hydrogen abstraction (Lederer, 1974; Pompon et al., 1980). The b-type heme is contained within the smaller domain (residues 1−99), which is the site of cytochrome *c* reductase activity (Baudras et al., 1971; 1972; Capeillère-Blandin, 1982; Janot, 1990; Tegoni et al., 1983, 1990, 1993; Thomas et al., 1983; Vanderkooi et al., 1980). The two domains are connected by a strand of polypeptide comprised of approximately 10 amino acids, referred to as the hinge region (Xia et al., 1987; Xia & Mathews, 1990). Recently, it has been shown that the structure of this hinge is vital to the catalytic properties of the *S. cerevisiae* enzyme (White et al., 1993). Furthermore, the primary and secondary structure of the hinge region may be what distinguishes the *S. cerevisiae* flavocytochrome from the structurally homologous, but kinetically distinct, enzyme from *Hansenula anomala* (Black et al., 1989; White et al., 1993). The most commonly accepted mechanism for the physiological catalytic cycle of the *S. cerevisiae* enzyme is as follows (cf. Chapman et al. 1991 and references therein):

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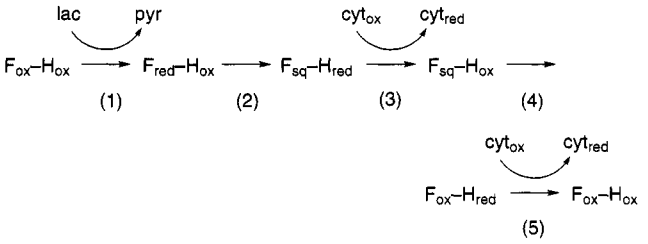

where F_{ox},[1] F_{red}, and F_{sq} are the oxidized, fully reduced, and semiquinone species of FMN, while H_{ox} and H_{red} correspond to the oxidized and reduced cytochrome b_2 heme. The rate-determining step for catalysis of the flavocytochrome b_2 from *Saccharomyces* has been suggested to be the dehydrogenation of L-lactate in reaction 1 (Lederer, 1974; Capeillère-Blandin et al., 1975). However, based on a decrease in the kinetic isotope effect for heme reduction relative to that for FMN reduction when cytochrome *c* is used for an electron acceptor in stopped-flow experiments, intramolecular electron transfer from reduced FMN to the heme (reaction 2) also has been suggested to have a limiting effect on the overall catalytic cycle (Pompon et al., 1980; Miles et al., 1992; Rouvière-Fourmy et al. 1994). Reactions 2 and 4 correspond to the two intramonomer electron transfer steps, while the reduction of the two cytochrome *c* molecules occur in reactions 3 and 5. Furthermore, it should be noted that in the above scheme, the enzyme cycles between either the two- or one-electron reduced and the fully oxidized species (Capeillère-Blandin et al., 1975), and never achieves full, i.e., three equivalent, reduction. The enzyme can, however, be fully reduced by titration with 1.5 equiv of lactate per monomer, in which case three reducing equivalents are present, two at the FMN and one at the heme (Capeillère-Blandin et al., 1975). In such a titration, due to the significantly lower redox potential of the FMN (−64 mV) relative to the heme (+10 mV) (Walker and Tollin, 1991; Kay & Lippay, 1992), reduction of the heme always precedes complete reduction of the FMN.

It has been shown that pyruvate, the product of lactate dehydrogenation, acts as a competitive inhibitor ($K_i = 3$ mM) for catalytic turnover (Lederer, 1978). Prior to elucidation of the three-dimensional structure of the enzyme, it was suggested for the *H. anomala* protein that this occurs by thermodynamic stabilization of the FMN semiquinone (Tegoni et al., 1984a; 1986; 1990), resulting in an inhibition of the interdomain electron transfer between this species and the oxidized heme (reaction 4) following reduction of the first cytochrome *c*. Thus, reduction of the second cytochrome *c* molecule cannot occur (reaction 5), and the catalytic cycle would be inhibited. Indeed, it has been shown by several investigators (for recent discussions of this phenomenon see Walker & Tollin, 1991; 1992; Kay & Lippay, 1992) that the F_{ox}/F_{sq} reduction potential of the flavin increases by approximately 70 mV in the presence of pyruvate, making this couple isopotential with the heme. Additionally, it has been suggested that pyruvate preferentially binds to the redox state of the enzyme which contains the semiquinone species of the FMN (Tegoni et al., 1986, 1990). Thus, the prevailing idea concerning the factor controlling electron transfer between the FMN and the b_2 heme has been thermodynamic stabilization of an intermediate produced at the end of the reaction scheme.

An interesting aspect of the crystallography of flavocytochrome b_2 is that two of the four molecules in the tetramer contain bound pyruvate (Xia et al., 1987; Xia & Mathews, 1990). In the pyruvate-bound monomers, increased disorder of the cytochrome domains did not permit determination of their structure, whereas the flavin domains were unaffected. In the pyruvate-free monomers, the structures of both the flavin and the heme domains were resolved. The interpretation of this aspect of the enzyme structure is important in terms of understanding the structural basis for intramolecular electron transfer within the enzyme. NMR experiments (Labeyrie et al., 1988) suggest that, in solution and in the *absence* of pyruvate, the cytochrome domain is quite dynamic relative to what would be expected if it were tightly complexed to the bulkier flavin domain. Furthermore, Tegoni and Mathews (1988) have suggested that the crystal packing forces in the pyruvate-free enzyme may minimize the intrinsic flexibility of the cytochrome domain.

Despite this, it has been argued that the geometries of the two prosthetic groups are "optimized" for electron transfer in the pyruvate-free structure, based on the relatively short distance (9.7 Å) and coplanar orientation of the prosthetic groups. Mutation of Tyr 143 (which lies between the two prosthetic groups and supposedly plays a role in interdomain hydrogen bonding) to a Phe resulted in a marked decrease in the rate constant for electron transfer between the FMN and heme, in both stopped-flow (Miles et al., 1992) and flash photolysis experiments (Tollin et al., 1993). Further, changing the relative orientations of the prosthetic groups by splicing the shorter hinge region from *H. anomala* into the *S. cerevisiae* enzyme also resulted in a decrease in the rate constant for intramolecular heme reduction (White et al., 1993). However, as discussed in Tollin and Hazzard (1991), our relative lack of understanding of intermolecular and interdomain electron transfer in redox proteins makes it difficult to propose "optimal" electron transfer pathways, based on static crystal structures, especially when protein−protein (or domain−domain) dynamics are known to be intrinsic to the system. Despite these uncertainties, the elucidation of the crystal structure of flavocytochrome b_2 has provided us with a basis for understanding the *kinetic* effect of pyruvate on the inhibition of enzymatic turnover.

