Deletions in the Interdomain Hinge Region of Flavocytochrome b_2: Effects on Intraprotein Electron Transfer†

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Received February 28, 1995; Revised Manuscript Received October 5, 1995⊗

ABSTRACT: The two distinct domains of flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) are connected by a typical hinge peptide. To probe the importance of the structural integrity of the hinge region for efficient intraprotein electron transfer, three mutant enzymes have been constructed: HΔ3 [Sharp, R. E., White, P., Chapman, S. K., & Reid, G. A. (1994) *Biochemistry 33*, 5115−5120], HΔ6, and HΔ9 in which three, six, and nine amino acids, respectively, were deleted from the hinge region. Intraprotein electron transfer was investigated by steady-state and stopped-flow kinetic analyses. All three hinge-deletion enzymes remained good L-lactate dehydrogenases, as was evident from steady-state experiments with ferricyanide as the electron acceptor and from stopped-flow experiments monitoring flavin reduction. The global effect of these deletions is to lower the enzyme's effectiveness as a cytochrome *c* reductase. This property of HΔ6 and HΔ9 flavocytochromes b_2 is manifested at the first interdomain electron-transfer step (fully reduced FMN → heme electron transfer), where the rate of heme reduction is the same within experimental error as the steady-state rate of cytochrome *c* reduction. Thus, interdomain electron transfer is rate limiting in the case of these two hinge-deletion enzymes compared to the wild-type enzyme, where αH abstraction from C-2 of L-lactate still contributes substantially to rate limitation. The situation for HΔ3 is more complicated, with more than one interdomain electron-transfer step being affected. Kinetic data, along with the measured deuterium kinetic isotope effects, are discussed in the context of the flavocytochrome b_2 catalytic cycle and show that complete structural integrity within the hinge region is essential for efficient interdomain communication.

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) is an excellent paradigm for the investigation of biological electron transfer [see Chapman *et al.* (1991) for a review]: the gene encoding the enzyme has been cloned and sequenced (Guiard *et al*., 1985), allowing site-directed mutants to be constructed (Reid *et al*., 1988) and the protein to be overexpressed in the bacterium *Escherichia coli* (Black *et al*., 1989b). The crystal structures of the native wild-type (Xia & Mathews, 1990) and several recombinant mutant enzymes (Tegoni & Cambillau, 1994b) are known to high resolution; the protein is soluble and is an elegant model system for the investigation of both intra- and interprotein electron transfer. The enzyme, isolated from baker's yeast (*Saccharomyces cerevisiae*), is a homotetramer of subunit molecular weight 57 500 (Jacq & Lederer, 1974). It is a soluble component of the mitochondrial intermembrane space (Daum *et al*., 1982), where it catalyzes the oxidation of L-lactate to pyruvate with subsequent electron transfer to cytochrome *c* (Appleby & Morton, 1954). The catalytic cycle for this reaction consists of several identifiable steps and is illustrated in Figure 1. The crystal structure of flavocytochrome b_2 solved to 0.24 nm resolution (Xia & Mathews, 1990) shows that each subunit consists of two

