Laser Flash Photolysis Study of the Kinetics of Electron Transfer Reactions of Flavocytochrome b_2 from *Hansenula anomala*: Further Evidence for Intramolecular Electron Transfer Mediated by Ligand Binding†

Mark C. Walker‡ and Gordon Tollin*
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721
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ABSTRACT: Intramolecular electron transfer between the heme and flavin cofactors of flavocytochrome b_2 is an obligatory step during the enzymatic oxidation of L-lactate and subsequent reduction of cytochrome *c*. Previous kinetic studies using both steady-state and transient methods have suggested that such intramolecular electron transfer is inhibited when pyruvate, the two-electron oxidation product of L-lactate, is bound at the active site of *Hansenula anomala* flavocytochrome b_2. In contrast to this, we have recently demonstrated using laser flash photolysis that intramolecular electron transfer could be observed in the flavocytochrome b_2 from *Saccharomyces cerevisiae only* when pyruvate was present [Walker, M., & Tollin, G. (1991) *Biochemistry 30*, 5546–5555], despite a large thermodynamic driving force of 100 mV and apparently favorable cofactor geometry as indicated by crystallographic studies. In the present study, we have utilized laser flash photolysis to investigate intramolecular electron transfer in the flavocytochrome b_2 from *H. anomala* in an effort to address these apparently conflicting interpretations with respect to the influence of pyruvate on enzyme properties. The results obtained are closely comparable to those we reported using the protein from *Saccharomyces*. Thus, in the absence of pyruvate, bimolecular reduction of both the heme and FMN cofactors by deazaflavin semiquinone occurs ($k \approx 10^9$ M^{-1} s^{-1}), followed by a protein concentration dependent *inter*molecular electron transfer from the semiquinone form of the FMN cofactor to the heme ($k \approx 10^7$ M^{-1} s^{-1}). In the presence of 5 mM pyruvate, FMN reduction is prevented, and only bimolecular heme reduction by the deazariboflavin semiquinone is observed, with a slightly larger rate constant than in the absence of pyruvate. This is followed by an *intra*molecular electron transfer from the heme to the FMN ($k = 1650$ s^{-1}). These observations differ with those obtained with the *Saccharomyces* enzyme only in the magnitude of the intramolecular rate constant, which is $\approx$600 s^{-1} for the latter enzyme under comparable conditions. Thus, the effects of pyruvate on intramolecular electron transfer as observed during laser flash photolysis are not appreciably modified by any structural differences which exist between the respective proteins obtained from these two sources.

Yeast flavocytochrome b_2 (L-lactate dehydrogenase, EC 1.1.2.3) is a bifunctional enzyme (Jacq & Lederer, 1974) that catalyzes the two-electron oxidation of L-lactate to pyruvate and subsequent one-electron transfers to two cytochrome *c* molecules (Labeyrie et al., 1978; Capeillere-Blandin et al., 1980; Labeyrie, 1982). The enzyme from *Saccharomyces cerevisiae* is folded into two functionally distinct domains (Xia et al., 1987; Mathews & Xia, 1987; Xia & Mathews, 1990): a flavodehydrogenase domain which contains the FMN[1] cofactor, substrate-binding pocket, and amino acid residues pertinent to the oxidation of lactate (Reid et al., 1988); a cytochrome domain which contains the b_2 heme cofactor by which the protein is reoxidized at the expense of two molecules of oxidized cytochrome *c*.

Reoxidation of lactate-reduced flavocytochrome b_2 by its physiological electron acceptor requires that the two electrons acquired by the $FMNH_2$ cofactor be transferred to the b_2 heme in two discrete one-electron steps. From the crystal structure of the *S. cerevisiae* protein (Xia et al., 1987; Mathews & Xia, 1987; Xia & Mathews, 1990), the geometries of the heme and flavin cofactors within the protein appear to be well suited for efficient electron transfer. However, direct measurement of this process has proven difficult (Morton & Sturtevant, 1964; Ogura & Nakamura, 1966; Suzuki & Ogura, 1970; Capeillere-Blandin, 1975; Capeillere-Blandin et al., 1975, 1984, 1986; Pompon et al., 1980; Tegoni et al., 1984c).

We recently reported a direct observation of intramolecular electron transfer between the heme and flavin cofactors of the *S. cerevisiae* flavocytochrome b_2, accomplished using the laser flash photolysis technique (Walker & Tollin, 1991). The results demonstrated that intramolecular electron transfer was observed *only* in the presence of pyruvate, despite both the apparently favorable geometry between cofactors and a large thermodynamic driving force when pyruvate is not bound (>100 mV). This was interpreted in terms of a pyruvate-induced change in protein conformation which served as a gating mechanism for intramolecular electron transfer between the heme and flavin cofactors. Presumably, the substrate (L-lactate) could also induce such a structural change, although this could not be ascertained using this approach. It is important to note in this context that half of the monomers in

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* To whom correspondence should be addressed.
‡ Present address: Monsanto Agricultural Company, 800 N. Lindbergh Boulevard, St. Louis, MO 63167.

[1] Abbreviations: FMN and $FMNH_2$, oxidized and two-electron-reduced flavin mononucleotide: F_{ox}, F_{sq}, and F_{red}, oxidized, one-electron-reduced, and two-electron-reduced FMN; H_{ox} and H_{red}, oxidized and reduced b_2 heme; dLfS and dLfSH, oxidized and one-electron-reduced 5-deazalumiflavin-3′-propanesulfonate; SC, semicarbazide hydrochloride; k_{obs}, observed rate constant; k_f and k_r, forward and reverse microscopic rate constants; E_1, first one-electron reduction potential of the FMN cofactor ($FMN_{ox} + e^- \rightarrow FMN_{sq}$); E_2, second one-electron reduction potential of the FMN cofactor ($FMN_{sq} + e^- \rightarrow FMN_{red}$).