In the present work, we have investigated the kinetics of electron equilibration within the two-electron reduced state of yeast flavocytochrome b_2, following the rapid *in situ* oxidation of the three-electron reduced enzyme by the triplet state of 5-deazariboflavin. In the initial photoinduced reaction, parallel oxidation of the fully reduced FMN and ferrous heme of two separate enzyme monomers occurs (two-electron oxidation within a single monomer is unlikely because of the low intensity of the laser flash). In those molecules containing $FMNH_2$ and oxidized heme, subsequent intramolecular electron transfer results in the re-reduction of the heme by the fully reduced FMN, with an observed

[1] Abbreviations: cyt_{ox}, cyt_{red}, oxidized and reduced cytochrome *c*, respectively; dRF, oxidized 5-deazariboflavin; ^{3}dRF, triplet state of 5-deazariboflavin; dRFH$^\bullet$, 5-deazariboflavin semiquinone; EDTA, ethylenediaminetetraacetate; FMN, flavin mononucleotide; F$^\bullet$, neutral semiquinone of flavin mononucleotide ($FMNH^\bullet$); $F^{\bullet -}$, anionic semiquinone of flavin mononucleotide ($FMN^{\bullet -}$); F_{sq}, semiquinone of flavin mononucleotide (no ionization state specified); F_{red}, fully reduced or dihydroquinone form of flavin mononucleotide ($FMNH_2$); H_{ox}, H_{red}, oxidized and reduced cytochrome b_2 domain of flavocytochrome b_2; lac, L-lactate; pyr, pyruvate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

rate constant of 2200 s^{-1}. In the presence of pyruvate, this rapid reoxidation of the reduced FMN is *not* observed. Thus, the results indicate that pyruvate is capable of binding to the fully reduced flavin domain, and inhibition of electron transfer can occur earlier in the catalytic scheme (i.e., reaction 2) than previously believed. The intramolecular electron transfer properties and the effect of pyruvate are different from those reported by Walker and Tollin (1991, 1992) for the one-electron reduced enzyme, suggesting that intramolecular communication between the two redox moieties in flavocytochrome b_2 is also dependent upon the state of reduction of the enzyme.

MATERIALS AND METHODS

Flavocytochrome b_2 from *Saccharomyces cerevisiae* was purified according to procedures described for the intact noncrystalline form given by Labeyrie et al. (1978). The ratio of A_{280} to A_{424} for the reduced b_2 heme was 0.50, in good agreement with the literature value of 0.49 (Labeyrie et al., 1978). The average molar activity of the purified enzyme was 480 s^{-1}, as determined by the reduction of $K_3Fe(CN)_6$ by enzymatic oxidation of L-lactate at 30 °C, in 0.1 M phosphate (pH = 7.0) buffer, containing 10 mM L-lactate, 1 mM $K_3Fe(CN)_6$, and 1 mM EDTA, which is within the range of 450−550 s^{-1} given by Labeyrie et al. 1978). SDS-PAGE gels indicated a single band of 57 kD. The protein was stored anaerobically on ice as a 70% ammonium sulfate suspension in the presence of excess DL-lactate in a sealed culture tube.

In order to prepare samples for kinetic experiments, an aliquot was removed from the suspension and passed through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer, pH = 7.0. During the elution, resolubilization and oxidation of the enzyme occurs. Fractions containing oxidized flavocytochrome were collected and concentrated to final volume of ≤50 μL and a concentration of ≥200 μM. The concentration of the oxidized enzyme was determined from the absorbance at 413 nm using ϵ = 129.5 mM^{-1} cm^{-1} (Labeyrie et al., 1978). To determine the stability of the enzyme during this procedure we monitored two parameters. First, the L-lactate oxidase/ferricyanide reductase activity of the enzyme was determined after elution of the enzyme from the G-25 column and after concentration of the oxidized enzyme, which typically required 35 min using Amicon and Centricon concentrators. No significant loss of activity was found. Second, band ratios for the oxidized enzyme at 365, 412, and 530 nm were compared at the various steps in the procedure. Again, no significant changes were observed. Loss of FMN during these procedures would be expected to have changed both the spectral properties and the enzyme activity. Thus, the absence of such changes is evidence that the enzyme used in our laser flash photolysis experiments is fully functional.

All laser flash experiments were performed anaerobically at 24 °C in cuvettes containing 0.5 mL of a solution of 0.1 M phosphate buffer, pH = 7.0, containing 100 μM 5-deazariboflavin (dRF). Aliquots of the concentrated, oxidized enzyme were added to the above reaction buffer before deaeration. Deoxygenation of the reaction solution was achieved by alternate cycles of evacuation and argon flushing. Fully reduced enzyme was obtained by titrating the anaerobic solution containing oxidized enzyme with 1.5 equiv of L-lactate (Sigma) per flavocytochrome heme. The extent of reduction was determined from a reduced minus oxidized difference spectrum using $\Delta\epsilon_{556}$ = 21.5 mM^{-1} cm^{-1} (Labeyrie et al., 1978). The enzyme was considered fully reduced when no further spectral changes were observed at 438 and 556 nm. Titration of the enzyme by L-lactate results in the formation of an equimolar concentration of pyruvate. However, since the concentration of the added lactate is much smaller than K_i (~3 mM) for pyruvate (cf. Results), the amount of enzyme having bound pyruvate should be vanishingly small. Thus, in keeping with the nomenclature of Tegoni et al. (1984) we refer to this form of the enzyme as "pyruvate free". The lactate solution was made anaerobic prior to titration of the enzyme by bubbling with water-saturated, O_2-sparged Ar.