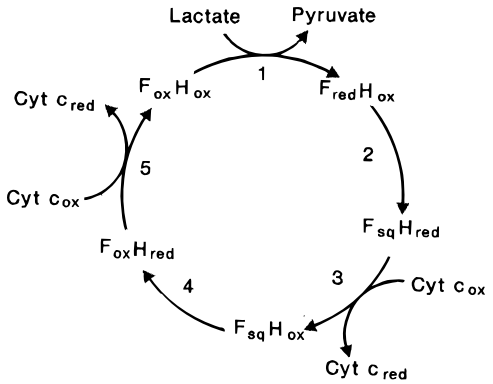

FIGURE 1: Catalytic cycle of flavocytochrome b_2. The redox states of cytochrome *c* and the flavocytochrome b_2 flavin (F) and heme (H) are indicated by the subscripts ox and red for the oxidized and reduced forms, respectively. The flavin semiquinone is shown as F_{sq}. (1) Oxidation of lactate to pyruvate and reduction of FMN; this is the major rate-limiting step in the case of the wild-type enzyme. (2) Electron transfer from fully reduced FMN to heme, resulting in the semiquinone form of FMN and reduced heme (this is the rate-limiting step in the case of the HΔ6 and HΔ9 enzyme). (3) Reduction of the first cytochrome *c* molecule by electron transfer from the heme group of flavocytochrome b_2. (4) Electron transfer from the semiquinone form of FMN to heme, resulting in fully oxidized FMN and reduced heme (this is probably the rate-limiting step in the HΔ3 enzyme). (5) Reduction of a second cytochrome *c* molecule by electron transfer from the heme group, which results in the regeneration of the fully oxidized enzyme. The enzyme is now ready to repeat the cycle.

† This work was supported by research grants from the Biotechnology and Biological Sciences Research Council (U.K.), which also provided a studentship for R.E.S., and by the European Commission (FLAPS Network).

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⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1996.

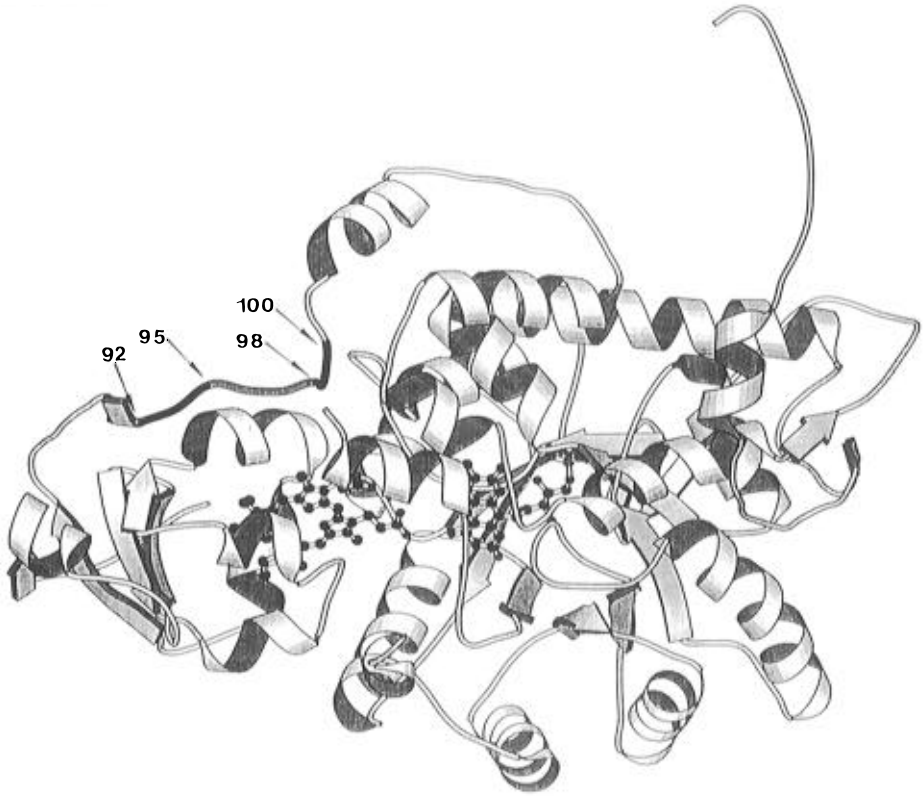

FIGURE 2: Graphical representation of a single wild-type flavocytochrome b_2 subunit generated by using the *MOLSCRIPT* molecular graphics program (Kraulis, 1991). α-Helices and β-sheets are shown as ribbons, the remaining α-carbon backbone is shown as wire, and ball and stick atoms represent the prosthetic groups. The arrow indicates the hinge region. The positions of the residues deleted from the hinge mutants are highlighted as follows: black shaded region flanked by positions 98 → 100, HΔ3; black and gray shaded regions flanked by positions 95 → 100, HΔ6; black, gray, and black shaded regions flanked by positions 92 → 100, HΔ9.