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the crystal structure as determined by Xia and Mathews (1990) have pyruvate bound at the active site and that in these subunits the cytochrome domain is disordered, implying a high degree of mobility in this region of the protein. It is not clear from the crystallography whether this has functional significance or is an artifact of the crystal packing forces. However, NMR line-broadening of the heme resonances indicates that this domain has appreciable mobility in solution (Labeyrie et al., 1988). Furthermore, pyruvate binding has been shown to shift the one-electron midpoint potentials of the FMN cofactor to more positive values in both the *S. cerevisiae* (Walker & Tollin, 1991) and *Hansenula anomala* (Tegoni et al., 1984a,b, 1986) enzymes, with resulting stabilization of the semiquinone form of the flavin. Interestingly, no change in heme potential occurs under these conditions.

The above structural interpretation of the effect of pyruvate binding on intramolecular electron transfer in *S. cerevisiae* flavocytochrome b_2 is not consistent with the current kinetic model for this enzyme, which postulates that pyruvate functions to inhibit intramolecular flavin-to-heme electron transfer. This model is based upon data obtained from potentiometric titrations (Tegoni et al., 1984a,b, 1986), temperature-jump (Tegoni et al., 1984c), and computer modeling of steady-state kinetic studies (Tegoni et al., 1990, 1991) performed using the *H. anomala* protein. Thus, it is possible that the apparent contradictions between these interpretations result from subtle structural differences between the b_2 flavocytochromes isolated from these different sources. In an effort to address this question, we have examined the kinetics of the electron transfer reactions of the flavocytochrome b_2 isolated from H. *anomala* using laser flash photolysis. Results qualitatively similar to those reported earlier (Walker & Tollin, 1991) were obtained, indicating that differences between the flavocytochromes isolated from these sources cannot account for the apparently discordant interpretations of the effects of pyruvate binding. This will be discussed further below.

Materials and Methods

H. anomala, strain C-317, was obtained from Dr. H. Phaff of the University of California, Davis. Precultures and 60-L liquid cultures were prepared as described by Labeyrie et al. (1978). Cells from 60-L growths were harvested within 20 min using a Millipore membrane concentrator and were promptly frozen. Flavocytochrome b_2 and cytochrome *c* were purified according to the procedure of Gervais et al. (1980). The molar activity of flavocytochrome preparations (expressed per monomeric heme) ranged from 1130 to 1200 s^{-1}, as determined by the reduction of $K_3Fe(CN)_6$ during the enzymatic oxidation of L-lactate at 30 °C (Labeyrie et al., 1978). The purified protein was stored as an ammonium sulfate precipitate at 4 °C in the presence of DL-lactate. Prior to laser flash experiments, protein aliquots were recovered from storage as previously described (Walker & Tollin, 1991). Protein concentrations were determined from absorbencies at either 413 nm (oxidized form) or 423 nm (reduced form) using extinction coefficients of 129.5 mM^{-1} cm^{-1} and 183 mM^{-1} cm^{-1}, respectively (Labeyrie et al., 1978). N^3-(propanesulfonate)deazalumiflavin (dLfS) was a generous gift from Dr. T. C. Bruice. Potassium phosphate buffer, pH 7.0, was used in concentrations as specified in the figure legends. For flash photolysis experiments, 1 mM semicarbazide (SC) was included in the buffer to act as sacrificial electron donor for excited state flavins. Sodium pyruvate was obtained from Sigma Chemical Co. (St. Louis, MO) and was recrystallized from water/ethanol as required.

Laser flash photolysis experiments were performed as described previously (Walker & Tollin, 1991). In this procedure, the laser flash (nitrogen-pumped dye laser; <1 ns pulse width, wavelength output 396 nm) generates the triplet state of dLfS which is then completely quenched by the sacrificial electron donor semicarbazide. This reaction produces the semiquinone of the flavin and a donor radical in <1 μs. The fate of the donor radical has not been established in the case of semicarbazide; however, with EDTA as the donor it has been shown (Traber et al., 1982a,b) that the radical is unstable and rapidly (<1 ms) undergoes a series of reactions, involving decarboxylation, an additional reduction of oxidized flavin to produce another molecule of semiquinone, and fragmentation, to generate stable products. Inasmuch as we have obtained similar results using these two donors in laser photolysis experiments, we presume that the semicarbazide radical undergoes an analogous set of reactions. The flavin semiquinone thus produced can either disproportionate to form oxidized and fully reduced flavins or, in the presence of a redox protein acceptor, can serve as an electron donor to form the reduced protein and reoxidized flavin. If the concentration of the redox protein is high enough, and if the rate constant for protein reduction is large enough, protein reduction takes precedence over flavin disproportionation. In the present experiments, this was the case.

The redox status of heme and flavin cofactors of flavocytochrome b_2 were monitored independently at 557 and 438.5 nm, respectively. Reoxidation of the deazaflavin semiquinone generated by the laser flash was followed at 510 nm, which corresponds to an isosbestic point for reduction of the protein. All kinetic experiments were performed under pseudo-first-order conditions, in which the concentration of protein acceptor (>2 μM) was in excess over the amount of semiquinone produced per flash (localized concentration <0.6 μM). This assures that only a single electron enters each protein molecule. Transient absorbance changes were analyzed using a computer fitting procedure (SIFIT, obtained from OLIS Co., Jefferson, GA). Unless quantitation was required (e.g., for absorbance change measurements), the number of flashes averaged per kinetic trace varied.

Results and Discussion

During the laser flash-induced reduction of *H. anomala* flavocytochrome b_2, a biphasic increase in absorbance was observed at 557 nm immediately following the laser flash (Figure 1a,b). The slow phase of heme reduction accounted for approximately 25–30% of the total signal observed at 557 nm. This behavior is identical to that obtained with the *S. cerevisiae* enzyme (Walker & Tollin, 1991), and as before the absorbance changes were ascribed to the reduction of the b_2 heme cofactor. The observed rate constants (k_{obs}) for both kinetic phases were dependent on protein concentration (insets to Figure 1a,b), yielding second-order rate constants of 0.9×10^9 M^{-1} s^{-1} and 1.3×10^7 M^{-1} s^{-1} for the fast and slow phases, respectively. These values are, within experimental error, equal to those reported earlier for the *S. cerevisiae* flavocytochrome. This comparison suggests that the accessibility of the heme cofactor to exogenous chemical reductants is not significantly different for the two flavocytochromes.