For reactions involving titration with excess pyruvate, addition was made following reduction of the enzyme with L-lactate using a freshly prepared stock solution, which had been made anaerobic in a manner similar to that used for lactate. All transfers of pyruvate were made using stringent anaerobic techniques with Hamilton gas-tight syringes. The maximum volume of pyruvate solution added was 10 μL. The visible spectrum of the sample taken immediately after pyruvate addition gave no indication of oxidation of the enzyme due to oxygen leakage into the solution, which would result in the generation of partially oxidized flavocytochrome species. During the course of our investigations, we found that there was variability in the magnitude of the effect of pyruvate in the laser flash photolysis experiments, depending upon its commercial source. Walker and Tollin (1991, 1992) also observed such a dependence in their experiments, and we have confirmed these results as well. The observations reported by Walker and Tollin were made using pyruvate which had been recrystallized from ethanol. We have found that the pyruvate obtained from Sigma (Cat. No. P-2256, Lot. No. 92H0071) gave kinetic results closely similar to those obtained with recrystallized pyruvate, in both the experimental procedure of Walker and Tollin (1991) and in the present experiments.

The possible involvement of dRFH•, generated during oxidation of the enzyme by the deazaflavin triplet, in reactions with the partially oxidized enzyme, was determined by placing a neutral density filter in the pathway of the laser pulse. This served to decrease the amount of semiquinone produced by the laser flash and would be expected to alter the kinetic behavior of electron transfer reactions involving this species. Intramolecular processes, as well as the initial oxidation reaction which proceeded in the presence of a large excess of reduced enzyme, should be unaffected by this procedure. All kinetic traces obtained were analyzed by computer fitting using SI-FIT (On-Line Instruments Systems) or by manual semilog plots of signal vs time. Both procedures gave equivalent results.

Laser photoexcitation of dRf was carried out with a nitrogen laser-pumped dye laser (LN-102, Photochemical Research Associates, Inc.), as described in Tollin and Hazzard (1991). All spectra were obtained using a Cary-15 UV−visible spectrophotometer, equipped with an On-Line Instrument System modification.

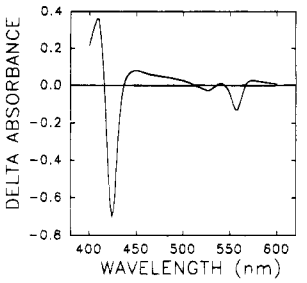

FIGURE 1: Steady-state oxidized minus reduced difference spectrum of flavocytochrome b_2. Flavocytochrome b_2 (6 μM) was titrated with L-lactate until no change in the visible spectrum was observed. All solutions were prepared using 100 mM phosphate buffer, pH 7.0.

RESULTS

A. Oxidation of Fully Reduced Flavocytochrome by ^{3}dRF

1. Steady-State Difference Spectrum. Figure 1 shows a steady-state oxidized minus reduced difference spectrum of flavocytochrome b_2 which was obtained by subtracting the spectrum of the fully reduced enzyme, produced by titration with L-lactate, from that of the oxidized enzyme. The most significant feature of this spectrum, for the purposes of the discussion of the laser flash photolysis experiments described below, is that it is clearly dominated by the heme contribution in the Soret (424 nm) and α-band (557 nm) regions, although the FMN cofactor contributes as much as the heme to the changes observed in the 450 nm region. This is due to the much larger absorptivity of the heme relative to either the oxidized or fully reduced FMN. However, it should be noted that the FMN semiquinone species (both neutral and anionic), which are relevant to the flash photolysis experiments discussed below, are *not* observed in this spectrum.

2. Flash-Induced Kinetics. Figure 2A shows a typical kinetic trace, monitored at 557 nm, occurring within 5 ms after a solution containing fully reduced flavocytochrome (produced by titration of the oxidized enzyme with a 1.5-fold molar excess of L-lactate under anaerobic conditions) and dRF was irradiated with a laser flash at 400 nm, a wavelength which excites the oxidized dRF to the triplet state (^{3}dRF). The large decrease in absorbance observed at 557 nm is consistent with the oxidation of the reduced heme cofactor (see below). The oxidation reaction is rapid with a bimolecular[2] rate constant of 1.7×10^9 M^{-1} s^{-1} (see insert in Figure 2A). This value is at the limits of diffusional control and agrees well with data obtained for oxidation of other redox proteins by free triplet state flavins (Roncel et al., 1990; Navarro et al., 1991; Hazzard et al., 1994). At reduced protein concentrations which are >10 μM, the oxidation reaction is over within a few microseconds and does not interfere with any subsequent slower reactions.

In order to characterize the transient species formed in this initial oxidation reaction, a time-resolved difference

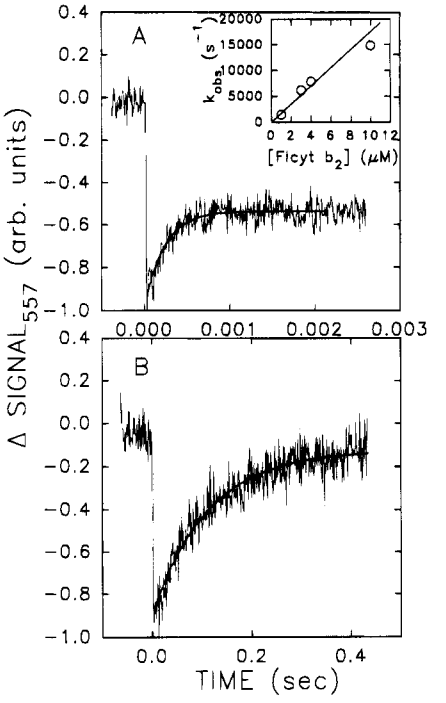

FIGURE 2: Transient absorbance changes at 557 nm upon laser flash photolysis of fully reduced flavocytochrome b_2 (15 μM) in 100 mM phosphate buffer (pH 7.0) containing 100 μM dRF. Laser excitation was at 406 nm. (A) The rapid loss of absorbance at t = 0 corresponds to oxidation of the flavocytochrome heme by ^{3}dRF. The subsequent increase in absorbance corresponds to re-reduction of the heme. The smooth curve through the data represents a single exponential fit with a rate constant of 2200 s^{-1}. The trace corresponds to the sum of five laser flashes. (Insert) Plot of observed rate constant vs enzyme concentration for the initial oxidation of the reduced enzyme by the flavin triplet. (B) The return of the signal at 557 nm to the preflash baseline on a 400 ms time scale for the same sample as shown in A. The smooth curve represents a single exponential fit to the data giving a rate constant of 12 s^{-1}.