	89	P	P	E	L	V	C	P	P	Y	A	P	G	E	T	K	103
HΔ3		P	P	E	L	V	C	P	P	Y				E	T	K	
HΔ6		P	P	E	L	V	C							E	T	K	
HΔ9		P	P	E										E	T	K	

FIGURE 3: Construction of the hinge-deletion flavocytochromes b_2. The amino acid sequence of the interdomain hinge region from the *S. cerevisiae* enzyme is shown, along with the truncated hinge region of the deletion mutants (gaps in the sequence indicate the residues deleted). Key: H indicates the hinge region; Δ indicates a deletion; the number indicates the number of amino acid residues deleted.

distinct domains: an N-terminal domain containing protoheme IX and a C-terminal domain containing flavin mononucleotide (Figure 2).

The two domains of *S. cerevisiae* flavocytochrome b_2 are connected by a typical hinge sequence that contains proline, glycine, and various charged residues (Figure 3). It has been proposed that the most likely role of this hinge region is to confer domain mobility, allowing movement of the cytochrome domain with respect to the flavin domain (White *et al*., 1993). This proposal is supported by a number of observations from crystallography and NMR[1] spectroscopy. In the three-dimensional structure of *S. cerevisiae* flavocytochrome b_2, two crystallographically distinct subunits are visible in the asymmetric unit. In one subunit, neither product nor substrate is bound at the active site and the cytochrome domain is resolved. In the other, pyruvate is bound at the active site and no electron density is observed for the cytochrome domain, implying that it is positionally disordered (Xia & Mathews, 1990). However, the fact that two of the cytochrome domains in the crystallized tetramer are positionally ordered may be due to restricted mobility imposed by crystal packing forces. In solution, NMR spectroscopy shows that the cytochrome domain is substantially more mobile than expected for a protein as large as the flavocytochrome b_2 tetramer; the observed linewidths for the heme group resonances in the cytochrome domain are not broadened to the degree anticipated for a large protein, implying considerable flexibility of this domain (Labeyrie *et al*., 1988).

Previous kinetics experiments involving a "hinge-swap" mutant enzyme, in which the hinge region of *S. cerevisiae* flavocytochrome b_2 was replaced by the equivalent region of the *Hansenula anomala* enzyme, showed that the structural integrity of this peptide was essential for efficient interdomain electron transfer (White *et al*., 1993). The rate of FMN → heme electron transfer had fallen 1000-fold in the hinge-swap enzyme compared to wild-type flavocytochrome b_2. A more subtle mutation, in which three amino acids were deleted from the hinge region of *S. cerevisiae* flavocytochrome b_2 (HΔ3 mutant, Figure 3), again illustrated the importance of maintaining the structural integrity of the hinge for efficient interdomain communication. In this case, the rate of fully reduced FMN → heme electron transfer was

[1] Abbreviations: FMN, flavin mononucleotide; k^F, pre-steady-state rate constant for flavin reduction; k^H, pre-steady-state rate constant for heme reduction; K_m^F, apparent K_m for pre-steady-state flavin reduction; K_m^H, apparent K_m for pre-steady-state heme reduction; KIE, kinetic isotope effect; [^{1}H]Lac, L-[2-^{1}H]lactate; [^{2}H]Lac, L-[2-^{2}H]lactate; ferri, ferricyanide; cyt *c*, cytochrome *c*; SHE, standard hydrogen electrode; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

16-fold lower than that of the wild-type enzyme (Sharp *et al*., 1994).