When protein reduction was monitored at 438.5 nm, a rapid absorbance bleach was observed immediately following the laser-induced formation of the deazaflavin semiquinone (Figure 2a), which was followed by a slower reappearance of absorbance (Figure 2b). This wavelength corresponds to an isosbestic point for the heme cofactor; therefore, these absorbance changes reflect the rapid reduction and slower reoxidation of the FMN cofactor of the flavocytochrome (however, see be-

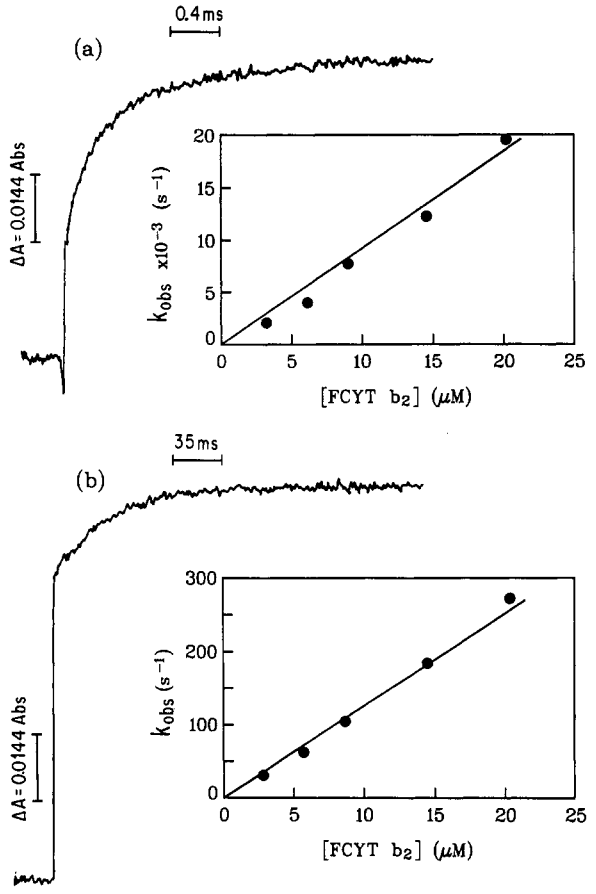

FIGURE 1: Transient absorbance changes observed at 557 nm for (a) the fast and (b) the slow phases of heme reduction obtained upon laser flash photolysis in the absence of pyruvate with *H. anomala* flavocytochrome b_2 (3.1 μM). The initial rapid rise in panel a corresponds to dLfS semiquinone formation; the subsequent slower absorbance increase is due to heme reduction. Each curve represents the sum of six flashes. Buffer conditions were 0.1 M phosphate, pH 7.0, containing 1 mM semicarbazide and 130 μM dLfS. Insets to each figure demonstrate the dependence of the observed rate constant on the protein concentration.

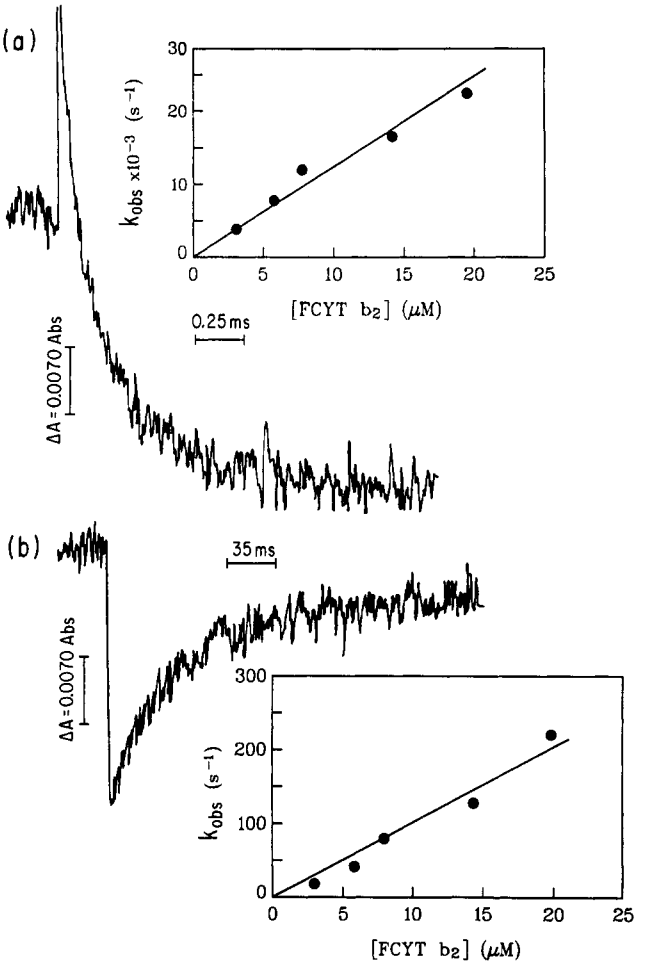

FIGURE 2: Transient absorbance changes observed at 438.5 nm upon laser flash photolysis of *H. anomala* flavocytochrome b_2 (3.1 μM) demonstrating (a) the rapid reduction and (b) slower reoxidation of the FMN cofactor in the absence of pyruvate. The initial rapid increase in absorbance in panel a corresponds to dLfS semiquinone formation; the decay below the preflash baseline is due to FMN reduction. Each curve represents the sum of 10 flashes. Buffer conditions are identical to those given in the legend to Figure 1. The inset demonstrates the dependence of the observed rate constant on the protein concentration.

low). Again, these results are similar to those observed with the *S. cerevisiae* protein (Walker & Tollin, 1991). Both the reduction and reoxidation of the FMN cofactor were found to be concentration dependent, as illustrated by the insets to Figure 2. This demonstrates that these absorbance changes represent bimolecular (i.e., *inter*molecular) electron transfer processes. Second-order rate constants of 1.2×10^9 M^{-1} s^{-1} and 1.1×10^7 M^{-1} s^{-1} were obtained for the fast reduction and slow reoxidation of the FMN cofactor. However, due to the nature of the heme isosbestic point near 438 nm and the relatively large spectral bandwidth (4–8 nm) used to monitor these absorbance changes during flash photolysis experiments, one cannot be assured that all spectral contributions resulting from heme reduction have been eliminated. Consequently, the second-order rate constants obtained for the reduction and reoxidation of the FMN cofactor should be considered as approximate values [see also Walker and Tollin (1991)]. The important feature which is clearly demonstrated by the transients at this wavelength is the *reappearance* of absorbance following a rapid absorbance loss. Thus, these changes must represent redox processes which are distinct from those monitored at 557 nm, for which the two phases proceed in the same direction.