spectrum was obtained over the wavelength range accessible in the present system (wavelengths below 450 nm cannot be measured due to dRF excitation by the monitoring light). This difference spectrum is shown in Figure 3A (open circles). There are two prominent spectral features to be noted. First, the sharp absorbance decrease at 557 nm, corresponding to a loss in intensity at the α-band of the cytochrome heme resulting from its oxidation by ^{3}dRF, is quite obvious. The isosbestic points are somewhat different than those expected based on the steady-state difference spectrum shown in Figure 1. This is due, in part, to the significantly larger slit width of the laser apparatus (2 mm) relative to that of the spectrophotometer (<0.1 mm) used to acquire the steady-state spectrum. Furthermore, the β-band of the heme at 535 nm does not bleach as much as would be expected if the oxidation reaction involved the heme moiety alone. Second, there is an underlying broad spectral species which has a positive absorbance change throughout the entire range of the difference spectrum. In order to identify this latter species, a normalized steady-state oxidized minus reduced difference spectrum of deflavocytochrome b_2 (Figure 3A, solid curve)[3] was subtracted from the transient spectrum, the result of which is shown by the closed triangles

[2] When freshly prepared samples of enzyme were used for these studies, oxidation of the enzyme observed at 557 nm occurred by a single exponential process, from which k_{obs} values were obtained for the insert to Figure 2A. However, with enzyme samples that were stored as ammonium sulfate suspensions for extended periods of time, the oxidation reaction became biphasic in nature. Concomitant with this was a decrease in the steady-state activity of the enzyme and an elimination of the rapid electron transfer observed subsequent to the initial oxidation (see below). We have taken this behavior to be diagnostic of enzyme degradation.

[3] Normalization was accomplished using the peak to trough ratios of A_{575}/A_{556} for the deflavo-enzyme and the 5 ms transient spectrum.

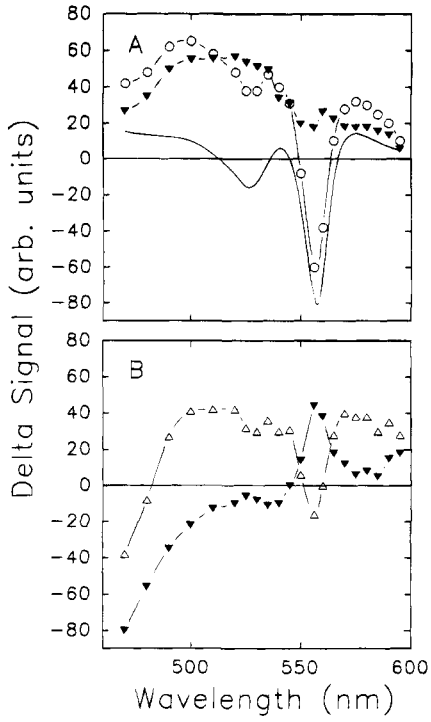

FIGURE 3: Time-resolved difference spectra for the oxidation and re-reduction of fully reduced flavocytochrome b_2 in the absence of pyruvate. All conditions were as in Figure 2. (A) (open circles) Difference spectrum obtained at $t = 0$ following laser excitation of the sample (cf. Figure 2A); (solid line) normalized oxidized minus reduced difference spectrum (see Results) for deflavocytochrome b_2 (from Walker and Tollin, 1991); (closed triangles): difference spectrum generated by subtraction of the deflavocytochrome b_2 spectrum from the $t = 0$ difference spectrum. (B) (open triangles) Difference spectrum obtained at $t = 1.5$ ms after laser excitation; (closed triangles): difference spectrum obtained by subtracting the initial transient spectrum from the $t = 1.5$ ms spectrum. Both the $t = 0$ and $t = 1.5$ ms spectra were measured relative to the pre-flash baseline.

in Figure 3A. The λ_{max} in the 490−510 nm region is consistent with the formation of the neutral semiquinone of dRF (dRFH$^\bullet$) (Walker & Tollin, 1991), which is expected to be generated by oxidation of the heme by the triplet deazaflavin:

$$H_{red}-F_{red} + {}^3dRF + H^+ \rightarrow H_{ox}-F_{red} + dRFH^\bullet \quad (6)$$

However, the relative amplitude of the positive signal at 510 nm (which is an isosbestic point for the heme redox difference spectrum) is too large to be attributed only to the 1 mol of dRFH$^\bullet$ produced per mole of heme oxidized. On the basis of previous determinations (Pan et al., 1991), attenuation of the α-band of a typical cytochrome heme is known to be ~50% at the slit width used for these experiments, whereas broad-banded spectra are much less prone to band attenuation.[4] Correcting the observed absorbance change at 556 nm for this attenuation, we calculate a corrected ratio of absorbances at 510 to 557 nm of 1:2. dRFH$^\bullet$ has a $\Delta\epsilon_{510} = 3.5$ mM^{-1} cm^{-1} (Visser & Fendler, 1982; Heelis et al., 1989), whereas the heme has a $\Delta\epsilon_{557} = 21.5$ mM^{-1} cm^{-1} (Labeyrie et al., 1978; there is very little dRF or FMN contribution at 557 nm, ≤4%), giving a ratio of difference extinction coefficients of 1:6. Thus, the observed absorbance ratio, when corrected for heme α-band attenuation, is clearly inconsistent with a mole ratio of one dRFH$^\bullet$ produced per heme oxidized.

In addition to having characteristics attributable to dRFH$^\bullet$ formation, the transient spectrum in Figure 3A is also similar to that of the anion semiquinone of FMN (F$^{\bullet-}$), which is the semiquinone form stabilized by flavocytochrome b_2 and which has a maximum absorbance at 490−510 nm and a shoulder at 550 nm (Edmondson & Tollin, 1983; Tegoni et al., 1984b, 1986). In contrast, the neutral FMN semiquinone (F$^\bullet$) has a λ_{max} at 580−590 nm, which is considerably red-shifted relative to the anionic species (Edmondson & Tollin, 1983). Thus, if the initial oxidation of the enzyme by ^{3}dRF also oxidized $FMNH_2$, then two additional flavin semiquinone species would be generated, namely F$^{\bullet-}$ and a second molecule of dRFH$^\bullet$. At the resolution of the laser flash experiment, the spectra of dRFH$^\bullet$ and F$^{\bullet-}$ would be virtually indistinguishable. Therefore, assuming equal amounts of heme and $FMNH_2$ oxidation, the total flavin contribution to the absorbance change at 510 nm would be comparable with the absorbance change for the heme, in good agreement with the corrected absorbance ratio observed, i.e., 1:2. Thus, we conclude that the following additional reaction also occurs in the initial oxidation of the enzyme:

$$H_{red}-F_{red} + {}^3dRF \rightarrow H_{red}-F^{\bullet-} + dRFH^\bullet + H^+ \quad (7)$$

Inasmuch as the reduced enzyme concentration is much larger than that of the ^{3}dRF generated by the laser flash, the oxidation of heme and of F_{red} must occur in separate molecules of the flavocytochrome. It should be noted that the simultaneous, but parallel, oxidation of the heme and flavin cofactors of two reduced flavocytochrome molecules is analogous to the simultaneous reduction of these two moieties observed upon dRFH$^\bullet$ reduction of the oxidized enzyme (Walker & Tollin, 1991), thus suggesting that both cofactors are approximately equally accessible to exogenous flavins in both oxidation states.