To further our understanding of the role of the hinge region in intraprotein (interdomain) electron transfer, we have constructed a set of two additional hinge mutant flavocytochromes b_2: HΔ6 and HΔ9, with six (residues 95−100) and nine (residues 92−100) amino acids deleted from the primary structure, respectively (Figures 2 and 3). The electron-transfer properties of the HΔ6 and HΔ9 enzymes are described here in the context of the previously characterized HΔ3 (Sharp *et al*., 1994) and wild-type flavocytochromes b_2 (Miles *et al*., 1992). These deletions were designed to start at the C-terminal end of the hinge region and progress toward the cytochrome domain, essentially deleting the central portion of the defined hinge region (Black *et al*., 1989a). In addition, these deletions are expected to have no effect upon the net charge.

MATERIALS AND METHODS

DNA Manipulation, Strains, and Growth. Site-directed mutagenesis was performed by the Kunkel method of nonphenotypical selection (Kunkel, 1985) using the oligonucleotides *327N* (AACTTGTCTGTGAAACTAAGGA) to construct HΔ6 and *328N* (TGCCTCCTGAAGAAACTAAGGA) to construct HΔ9 (Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland). Standard methods for the growth of *E. coli*, plasmid purification, DNA manipulation, and transformation were performed as described in Sambrook *et al*. (1991). *E. coli* strain AR120 was used for the expression of mutant flavocytochromes b_2.

Enzymes. Wild-type and hinge-deletion flavocytochromes b_2 expressed in *E. coli* were isolated from cells that had been stored at −20 °C. The purification procedure was essentially as previously described (Black *et al*., 1989b), except that throughout all stages of the procedure flavin mononucleotide (FMN) was present at a concentration of 5 μM in the purification buffers. Exogenous FMN was added to prevent dissociation of endogenous enzyme-bound FMN from the hinge-deletion flavocytochromes b_2 during purification. Purified enzyme preparations were stored under a nitrogen atmosphere at 4 °C as precipitates in 70% $(NH_4)_2SO_4$. Under these conditions the enzymes retained full activity for several weeks. Enzyme concentrations were calculated by using previously published extinction coefficients (Pajot & Groudinsky, 1970).

Kinetic Analysis. All kinetic experiments were carried out at 25 ± 0.1 °C in 10 mM Tris-HCl at pH 7.5 and $I = 0.10$ M (I adjusted to 0.10 M by the addition of NaCl). Steady-state kinetic measurements involving the enzymatic oxidation of L-lactate were performed by using a Shimadzu UV2101PC or a Beckman DU62 spectrophotometer. Horse heart cytochrome *c* (type VI, Sigma) or ferricyanide (potassium salt, BDH chemicals) was used as the electron acceptor, as previously described (Miles *et al*., 1992). Stopped-flow measurements involving the single turnover of L-lactate were carried out with an Applied Photophysics SF.17 MV stopped-flow spectrofluorimeter (dead time, 1 ms; 1 cm path length; mixing volume, 100 μL; dilution factor, 2) as previously described (Sharp *et al*., 1994). Flavin reduction was monitored at 438.3 nm (a heme isosbestic point), and heme reduction was monitored at either 423 or 557 nm (the measured rate constants were identical at both wavelengths). Collection and analysis of data were as previously described (Miles *et al*., 1992). K_m values and rate constants were determined by using nonlinear regression analysis. Kinetic isotope effect analysis was performed with L-[2-^{2}H]lactate. Purification and preparation of deuterated substrates (Pompon *et al*., 1980) and measurements of kinetic isotope effects, using this substrate, were as previously described (Miles *et al*., 1992).