The rapid reductions of the heme and flavin cofactors are interpreted as the result of direct electron transfers from the deazaflavin semiquinone generated during the laser flash. The deazaflavin cofactor was conveniently monitored by the absorbance changes at 510 nm, which corresponds to an isosbestic point for the flavocytochrome b_2 (Walker & Tollin, 1991). Complete reoxidation of the deazaflavin semiquinone occurred on a time scale comparable to that used to follow the fast phase of reduction of both the heme and flavin cofactors (data not shown), consistent with this interpretation. No additional spectral changes were observed at 510 nm on time scales comparable to the slow absorbance changes observed at 438.5 or 557 nm.

The slow phases of heme reduction and FMN semiquinone reoxidation had similar second-order rate constants. Inasmuch as the deazaflavin semiquinone was completely reoxidized before these slower absorbance changes at 557 and 438.5 nm were observed, we interpret these as being due to *inter*molecular electron transfer from the FMN semiquinone of one protein molecule to the oxidized b_2 heme of a second protein molecule. Similar results were obtained for the *S. cerevisiae* flavocytochrome b_2 (Walker & Tollin, 1991). As was also the case for the *S. cerevisiae* flavocytochrome, no evidence for a protein *concentration independent* exchange of electrons between the FMN and heme cofactors was observed in the present flash experiments.

The results from flash photolysis experiments suggest that the apparent second-order rate constant for direct reduction of the FMN cofactor by deazaflavin semiquinone is approximately 30% larger than that obtained for the heme cofactor (see above). Consequently, a proportionally greater amount of the reducing equivalents generated during the laser flash leads to the formation of the FMN semiquinone. Potentiometric titrations and room temperature EPR studies have demonstrated that the midpoint potentials for the F_{ox}/F_{sq} and H_{ox}/H_{red} redox couples of the *H. anomala* flavocytochrome are approximately equal in the absence of pyruvate (Tegoni et al., 1984a,b, 1986). Therefore, equal distributions of reduced heme and FMN semiquinone are expected at equilibrium following the addition of substoichiometric amounts of reducing equivalents to the oxidized flavocytochrome. Thus, electron transfer from the FMN semiquinone to oxidized heme occurs as a result of the approach to equilibrium following the rapid introduction of reducing equivalents from the deazaflavin semiquinone, resulting in a nonequilibrium distribution. For reasons discussed above [see also Walker and Tollin (1991)], the signal amplitudes at 438.5 nm may not properly reflect the extent of FMN reduction and reoxidation, and thus the concentration of FMN semiquinone remaining at equilibrium could not be accurately determined from the observed absorbance changes.

Previous studies with the *H. anomala* flavocytochrome b_2 have demonstrated that the binding of pyruvate alters the spectral properties of the flavin cofactor (Lederer, 1978), stabilizes the FMN semiquinone (Tegoni et al., 1984a,b, 1986), and influences the kinetics of electron transfer reactions (Tegoni et al., 1984c, 1990, 1991). The presence of pyruvate at the substrate-binding site is also associated with differences in the X-ray crystal structures of the monomers of *S. cerevisiae* flavocytochrome (Xia et al., 1987; Mathews & Xia, 1987; Xia & Mathews, 1990). Furthermore, the inclusion of 5 mM pyruvate during previous flash photolysis experiments performed with the *S. cerevisiae* flavocytochrome resulted in the observation of *intra*molecular electron transfer between heme and FMN cofactors (Walker & Tollin, 1991). Therefore, it was of interest to examine the effects of pyruvate on the electron transfer reactions of the *H. anomala* flavocytochrome b_2.

In the presence of 5 mM pyruvate, transient absorbance changes at 557 nm again displayed an initial rapid increase corresponding to heme reduction (Figure 3a). However, in this case the absorbance increase was followed by a slower absorbance *decrease* due to heme reoxidation (Figure 3a; compare with Figure 1b). This behavior is comparable to that reported earlier for the *S. cerevisiae* protein (Walker & Tollin, 1991). The initial absorbance increase was protein concentration dependent, yielding a second-order rate constant for the reaction with deazaflavin semiquinone of 1.5×10^9 M^{-1} s^{-1} (inset to Figure 3). This value is slightly larger than that obtained in the absence of pyruvate, suggesting a possible increase in heme accessibility (a similar increase was not observed with the *S. cerevisiae* enzyme). The subsequent absorbance decrease observed at 557 nm was protein concentration *independent* (inset to Figure 3; $k = 1650$ s^{-1}), which demonstrates that the heme reoxidation is a unimolecular (i.e., *intra*molecular) process. A slow, protein concentration independent loss of absorbance was also observed at 438.5 nm on a time scale comparable to that used to monitor heme reoxidation (Figure 3b; concentration independence not shown). Although the transient changes at this wavelength also include some contribution from the reoxidation of the