Subsequent to the initial oxidation of the enzyme, there is a second kinetic phase that appears as a partial (60%) return of the negative signal at 557 nm toward the preflash baseline (Figure 2A). This reaction is complete within approximately 1 ms after the laser flash. Figure 3B shows the time-resolved difference spectrum (at $t = 1.5$ ms) for this second species (open triangles). A difference spectrum obtained by subtracting the $t = 0$ ms spectrum (Figure 3A) from the spectrum at $t = 1.5$ ms is also shown in Figure 3B (filled triangles). These difference spectra indicate that there are two distinct spectral species in this second kinetic process. First, the contribution of the heme redox difference spectrum is most obvious at 557 nm, where there is an increase in absorbance to a more positive value at $t = 1.5$ ms, indicating heme reduction. Furthermore, we consistently observe a large and significant increase in absorbance at 580−600 nm. The presence of an absorbance band in this region is diagnostic of the formation of a *neutral* FMN semiquinone (F$^\bullet$) (Edmondson & Tollin, 1983), inasmuch as the heme contribution in this region is minimal (see Figure 1). A

[4] The attenuation of the heme α-band is due to the fact that the half-band width is quite narrow, approximately 8 nm, and that there is a sign change on both the blue and red wavelength sides of the λ_{max}. Due to the small concentration of ^{3}dRF produced per laser flash, a relatively wide slit width (2 mm) was used in order to maximize the signal/noise ratio. For the deazaflavin semiquinone, the spectrum is very broad and does not change sign in the spectral region of interest, and thus attenuation is not expected or observed.

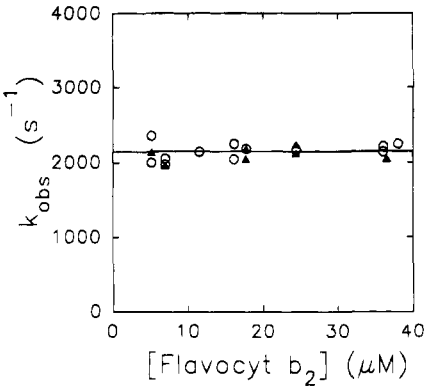

FIGURE 4: Plot of k_{obs} vs flavocytochrome concentration for the initial return of signal at 557 nm in the absence of pyruvate: (open circles) data obtained without neutral density filter; (closed triangles) data obtained using a 0.5 OD neutral density filter. Straight line represents the mean value obtained for all the data, $k = 2150\ s^{-1}$. Reaction conditions were as in Figure 2. For each concentration, titration of the flavocytochrome with L-lactate was performed immediately prior to the laser flash photolysis experiment.

mechanism for the formation of this species will be addressed in more detail below.

The rapid return of the signal at 557 nm can be adequately fit with a single exponential at all concentrations of enzyme studied. A plot of the dependence of the heme re-reduction rate constant as a function of enzyme concentration is shown in Figure 4. Note that the value of the observed rate constant, $2150 \pm 155\ s^{-1}$, is clearly enzyme concentration independent. In order to determine the effect of ^{3}dRF concentration (and thus dRFH$^\bullet$ concentration) on the rate constant for this kinetic process, neutral density filters were used to decrease the intensity of the laser beam which initiates the photochemistry within the reaction cuvette.[5] If the rapid re-reduction of the heme were due to a bimolecular reaction with dRFH$^\bullet$ generated in the initial oxidation reaction, the value of k_{obs} would be expected to decrease upon insertion of a 0.5 OD neutral density filter. As shown in Figure 4 (filled symbols), there was no significant effect of the neutral density filter on the values for the rate constant at the concentrations indicated. Taken together, these results clearly indicate that the rapid heme reduction process shown in Figure 2A occurs via an *intramolecular* reaction.

Intramolecular re-reduction of the transiently oxidized cytochrome b_2 heme can occur by two processes. First, if the dRFH$^\bullet$ produced in the oxidation reaction were still bound to the enzyme, then a concentration-independent re-reduction could be observed. However, the results of Walker and Tollin for the reduction of oxidized *S. cerevisiae* (1991) and *H. anomala* (1992) flavocytochromes by dRFH$^\bullet$ showed no evidence for the formation of a complex between the free flavin and the enzyme. Thus, we consider this possibility to be highly unlikely in these experiments as well. A second means of obtaining intramolecular re-reduction of the heme would involve reaction with a reduced prosthetic group within the enzyme itself. As noted above, there is a large increase in absorbance in the region from 580 to 600 nm, due to the formation of F$^\bullet$, concomitant with the return of the signal at 557 nm. Within the limits of the experiment, the rate constants obtained at both wavelengths are equivalent. We interpret this to mean that the rapid re-reduction of the heme prosthetic group is a result of an intramolecular electron transfer from the F_{red} cofactor.

As shown in Figure 2A and noted above, the return of the signal on a fast time scale corresponds to 60% of the initial loss of absorbance due to oxidation of the heme. This suggests that an equilibrium is being established, and thus the value for k_{obs} must represent the sum of the rate constants for the forward and reverse reactions (k_f and k_r, respectively) for the intramolecular electron transfer (see eq 8). Assuming that the initial oxidation reaction generates equimolar amounts of oxidized heme and flavin semiquinone within the separate enzyme molecules (i.e., 50% $H_{ox}-F_{red}$ and 50% $H_{red}-F_{sq}$), a 60% return of the heme absorbance would correspond to a final equilibrium consisting of 80% reduced cytochrome b_2 (i.e., $H_{red}-F_{sq}$, see eq 8 below). Allowing this to be a measure of the equilibrium constant, K_{eq} ($= k_f/k_r$), we calculate a value of $k_f = 1900\ s^{-1}$. Under the conditions of these experiments (100 mM phosphate buffer, pH 7.0), the rate constant observed for the heme re-reduction is approximately 1 order of magnitude larger than the turnover number for cytochrome *c* oxidation of the *S. cerevisiae* enzyme (TN = 180−200 s^{-1}, Labeyrie et al. 1978; Walker & Tollin, 1991; Miles et al., 1992). This rate constant is also at least 2 times larger than that obtained upon stopped-flow reduction of the heme of the fully oxidized *S. cerevisiae* flavocytochrome using [^{1}H]-L-lactate (Black et al., 1991; Miles et al., 1992). Thus, our data are in agreement with previous conclusions that the electron transfer from $FMNH_2$ to oxidized heme, produced by cytochrome *c* oxidation of the enzyme, cannot be rate-limiting during enzymatic turnover. In addition, it is interesting that the rate constant we obtain is larger than that obtained following reduction of the fully oxidized enzyme (Walker & Tollin, 1991).