Measurement of Redox Potential. The midpoint potentials of the heme groups of the mutant flavocytochromes b_2 were determined spectrophotometrically by using a previously published redox potentiometry method (Dutton, 1978). The mediators, *N*-ethylphenazonium sulfate (14 μM), *N*-methylphenazonium sulfate (14 μM), 2,3,5,6-tetramethylphenylenediamine (14 μM), 2-hydroxy-1,4-naphthoquinone (14 μM), flavin mononucleotide (14 μM), and iron(III) sulfate/EDTA (20 μM), were used as previously described (Dutton, 1978) at the indicated concentrations. The enzyme concentration was 10 μM. Reduction was achieved by titrating with sodium dithionite under anaerobic conditions, and oxidation was achieved by titrating with potassium ferricyanide (Brunt *et al*., 1992). Changes in heme absorbance at 557 nm (where the absorbance due to the mediators was negligible) were measured with changing electrode potential. The midpoint potentials were determined with a platinum electrode relative to the Ag^+/AgCl cell and were corrected for SHE by the addition of 196 mV to the measured potential. The electrode was calibrated with respect to the known potential of the $Fe^{II}-Fe^{III}$EDTA couple (Kolthoff & Auerbach, 1952). The system was buffered with the same Tris buffer that was used for kinetic experiments. The Nernst plots for both reductive and oxidative sequences showed no hysteresis, implying that the system was at equilibrium.

RESULTS

Steady-State Kinetic Properties of the Hinge-Deletion Flavocytochromes b_2. Table 1 presents the results of the steady-state kinetic measurements on HΔ3, HΔ6, and HΔ9 flavocytochromes b_2 using L-[2-^{1}H]lactate and L-[2-^{2}H]lactate as substrates with ferricyanide and cytochrome *c* as electron acceptors. These results are compared to previously reported values for the wild-type enzyme.

From the data in Table 1A, it is clear that all three hinge-deletion mutants are good L-lactate dehydrogenases as judged by the k_{cat} value with ferricyanide as electron acceptor; only a 40% decrease is seen for HΔ3 and HΔ6, whereas in the case of HΔ9 the k_{cat} value is identical to that of wild-type flavocytochrome b_2. The ratio of k_{cat}/K_m gives a measure of the catalytic efficiency of an enzyme, and comparison of these values to that of wild-type flavocytochrome b_2 shows that HΔ3 and HΔ6 have catalytic efficiencies 40% and 70% of the value for the wild-type enzyme, respectively. The k_{cat}/K_m for HΔ9, however, is the same within experimental error as the value reported for wild-type flavocytochrome b_2.

The ferricyanide reduction data illustrate that there are no major kinetic differences between wild-type and hinge-deletion flavocytochromes b_2 when this inorganic anion is used as an electron acceptor. However, when the physiological electron acceptor cytochrome *c* is used, the values of k_{cat} for HΔ3, HΔ6, and HΔ9 flavocytochromes b_2 are significantly decreased by factors of 5-, 6-, and 26-fold with respect to the value for the wild-type enzyme (Table 1B). The deuterium KIE values determined with the HΔ3 and

Table 1: Steady-State Kinetic Parameters and Deuterium Kinetic Isotope Effects for Wild-Type and Hinge-Deletion Flavocytochromes b_2[a]

Part A

enzyme	k_{cat} (s^{-1}) [^{1}H]Lac	k_{cat} (s^{-1}) [^{2}H]Lac	K_m (mM) [^{1}H]Lac	K_m (mM) [^{2}H]Lac	k_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1}) [^{1}H]Lac	k_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1}) [^{2}H]Lac	KIE
wild type[b]	400 ± 10	86 ± 5	0.49 ± 0.05	0.76 ± 0.06	8.2 ± 1.1	1.1 ± 0.2	4.7 ± 0.4
HΔ3[c]	257 ± 10	70 ± 2	0.72 ± 0.07	0.94 ± 0.11	3.6 ± 0.5	0.75 ± 0.12	3.7 ± 0.4
HΔ6	276 ± 10	61 ± 6	0.49 ± 0.06	0.65 ± 0.11	5.6 ± 1.1	0.94 ± 0.3	4.5 ± 0.7
HΔ9	400 ± 10	120 ± 10	0.37 ± 0.04	0.47 ± 0.06	10.8 ± 3.3	2.6 ± 0.6	3.3 ± 0.4