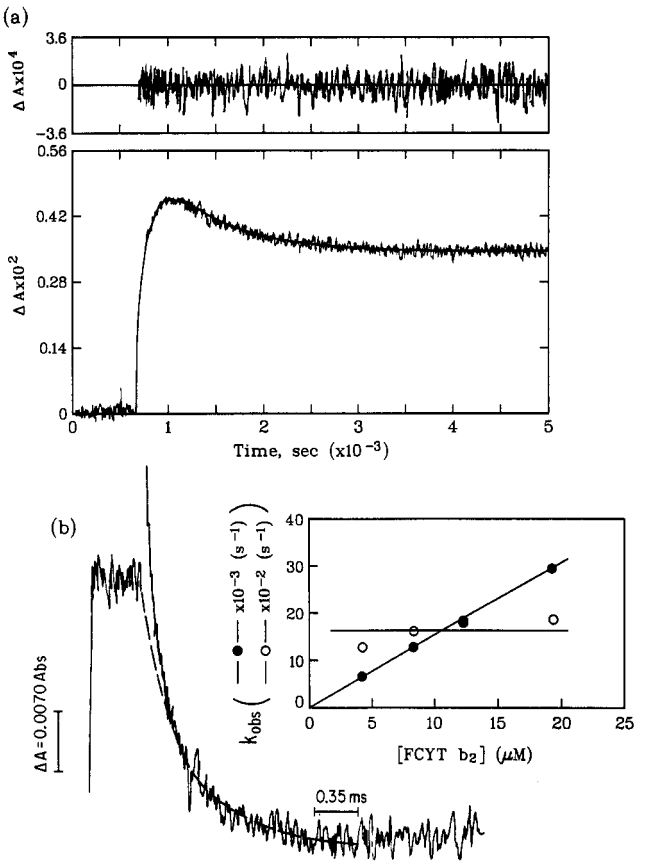

FIGURE 3: (a) Transient absorbance changes obtained at 557 nm upon laser flash photolysis of *H. anomala* flavocytochrome b_2 (4.4 μM) in the presence of 5 mM pyruvate showing the fast phase of heme reduction and the slower heme reoxidation. The initial rapid rise in absorbance corresponds to dLfS semiquinone formation; this is followed by a slower increase due to heme reduction and a decrease due to heme reoxidation. The solid line drawn through the latter two kinetic phases corresponds to a two-exponential fit with rate constants of 6800 s^{-1} and 1470 s^{-1}, respectively; the residuals are shown plotted in the upper box. Buffer conditions are identical to those given in the legend to Figure 1. The curve represents the sum of six flashes. (b) Transient absorbance changes observed at 438.5 nm upon laser flash photolysis of *H. anomala* flavocytochrome b_2 (4.4 μM) demonstrating the slow reduction of the FMN cofactor in the presence of 5 mM pyruvate. The dashed curve corresponds to a single exponential with a rate constant of 1600 s^{-1}; because the absorbance changes for the two kinetic phases (dLfS semiquinone decay and FMN reduction) both correspond to absorbance decreases, computer deconvolution is difficult. Buffer conditions are identical to those given in the legend to Figure 1. The curve represents the sum of 10 flashes. The inset to panel b demonstrates the dependence of the observed rate constant on the protein concentration for both the initial heme reduction (closed circles) and the slower heme reoxidation (open circles).

deazaflavin semiquinone, it is possible to deconvolute the two processes and obtain a single exponential for FMN reduction (dashed line in Figure 3b) with a rate constant similar to that found for the slow phase of heme reoxidation at 557 nm. Thus, these slower absorbance changes can be interpreted as resulting from *intra*molecular electron transfer from reduced b_2 heme to oxidized FMN in the presence of pyruvate. Note that little or no rapid absorbance bleach due to direct reduction of the FMN cofactor by deazaflavin semiquinone was observed at 438.5 nm in the presence of pyruvate (Figure 3b; compare with Figure 2b). This latter result is also similar to that reported earlier for the *S. cerevisiae* flavocytochrome (Walker & Tollin, 1991) and is consistent with steric restriction of the FMN site by the bound pyruvate.

It is clear from the above results that intramolecular electron transfer between the heme and flavin cofactors of *H. anomala*

flavocytochrome b_2 was observed only in the presence of pyruvate, similar to the situation found for the *S. cerevisiae* protein (Walker & Tollin, 1991; $k \approx 600\ s^{-1}$). This suggests that electron transfer between the heme and FMN cofactors is inhibited in the absence of pyruvate. The simplest explanation for these results is that pyruvate binding induces a conformational change within the flavocytochrome which permits electron transfer between the respective cofactors. As noted above, significant differences exist in the crystal structures of the pyruvate-free and pyruvate-bound subunits of *S. cerevisiae* flavocytochrome b_2 (Xia & Mathews, 1990). In those subunits that contain pyruvate at the active site of the flavodehydrogenase domain, the cytochrome domain is disordered and unresolved, which implies a high degree of mobility in this region of the protein. More subtle differences in protein conformation were observed in the flavodehydrogenase domain. Such changes in protein conformation, induced upon ligand binding, may serve as a gating mechanism for efficient electron transfer between the respective cofactors (Hoffman & Ratner, 1987, 1988; Brunschwig & Sutin, 1989).

Although the observed direction of intramolecular electron transfer was from reduced heme to oxidized FMN, whereas during enzymatic turnover electron transfer occurs in the reverse direction, this does not mean that the process we observe is physiologically irrelevant. First of all, the events which occur during the present experiments and during reduction of enzyme by substrate proceed from distinctly different initial conditions, although the final equilibrium states are similar. Thus, in the laser flash experiment performed in the presence of pyruvate, initially all of the electrons reside on the heme cofactor, whereas during enzyme turnover the electrons initially reside on the FMN. Thus, in the first case the net direction of electron transfer must be from reduced heme to oxidized FMN, whereas in the latter situation the direction is from reduced FMN to oxidized heme. Furthermore, substrate reduction initially produces a two-electron-reduced FMN, whereas only a single electron enters the protein during the flash experiment. Inasmuch as the E_1 and E_2 potentials of the FMN cofactor are not identical (see below), the driving forces for the two possible one-electron transfer reactions also will not be identical. It should further be noted that the presence of a terminal electron acceptor (i.e., cytochrome *c*) during enzyme turnover will pull the equilibrium in favor of the products (i.e., oxidized flavocytochrome b_2 and reduced cytochrome *c*).

In the present study, a concentration of 5 mM pyruvate was used in order to match the conditions of the previous flash photolysis experiments performed with the *S. cerevisiae* protein (Walker & Tollin, 1991). A K_i for pyruvate of 1 mM has been obtained from the inhibition of the cytochrome *c* dependent oxidation of lactate catalyzed by the *H. anomala* flavocytochrome b_2 (Tegoni et al., 1990). Consequently, under the conditions of the laser flash photolysis experiments, approximately 84% of the total protein is expected to contain bound pyruvate. It is worth noting that, in the case of the *S. cerevisiae* flavocytochrome b_2, alterations in kinetic behavior and changes in the midpoint potentials of the FMN cofactor were fully titrated at a concentration of 5 mM pyruvate (Walker & Tollin, 1991), even though a K_i of 3 mM for competitive inhibition had been determined (Lederer, 1978; Walker & Tollin, 1991). This suggests that the K_d for pyruvate is lower than the K_i value predicts. This is not surprising when one considers the complex nature of pyruvate binding by flavocytochrome b_2 (Hinkson & Mahler, 1963; Lederer, 1978; Tegoni et al., 1990). This point is addressed further below.