There are only two mechanisms by which oxidized heme could be re-reduced intramolecularly in the present system, i.e., reduction via intramolecular electron transfer from $FMNH_2$ within the same monomeric unit of the enzyme (intramonomer electron transfer); reduction via intramolecular transfer from $FMNH_2$ in another monomeric subunit within the same enzyme tetramer (intermonomer electron transfer). On the basis of the significantly shorter distances involved in intramonomer electron transfer (9.7 Å) than for intermonomer electron transfer (38 Å; Xia & Mathews, 1990), and the presumed presence of a physiological electron transfer pathway connecting the two redox centers within the same monomer, it seems likely that the intramonomer reaction is the more probable mechanism for the rapid re-reduction of the flavocytochrome heme. This reaction can be written as follows:

$$H_{ox}-F_{red} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} H_{red}-F^\bullet + H^+ \qquad (8)$$

For reasons stated above, eq 8 is written as an equilibrium. Given that the final proportion of $H_{red}-F^\bullet$ is 80%, we can calculate that the difference in midpoint reduction potentials

[5] The effect of the neutral density filter on the magnitude of the signal produced when the laser beam initiates a reaction in the cuvette was calibrated using the intensity of the signal at 465 nm generated by excitation of chlorophyll (in an ethanol solution) to the triplet state. A 65% decrease in the chlorophyll triplet signal was observed when a 0.5 OD neutral density filter was used, which corresponds directly to the decrease in concentration of the excited chlorophyll generated by the laser flash.

of the two prosthetic groups is approximately 36 mV. On the basis of the midpoint potentials of the heme (+ 10 mV) and the $F^{\bullet-}/F_{red}$ couple (−34 mV) (Walker & Tollin, 1991), a 44 mV driving force would be expected to produce a final equilibrium containing 84% reduced heme. Thus, the final partitioning of reduced prosthetic groups which we observe is consistent with what is predicted based upon the midpoint potentials for the individual redox centers.

Also shown in Figure 2B is a kinetic trace taken on a much longer time scale (400 ms). During this latter process the signal returns completely to the preflash baseline, which can be interpreted in terms of a slower re-reduction of the remaining oxidized heme. The observed rate constant for this process, ~10 s^{-1}, is significantly smaller than enzyme turnover under similar conditions and thus likely occurs via a physiologically irrelevant pathway. This rate constant is independent of laser intensity, which indicates it does not reflect direct reduction of the enzyme by dRFH•, which would be highly unlikely anyway since the time scale of the reaction is much greater than the lifetime of the 5-deazariboflavin semiquinone. Similarly, we do not observe a clear dependence on enzyme concentration. Although we have not investigated this reaction any further, we can speculate that it could be the result of a complicated series of reactions involving FMN semiquinone disproportionation, collisional processes involving enzyme tetramers, or, perhaps most likely, intermonomer electron tranfser within the same enzyme tetramer, for which a similar rate constant was determined by steady-state kinetics (Capeillére-Blandin et al., 1975; Capeillére-Blandin, 1975).

B. Oxidation of Fully Reduced Flavocytochrome in the Presence of Pyruvate

Figure 5A shows the transient signal obtained at 557 nm on a 2.5 ms time scale for the oxidation of reduced flavocytochrome in the presence of excess pyruvate. As was observed for the reduced enzyme in the absence of pyruvate, an initial oxidation of the heme is observed. Pyruvate addition had no significant effect on the bimolecular rate constant for heme oxidation (data not shown). However, in the presence of increasing amounts of pyruvate we observe corresponding large decreases in the magnitude of the rapid re-reduction of the heme, as shown in Figures 5 and 6. At pyruvate concentrations for which there is a mixture of free and bound enzyme, although the magnitude of the heme re-reduction is diminished, the apparent rate constant is not affected (data not shown). Thus, it appears that pyruvate binding results in an enzyme for which the rate constant for intramolecular electron transfer is slowed sufficiently so that we do not observe any biphasicity on a fast time scale. In order for this interpretation to be consistent with catalysis, the off constant for bound pyruvate must be at least as large as k_{cat}. From the data shown in Figure 6, we can calculate a value for K_i = 3.8 mM, which is in reasonable agreement with the value determined from steady-state kinetics (Lederer, 1978). The time-resolved difference spectrum for the transient produced immediately after ^{3}dRF oxidation of the reduced enzyme in the presence of excess pyruvate (data not shown) is essentially the same as that obtained in the absence of pyruvate (Figure 3), again suggesting oxidation of both the FMN and the heme. Furthermore, there is no significant absorbance change in the spectral region from