Part B

enzyme	k_{cat} (s^{-1}) [^{1}H]Lac	k_{cat} (s^{-1}) [^{2}H]Lac	K_m (mM) [^{1}H]Lac	K_m (mM) [^{2}H]Lac	k_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1}) [^{1}H]Lac	k_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1}) [^{2}H]Lac	[^{1}H]Lac inhibition (mM) [S_{opt}][d]	[^{1}H]Lac inhibition (mM) K_i	KIE
wild type[b]	207 ± 10	70 ± 10	0.24 ± 0.04	0.48 ± 0.10	8.6 ± 2.3	1.5 ± 0.6	6.2 ± 0.6	173 ± 13	3.0 ± 0.6
HΔ3[c]	39 ± 1	20 ± 1	0.25 ± 0.03	0.61 ± 0.07	1.6 ± 0.2	0.3 ± 0.1	4.5 ± 0.3	92 ± 5	2.0 ± 0.1
HΔ6	33.5 ± 1.4	14.0 ± 0.4	0.09 ± 0.01	0.13 ± 0.02	3.7 ± 0.7	1.1 ± 0.2	1.2 ± 0.4	21.1 ± 2.2	2.4 ± 0.2
HΔ9	7.9 ± 0.4	4.6 ± 0.3	0.02 ± 0.01	ND	3.4 ± 2.1	ND	0.6 ± 0.2	14.0 ± 1.5	1.7 ± 0.2

Part C[e]

enzyme	electron acceptor K_m: ferricyanide (mM)	electron acceptor K_m: cytochrome *c* (μM)
wild type[b]	≪0.1	10.0 ± 1.0
HΔ3[c]	0.15 ± 0.06	7.0 ± 1.0
HΔ6	0.40 ± 0.07	3.4 ± 0.6
HΔ9	0.58 ± 0.10	2.2 ± 0.3

[a] Steady-state kinetic parameters and deuterium kinetic isotope effects for wild-type and hinge-deletion flavocytochromes b_2. All experiments were performed at 25 °C in 10 mM Tris-HCl buffer (pH 7.5), with ionic strength (*I*) adjusted to 0.10 M by the addition of NaCl. (A) and (B) show data for ferricyanide and cytochrome *c* reduction, respectively, while (C) shows the K_m values for these electron acceptors. Concentrations of electron acceptors used with the hinge-deletion and wild-type flavocytochromes b_2 were as follows. [ferricyanide]: wild-type = 1 mM, HΔ3 = 2 mM, and HΔ6 and HΔ9 = 8 mM (these concentrations were greater than 90% saturating in all cases). [cytochrome *c*]: wild-type = 30 μM (>75% saturating) and HΔ3, HΔ6, and HΔ9 = 35 μM (>80% and >90% saturating for HΔ3 and HΔ6/HΔ9, respectively). The k_{cat} values are expressed as moles of electrons transferred per second per mole of enzyme (as L-lactate is a two-electron donor, these values can be halved to express them in terms of moles of substrate reduced per second). [b] Miles *et al*. (1992). [c] Sharp *et al*. (1994). [d] $[S_{opt}]$, optimal [substrate] for the fastest observed rate. ND, not determined. [e] For (C), the L-lactate concentration was 10 mM throughout, except for experiments with HΔ6 and HΔ9 flavocytochromes b_2, where cytochrome *c* was used as the electron acceptor. In these cases, [L-lactate] was 1 mM, which is close to the optimum substrate concentration.

HΔ9 enzymes are significantly lower than those with wild-type flavocytochrome b_2 for both electron acceptors. This implies that electron-transfer reactions following αH abstraction from C-2 of L-lactate contribute to the overall rate limitation in these enzymes to a greater extent than in the wild-type enzyme. Interestingly, the HΔ6 enzyme has the same KIE values within experimental error as wild-type flavocytochrome b_2 for both electron acceptors. However, electron flow to cytochrome *c* has been affected in HΔ6, as is evident from the lower k_{cat} for cytochrome *c* reduction.