Other workers have reported difficulty in maintaining *H. anomala* flavocytochrome b_2 in the fully oxidized form under anaerobic conditions in the presence of 10 mM pyruvate (Tegoni et al., 1986). In the present experiments, UV–vis spectra were recorded before and after each series of laser flashes. From an examination of the absorbance values at 450 and 557 nm obtained during experiments in which pyruvate was included, it is estimated that accumulation of FMN semiquinone during the course of the experiment represented less than 20% of the total FMN in the sample.

Previously it was reported (Walker & Tollin, 1991) that the rate constant for intramolecular electron transfer between FMN and heme cofactors of *S. cerevisiae* flavocytochrome b_2 displayed a dependence on ionic strength, corresponding to an attractive electrostatic interaction. This was interpreted as the result of a single charge-pair interaction at the interface between the flavodehydrogenase and cytochrome domains of the *S. cerevisiae* protein (Xia & Mathews, 1990), which could serve to orient the two domains for efficient electron transfer. Comparison of the amino acid sequences for the *S. cerevisiae* and *H. anomala* flavocytochromes (Black et al., 1989) suggests that there are significant differences in net charge in the vicinity of the interface region between these two functional domains. Therefore, it was of interest to examine the ionic strength dependence of the intramolecular electron transfer within the *H. anomala* flavocytochrome. Unfortunately, we were unable to follow the kinetics of intramolecular electron transfer within the *H. anomala* protein at lower ionic strengths. When the concentration of phosphate buffer was decreased to 50 mM, complex absorbance changes were observed at 557 nm which were consistent with the rapid reduction of the heme cofactor followed by partial reoxidation (<10%) and then a much slower phase of heme reduction (data not shown). At a concentration of 4 mM phosphate, the partial reoxidation of heme was no longer observed, and the rapid reduction of heme was directly followed by a slower phase of heme reduction, as occurred in the absence of pyruvate (data not shown). These results suggest that intramolecular electron transfer between the heme and FMN cofactors of the *H. anomala* flavocytochrome is inhibited at the lowest ionic strengths, while, at intermediate ionic strengths, mixed kinetic behavior is observed. It is not possible at the present time to fully rationalize these observations in terms of protein structure; however, the results do reflect differences in the electrostatic properties of the *S. cerevisiae* and *H. anomala* proteins. Furthermore, the inability to observe intramolecular electron transfer at low ionic strengths correlates with a reported loss of enzyme activity which results from destabilization of the tetrameric assembly of *H. anomala* flavocytochrome b_2 and formation of inactive monomers at low ionic strengths (Labeyrie & Baudras, 1972; Baudras, 1973; Prats, 1978).

The observed rate constant of $1650\ s^{-1}$ represents the sum of the forward and reverse microscopic rate constants for intramolecular electron transfer, i.e.,

$$F_{sq}\text{–}H_{ox} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} F_{ox}\text{–}H_{red}$$

Values for k_f and k_r can be obtained from comparison of the observed rate constant ($k_{obs} = k_f + k_r = 1650\ s^{-1}$) and the equilibrium constant for the reaction ($K_{eq} = k_f/k_r$). The midpoint potential for the H_{ox}/H_{red} couple of the flavocytochrome b_2 has been established as −19 mV (Tegoni et al., 1984a, 1986; Capeillere-Blandin et al., 1986). The midpoint potential for the F_{ox}/F_{sq} couple has proven significantly more difficult to measure; values of +36 mV (Tegoni et al., 1984a)

and +52 and +71 mV (Tegoni et al., 1986) have been obtained using both room temperature EPR and potentiometric titrations performed in the presence of pyruvate. Based upon this range of midpoint potentials, the following range of values for K_{eq}, k_f, and k_r can be calculated:

$$0.030 \leq K_{eq} \leq 0.120$$

$$49\ s^{-1} \leq k_f \leq 195\ s^{-1}$$

$$1460\ s^{-1} \leq k_r \leq 1600\ s^{-1}$$

A rate constant of 125 s^{-1} for the enzymatic oxidation of L-lactate at 18 °C has been reported using *H. anomala* cytochrome *c* as the final electron acceptor (Tegoni et al., 1990). Therefore, given the range of values reported for the first one-electron reduction potential of the FMN cofactor (E_1), the individual microscopic rate constants for electron transfer between the FMN and heme cofactors appear to be consistent with enzymatic turnover. Furthermore, since the transfer of electrons between these cofactors is a rapid equilibrium process, the extent to which this poses a rate limitation to overall catalysis is more accurately reflected by the rate at which this equilibrium is attained (k_{obs} = 1650 s^{-1}), which is clearly faster than turnover. This conclusion is consistent with the inability to observe intramolecular electron transfer between heme and flavin cofactors during stopped-flow studies of the reductive half-reaction at ambient (Morton & Sturtevant, 1964; Suzuki & Ogura 1970; Capeillere-Blandin et al., 1975) or low temperatures (Pompon et al., 1980; Capeillere-Blandin et al., 1986). Additionally, it should be recognized that these calculations assume that the K_{eq} for the intramolecular electron transfer reaction observed during laser flash photolysis experiments is identical to that obtained from potentiometric titrations performed in the presence of pyruvate (Tegoni et al., 1984a, 1986; Capeillere-Blandin et al., 1986). As discussed by Brunschwig and Sutin (1989), one of the consequences of a conformational gating mechanism may be *directional* electron transfer, in which the observed forward and reverse rate constants for electron transfer may not be the same in both directions. This is an important point which requires further study.