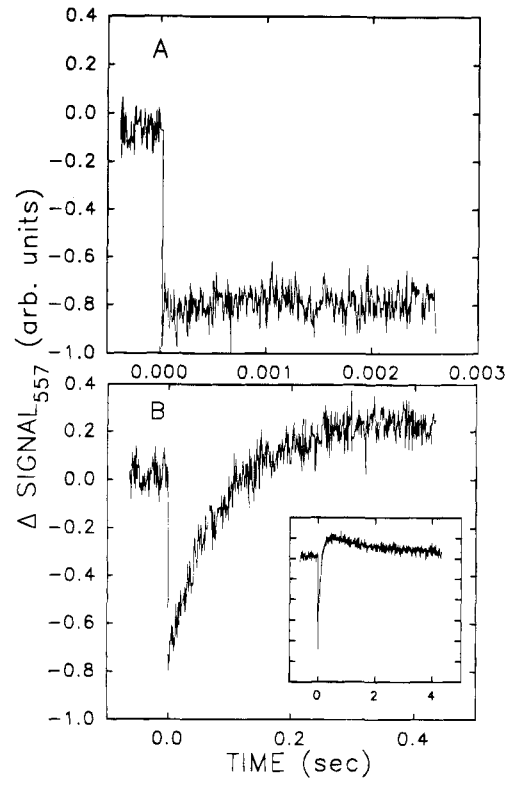

FIGURE 5: Kinetic traces of flavocytochrome b_2 oxidation and subsequent re-reduction in presence of pyruvate. The reaction solution contained 15 μM flavocytochrome, 15 mM pyruvate, and 100 μM 5-deazariboflavin in 100 mM phosphate buffer (pH 7.0). Samples were prepared by titration of the flavocytochrome with L-lactate (1.5 M excess), flashing the sample a minimum number of times (typically two or three to verify that re-reduction of the enzyme could be observed as in Figure 2, followed by anaerobic addition of pyruvate. (A) Kinetic trace obtained on 2.5 ms time scale illustrating inhibition of heme re-reduction. (B) Kinetic trace obtained on a 400 ms time scale showing return of signal above the preflash baseline. (Insert) Return of positive signal to preflash baseline on a 4 second time scale.

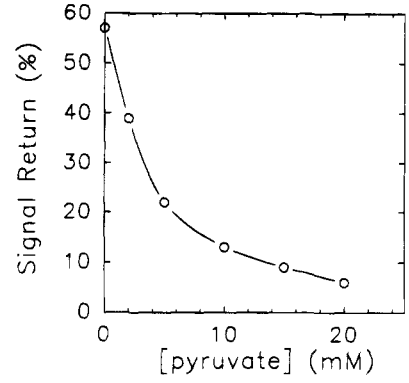

FIGURE 6: The magnitude of the return of the signal at 557 nm as a function of increasing pyruvate concentration. All reaction conditions and procedures were as in Figure 5. Flavocytochrome concentration was 15 μM. The sample was titrated with a concentrated pyruvate solution (500 mM) in order to minimize volume changes. From the plot, a value of K_i = 3.8 mM can be determined.

470 to 600 nm within 1.5 ms after the initial oxidation of the enzyme, consistent with the data shown in Figure 5A.

The kinetics observed on a slow time scale in the presence of pyruvate are also different from those seen without pyruvate. Thus, return of the absorbance at 557 nm occurs on a 400 ms scale and goes *above* the preflash baseline

(Figure 5B). The rate constant for this process (14 s^{-1}) is similar to that observed in the pyruvate-free sample, and again does not vary with laser intensity. The possibility that this slower spectral change is due to incomplete reduction of the enzyme *prior* to the laser flash can be ruled out by the fact that addition of lactate in excess of that used to titrate the enzyme had no effect on either the steady-state visible spectrum or the transient spectral properties observed in the laser flash experiment. Eventually the positive signal decays back to the preflash baseline on a 4 s scale (see insert in Figure 5B), with a rate constant of 0.4 s^{-1}. Since both of these transients lie outside the time domain relevant to catalytic turnover, we have not investigated them further.

DISCUSSION

The results presented in this paper can be summarized as follows. Rapid one-electron oxidation of fully reduced *Saccharomyces* flavocytochrome b_2 by ^{3}dRF results in the parallel formation of two enzyme species. In one of these, the $FMNH_2$ has been oxidized to the anion semiquinone, $F^{\bullet -}$, and the heme remains reduced. In the second species, the heme has been oxidized to the ferric state while the FMN remains fully reduced. In the latter species, a rapid re-reduction of the ferric heme by the $FMNH_2$ of the same monomeric subunit occurs, in which the flavin is converted to the neutral semiquinone, $F^{\bullet}$, with an observed rate constant of 2150 s^{-1}. Pyruvate, at concentrations consistent with the K_i value for enzyme inhibition (Lederer, 1978; Walker & Tollin, 1991) blocks intramonomer electron transfer between the $FMNH_2$ and oxidized heme.

Parallel oxidation of both chromophores in flavocytochrome b_2 is consistent with results reported previously by Walker and Tollin (1991), in which reduction of both redox centers by 5-deazalumiflavin semiquinone occurred with similar rate constants. This aspect of the kinetics is interesting in that the solvent accessibilities of the two chromophores are quite different (Xia & Mathews, 1990). The exposure of the cytochrome b_2 heme edge is much greater than that of the FMN. In fact, using the coordinates deposited in the Brookhaven Data Bank, we have found it is impossible to bring a free flavin within van der Waals contact of any atom of the isoalloxazine ring of the FMN. The closest approach of the free flavin to the FMN was in the lactate binding channel, although there is a great deal of steric constraint for such a docking; this would be expected to have an inhibitory effect on the oxidation of the FMN relative to the heme. This was not observed. Similar steric constraints would be applicable to ferricyanide, which is also known to be capable of oxidizing both the reduced FMN and heme centers (Ogura & Nakamura, 1966; Iwatsubo et al., 1977). However, it has been clearly established that large substrates can gain access to highly buried redox groups, e.g., camphor bound within the protein matrix of cytochrome P-450 (Poulos et al., 1986). These data clearly suggest either that pathways exist for rapid efflux of electrons to exogenous oxidants on the protein surface via a multistep process or that dynamic processes influence the solvent accessibility of the cofactors. This deserves further study.

Oxidation of the b_2 heme is followed by rapid heme re-reduction, which occurs with an observed rate constant of 2150 s^{-1}. Taking into account the equilibrium concentration of reduced cytochrome b_2 heme, we calculate a value for k_f = 1900 s^{-1} in eq 8. This reaction is strictly independent of both protein and ^{3}dRF concentrations, indicating that electron transfer must occur intramolecularly within the tetrameric unit. The detection of an FMN radical component in the time-resolved difference spectrum further supports the notion that electron transfer between $FMNH_2$ and the oxidized heme had occurred. Since the magnitude of the rate constant is much larger than that determined for intermonomer electron transfer (Capeillére-Blandin, 1975; Pompon et al., 1980), this reaction most likely represents an intramonomer process. Under comparable conditions, the rate constant for intramolecular electron transfer between the heme and the FMN in the one-electron-reduced enzyme (in the presence of 5 mM pyruvate) is approximately 500 s^{-1} (Walker & Tollin, 1991). Both of these rate constants are significantly larger than the turnover number (~200 s^{-1}) obtained using cytochrome *c* as an acceptor (cf. Chapman et al., 1991; Walker & Tollin, 1991; Miles et al., 1992; White et al., 1993), which is consistent with the initial oxidation of L-lactate being the rate-determining step for the *S. cerevisiae* flavocytochrome b_2. It is also worth pointing out that computer simulations of steady-state data, which can only give lower limits for the rate constants (Capeillère-Blandin, 1975; Pompon et al., 1980), have yielded a value of 500−600 s^{-1} for the $FMNH_2 \rightarrow H_{ox}$ electron transfer. Tegoni et al. (1984b) have reported temperature jump measurements on partially reduced *H. anomala* flavocytochrome b_2, monitoring the reduction of the oxidized heme by reduced FMN (either semiquinone or hydroquinone). A mean relaxation rate constant of 190 s^{-1} was obtained for the pyruvate-free sample, which was proposed to be due to FMN semiquinone reduction of the oxidized heme (at higher percentages of reduction the data was best fit assuming a contribution from the FMN hydroquinone).

It is well-established that pyruvate acts as an inhibitor for enzyme activity in both a competitive (K_i = 3 mM) and noncompetitive (K_i = 33 mM) manner (Lederer, 1978). The most commonly accepted mechanism for this inhibition is the preferential binding to and stabilization of the FMN semiquinone-containing enzyme. According to the reaction scheme given above (see introduction), this would correspond to blockage of reaction 4. Consistent with this argument, Janot et al. 1990) reported a slow reduction (~5 s^{-1}) of a transiently oxidized cytochrome b_2 by the FMN in the flavocytochrome from *Hansenula*, which had been prepared by reduction of the enzyme in the presence of saturating concentrations of pyruvate. By this procedure, Janot et al. produced an enzyme in which the FMN would be mainly in the semiquinone state. Our experimental procedure differs from that of Janot et al. in that we reduced the enzyme *prior to* addition of pyruvate, and thus we conclude that both the flavin and the heme were fully reduced at the time of the photooxidation reaction. Thus, our data suggests that inhibition can also occur much earlier in the reaction sequence, i.e., reaction 2, since in our experiments the FMN cofactor was in the fully reduced state. On the basis of the effect of pyruvate (Figures 5 and 6), it appears that this compound can also bind to the enzyme containing the fully reduced FMN species and can block electron transfer between this form of the flavin and the oxidized heme.

Walker and Tollin (1991, 1992) have reported results which seemingly contradicted the conclusions drawn from steady-state and transient-state pyruvate inhibition studies.

In these experiments, fully oxidized enzyme was reduced by one electron using 5-deazalumiflavin semiquinone. Biphasic reduction of the b_2 heme was observed in the absence of the pyruvate, with the fast phase due to direct reduction by the free flavin semiquinone, and the slower (and minor) reaction to a bimolecular reaction between tetrameric ensembles. When pyruvate (5 mM) was added to the reaction mixture, direct reduction of the heme was followed by an *intramolecular* re-oxidation reaction with concomitant reduction of the FMN. In a similar manner, Tegoni et al. (1984b) reported that the addition of pyruvate to partially reduced *H. anomala* flavocytochrome b_2 resulted in an increase in the rate constant for reduction of the oxidized heme by reduced FMN in temperature jump studies. It is important to note that in both of those investigations, the heme was fully oxidized prior to initiation of the reaction. Walker and Tollin (1991, 1992) concluded that pyruvate facilitated electron transfer between the two domains of a single monomeric subunit and that such facilitation was linked to the increased dynamic motion of the two domains upon pyruvate binding as deduced from the crystal structure (Xia & Mathews, 1990). During the course of the present investigations, the results of Walker and Tollin could be routinely reproduced. We conclude, therefore, that the effect of pyruvate binding on intramolecular electron transfer is determined by the oxidation state of the enzyme, suggesting that there exists a redox state dependent "gate" within flavocytochrome b_2. Such a proposal is not without precedence. In at least two redox proteins, bovine cytochrome *c* oxidase (Colosimo et al., 1981; Antonini et al., 1985; Young & Palmer, 1986; Hendler et al., 1993) and zucchini ascorbate oxidase (Tollin et al., 1993), "priming" the resting enzyme by substrate reduction followed by oxidation has resulted in elevated enzyme activity and/or intramolecular electron transfer rates. In the case of ascorbate oxidase, it has also been shown that intramolecular electron transfer between the copper centers can increase from a value of $\sim 150\ s^{-1}$ for the one-electron reduced enzyme to $9500\ s^{-1}$ in the two-electron reduced species (Hazzard et al., 1994). For the flavocytochrome b_2 from *H. anomala*, it has been proposed that there is a 4-fold decrease in the rate constant for the fully reduced FMN to heme electron transfer, relative to the reaction involving the FMN semiquinone (Janot et al., 1990), based on rapid mixing of oxidized cytochrome *c* with fully or partially reduced flavocytochrome b_2.

Another interesting feature of the present studies is the ability of the $FMNH_2$ cofactor to be oxidized to two different semiquinone species, either $F^{\bullet}$ or $F^{\bullet -}$, depending on the nature of the oxidant (^{3}dRF or heme). This can be rationalized as follows. Conversion of the triplet deazariboflavin to the neutral semiquinone using EDTA as a sacrificial donor is believed to occur by hydrogen atom transfer. Thus, initially the following reaction would be expected:

$$^3\text{dRF} + \text{FMNH}_2 \rightarrow \text{dRFH}^{\bullet} + \text{FMNH}^{\bullet} \qquad (9)$$

which, if followed by a rapid deprotonation of the $FMNH^{\bullet}$, would produce the anionic species, $F^{\bullet -}$. Equation 9 is written based on the spectral changes observed in the laser flash experiment in which a neutral FMN semiquinone is produced (as noted above, this does not necessarily imply a *direct* electron transfer from the FMN to the deazariboflavin). In contrast, oxidation of $FMNH_2$ by the ferric heme within the same monomer is only an electron transfer reaction:

$$\text{H}_{ox}\text{–FMNH}_2 \rightarrow \text{H}_{red}\text{–FMNH}_2^{\bullet +} \qquad (10)$$

which, when followed by a deprotonation reaction, would produce H_{red}–$FMNH^{\bullet}$. Thus, although in both cases there is only a single deprotonation process, the production of the anionic semiquinone is caused by the additional loss of a proton in the initial oxidation step. Inasmuch as we did not investigate the fate of the neutral semiquinone on an extended time scale, we did not determine if deprotonation to the more stable anionic species occurred.

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