It must also be noted that a direct comparison of results from steady-state kinetic experiments and the present flash photolysis experiments is not straightforward. Enzymatic turnover does *not* demonstrate an absolute requirement for exogenous pyruvate. If indeed a conformational change is required for efficient intramolecular electron transfer, such a change may occur upon the initial binding of the substrate L-lactate. Thus, pyruvate may function only as a poor substitute for L-lactate with respect to inducing the change in kinetic behavior observed in the presence of this ligand during flash photolysis experiments. Consistent with this, previous flash photolysis experiments performed with the *S. cerevisiae* flavocytochrome demonstrated that both oxalate and D-lactate were unable to facilitate intramolecular electron transfer (Walker & Tollin, 1991) even though they are more potent inhibitors of this enzyme than is pyruvate (K_i = 1.1, 1.4, and 3 mM, respectively; Lederer, 1978).

Only a partial reoxidation of the heme cofactor (approximately 30%; cf. Figure 3a) was observed in the presence of 5 mM pyruvate. On the basis of the thermodynamic driving force for electron transfer from the reduced heme cofactor to the oxidized FMN in the presence of exogenous pyruvate (>55 mV; Tegoni et al., 1984a, 1986; Capeillere-Blandin et al., 1986), complete reoxidation of the b_2 heme was expected at equilibrium. Consequently, it appears that complete equilibration was not attained on the time scale of the laser flash experiment (≈400 ms), i.e., only a fraction of the flavocytochrome present during the flash experiment was capable of supporting rapid intramolecular electron transfer in the presence of pyruvate. A possible explanation is as follows: protein molecules which contain bound ligand may exist in two distinct conformations, only one of which supports intramolecular electron transfer. Complete equilibration is achieved only via a slow process which may involve either intermolecular electron transfer from a pyruvate:F_{sq}-containing enzyme species to b_2 heme or a rate-limiting conformational change to an enzyme form which now supports intramolecular electron transfer between the heme and flavin cofactors. Furthermore, the equilibrium distribution between different protein conformations may be dependent on the nature of the bound ligand. This could account for the lack of a correlation between inhibitory potency and the ability of specific ligands to facilitate intramolecular electron transfer, which was noted above. These possibilities require further study.

The conclusions presented above are also consistent with other previous experimental results. Reoxidation of fully reduced flavocytochrome b_2 by cytochrome *c* has been examined using stopped-flow techniques in an effort to measure the rate constant for intramolecular electron transfer between the FMN and heme cofactors (Janot et al., 1990). During this study, biphasic reduction of cytochrome *c* by flavocytochrome b_2 was obtained (a third, much slower phase was also observed which was ascribed to the turnover of enzyme when in the presence of L-lactate). The fast phase (k_{obs} = 250 s^{-1}) was interpreted as being due to electron transfer from the reduced b_2 heme to oxidized cytochrome *c* within a transient protein–protein complex [Janot et al., 1990; see also Capeillere-Blandin et al. (1980) and Capeillere-Blandin (1982)]. However, the stoichiometry observed during this fast phase of electron transfer demonstrated that the reoxidation of 1.0 mol of b_2 heme was accompanied by the reduction of 1.5 mol of cytochrome *c* (Janot et al., 1990). This stoichiometry indicates that some of the reducing equivalents present in the flavin cofactor are capable of directly reducing cytochrome *c* in a rapid electron transfer process. This most likely involves intramolecular electron transfer from reduced $FMNH_2$ to b_2 heme, followed by a rate-limiting intermolecular electron transfer from the b_2 heme to cytochrome *c*. The slow phase of cytochrome *c* reduction observed during the approach to equilibrium was characterized by an apparent first-order rate constant of 5 s^{-1}; this was ascribed to intramolecular electron transfer from flavosemiquinone to b_2 heme. It should be noted, however, that this value is consistent with the second-order rate constant reported in the present study for *inter*molecular electron transfer between heme and flavin cofactors. Indeed, the authors point out that the slow phase of cytochrome *c* reduction can be interpreted using a kinetic model wherein direct electron transfer from FMN semiquinone to oxidized cytochrome *c* in a *concentration dependent* manner is permitted, although they discard this scheme as inappropriate (Janot et al., 1990). Unfortunately, the concentration dependence of this slower process was not examined, which would have distinguished between an intermolecular electron exchange and a rate-limiting intramolecular process.

It is not known at which point during catalytic turnover the dissociation of pyruvate from flavocytochrome occurs. If our interpretations are correct, it could suggest that the release of pyruvate occurs following the complete reoxidation of the FMN cofactor. However, pyruvate release could also precede reoxidation of the FMN cofactor, provided that the rate constant for interconversion between different enzyme con-

formations is relatively slow in the absence of ligands.

Pyruvate has been demonstrated to be a potent inhibitor of the steady-state turnover of L-lactate as catalyzed by flavocytochrome b_2 (Hinkson & Mahler, 1963; Lederer, 1978; Tegoni et al., 1990). The pattern of inhibition appears complex in nature, showing noncompetitive behavior at low concentrations of pyruvate and uncompetitive behavior at higher concentrations (Tegoni et al., 1990). This suggests that there are multiple sites on the enzyme which can bind pyruvate, and they may also reflect multiple effects on various kinetic processes relevant to catalysis. The facilitation of intramolecular electron transfer by pyruvate as observed in the present experiments, and the inhibition of steady-state turnover by exogenous pyruvate, most probably represent distinct, but not necessarily mutually exclusive, processes.

Tegoni et al. (1990, 1991) have concluded that the rate constant for intramolecular electron transfer between heme and flavin cofactors is inhibited in the presence of pyruvate. This conclusion is based primarily upon a thermodynamic stabilization of the FMN semiquinone following the binding of pyruvate to partially reduced flavocytochrome b_2 (Tegoni et al., 1984a,b, 1986), as well as upon computer modeling of steady-state (Tegoni et al., 1990, 1991) and pre-steady-state (Janot et al., 1990; Tegoni et al., 1991) kinetic data obtained in the absence and presence of pyruvate. According to this interpretation, the enzyme form containing the FMN semiquinone and bound pyruvate is a dead-end inhibitory complex. However, stabilization of the FMN semiquinone upon pyruvate binding does not a priori provide a mechanism for enzyme inhibition. As demonstrated above, the rate constant for intramolecular electron transfer between the FMN and heme cofactors in the presence of pyruvate is large enough to support enzymatic turnover, even though the equilibrium constant reflects a highly disfavored process. Furthermore, this interpretation of pyruvate inhibition does not take into account the presence of cytochrome *c* as the final electron acceptor during steady-state turnover experiments. The K_{eq} for the overall process of electron transfer from FMN semiquinone to oxidized cytochrome *c* (which has an $E_h \approx +260$ mV) is quite favorable, which serves to pull an otherwise unfavorable equilibrium for intramolecular electron transfer toward reoxidation of the FMN semiquinone ($K_{eq} > 1500$).

Rapid mixing of substrate and enzyme has been previously employed to examine the intramolecular electron transfer from the reduced FMN to heme cofactor of flavocytochrome b_2 (Morton & Sturtevant, 1964; Ogura & Nakamura, 1966; Suzuki & Ogura, 1970; Capeillere-Blandin, 1975; Capeillere-Blandin et al., 1975, 1986; Pompon et al., 1980). However, as was discussed previously (Walker & Tollin, 1991), the results of these studies are not directly comparable with those obtained from flash photolysis experiments. A fundamental difference between the stopped-flow and flash photolysis techniques is that, in the latter, a *substoichiometric* amount of reducing equivalents is rapidly introduced into the oxidized protein, resulting in a population containing only one-electron-reduced species. Consequently, the values of k_f and k_r obtained from simulations of reductive stopped-flow traces correspond to a one-electron transfer from $FMNH_2$ to H_{ox} (the first catalytically relevant intramolecular electron transfer process), whereas the values reported here represent electron transfer from FMN_{sq} to H_{ox}, which is the second intramolecular electron transfer step during catalysis.

Pulse radiolysis (Capeillere-Blandin et al., 1984) and temperature-jump equilibrium perturbation (Tegoni et al., 1984c) techniques have also been employed in order to examine intramolecular electron transfer reactions between the redox cofactors of the *H. anomala* flavocytochrome b_2. Spectral changes observed using the former method were consistent with the direct reduction of heme, with no indication of FMN reduction and thus no possibility for intramolecular electron transfer because of the thermodynamic barrier. It is interesting to note that the buffer conditions utilized in these studies were 20 mM (or less) phosphate, pH 7.0. Under similar conditions, we were unable to observe intramolecular electron transfer during flash photolysis experiments, even in the presence of 5 mM pyruvate (see above). Spectral changes obtained during the temperature-jump studies suggested that intramolecular reduction of heme by FMN semiquinone was occurring while in the presence of stoichiometric amounts of pyruvate; however, the kinetics were found to be complex and difficult to interpret (Tegoni et al., 1984c). Furthermore, the interpretation of the effects of exogenous pyruvate on rate constants and signal amplitudes was also not straightforward (Tegoni et al., 1984c).

In summary, laser flash photolysis examination of the electron transfer reactions of *H. anomala* flavocytochrome b_2 has demonstrated that intramolecular electron transfer between the FMN and heme cofactors is not observed in the absence of bound ligands, consistent with previous results obtained with the b_2 flavocytochrome from *S. cerevisiae* (Walker & Tollin, 1991). Intramolecular exchange of electrons between these cofactors could, however, be observed in the presence of pyruvate. Overall, our results and those obtained in other laboratories are consistent with the conclusion that the binding of pyruvate by flavocytochrome elicits changes in protein conformation which facilitate efficient electron transfer between the cofactors. Consequently, flavocytochrome b_2 may provide an example of a more general mechanism for conformational gating of intramolecular electron transfer in redox enzymes. Elucidation of the mechanism by which electron transfer is blocked in the absence of bound ligands in flavocytochrome b_2 requires further study.

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Inactivation of Factor VIII by Factor IXa

Donogh Paul O'Brien,* Daniel Johnson, Peter Byfield, and Edward G. D. Tuddenham
Haemostasis Research Group, Clinical Research Centre, Harrow, Middlesex HA1 3UJ, England
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ABSTRACT: Factor VIII (FVIII) is the nonproteolytic cofactor for FIXa in the tenase complex of blood coagulation. FVIII is proteolytically activated by thrombin and FXa in vitro to form a heterotrimer with full procoagulant activity. Activated protein C inactivates thrombin-activated FVIII through cleavage adjacent to position Arg 336 in the cofactor. We have investigated the interaction of FIXa and FVIII and subjected FVIII polypeptides to N-terminal amino acid sequence analysis. Contrary to previous reports, we were unable to demonstrate the activation of FVIII by FIXa. Incubation of these two proteins at equimolar or close to equimolar concentrations resulted in the inactivation of FVIII, coincident with cleavage of the FVIII heavy chain adjacent to Arg 336 and the light chain adjacent to Arg 1719. These cleavages were detected in the presence or absence of thrombin, indicating that FIXa does not stabilize thrombin-activated FVIIIa. APC cleaved FVIII at the same position in the heavy chain, and simultaneous incubation of FVIII, APC, and FIXa did not result in stabilization of the cofactor. We conclude that FIXa does not play a role in the stabilization or activation of FVIII.

Factor VIII (FVIII) is the nonproteolytic cofactor for FIXa, a serine protease which activates factor X (FX) in the central reaction of the coagulation cascade (Mann & Krishnaswarmy, 1990). FVIII must itself undergo limited proteolysis in order to participate in the intrinsic tenase complex, and this activation is effected in vitro by thrombin and by FXa (Eaton et al., 1986). Thrombin cleaves arginyl-serine bonds in FVIII at positions 372-373, 740-741, and 1689-1690 to generate a three-chain metal ion-bridged polypeptide with full cofactor activity. Site-directed mutagenesis of recombinant FVIII and characterization of naturally occurring variant molecules have demonstrated that the cleavages at positions 372-373 and 1689-1690 are essential for the expression of FVIII activity (Pittman & Kaufman, 1988; Eaton et al., 1986; O'Brien & Tuddenham, 1989; O'Brien et al., 1990; Arai et al., 1990). FXa also activates FVIII following cleavage adjacent to positions 372, 740, 1689, and 1721, and, in addition, the enzyme will further cleave FVIII at position Arg 336-Ser 337,

* To whom correspondence should be addressed.

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