An interesting observation regarding the kinetic behavior of HΔ6 and HΔ9 flavocytochromes b_2, when cytochrome *c* is used as the electron acceptor, is the marked substrate inhibition exhibited by these mutant enzymes over the L-lactate concentration range investigated (Table 1B). The degree of inhibition was much smaller over an equivalent concentration range for both HΔ3 and wild-type flavocytochrome b_2. Also, no significant inhibition was observed for any mutant or wild-type enzyme when ferricyanide was used as the electron acceptor. This kinetic phenomenon, which becomes more apparent as the length of the hinge region is progressively truncated, may be due to the greater accessibility of lactate to the active site for these hinge-deletion enzymes compared to wild-type flavocytochrome b_2, although the precise physical basis for this inhibition by excess substrate is unknown.

Another significant difference in the steady-state kinetic behavior between the hinge-deletion and wild-type flavocytochromes b_2 is the dependence of the reaction rates on electron acceptor concentration (Table 1C). The most obvious difference is found with ferricyanide. Under the experimental conditions used in this study, no concentration dependence was observed above 100 μM ferricyanide for the wild-type enzyme. The hinge-deletion enzymes, however, had K_m values for ferricyanide that increased as the length of the hinge region was progressively truncated (Table 1C). This necessitated kinetic assays being performed at higher ferricyanide concentrations for the hinge-deletion enzymes than is routine for wild-type flavocytochrome b_2 (Table 1). A similar phenomenon has been observed in other mutant forms of flavocytochrome b_2 in which electron transfer from flavin to heme has been impaired, and the reasons for the resulting ferricyanide concentration dependence are explained in Miles *et al*. (1992). In essence, ferricyanide is more readily reduced by the flavocytochrome b_2 heme than by the flavin hydroquinone. In the wild-type enzyme, the intrinsic rate of flavin to heme electron transfer is considerably faster than the oxidation of flavin hydroquinone by ferricyanide. However, by slowing down the rate of flavin to heme electron transfer in mutant enzymes, the heme can be effectively bypassed since the rate of the flavin hydroquinone to ferricyanide electron transfer becomes more significant. In the most extreme case, the separately expressed flavin domain (with no heme group present) shows a ferricyanide concentration dependence similar to that described earlier (Balme *et al*., 1995).

Pre-Steady-State Kinetic Parameters for L*-Lactate Oxidation*. Microscopic rate constants for the reduction of the FMN and heme prosthetic groups of hinge-deletion and wild-type flavocytochromes b_2 by [^{1}H]lactate and [^{2}H]lactate were determined directly by using stopped-flow spectrophotometry. The kinetic parameters are summarized in Table 2.

Table 2: Pre-Steady-state Kinetic Parameters and Deuterium Kinetic Isotope Effects for Reduction of FMN and Heme in Wild-Type and Hinge-Deletion Flavocytochromes b_2[a]

Part A

	k^F(s^{-1})		K_m^F(mM)		k^F/K_m^F($\times 10^5$ M^{-1} s^{-1})		
enzyme	[^{1}H]Lac	[^{2}H]Lac	[^{1}H]Lac	[^{2}H]Lac	[^{1}H]Lac	[^{2}H]Lac	KIE
wild type[b]	604 ± 60	75 ± 5	0.84 ± 0.20	1.33 ± 0.28	7.2 ± 3.2	0.6 ± 0.2	8.1 ± 1.4
HΔ3[c]	518 ± 17	71 ± 3	0.60 ± 0.08	1.46 ± 0.21	8.6 ± 1.7	0.5 ± 0.1	7.3 ± 0.6
HΔ6	514 ± 21	83 ± 3	0.43 ± 0.08	0.30 ± 0.05	12.0 ± 3.3	2.8 ± 0.6	6.2 ± 0.5
HΔ9	690 ± 16	64 ± 3	0.30 ± 0.03	0.44 ± 0.06	23.0 ± 3.2	1.5 ± 0.3	10.8 ± 0.8

Part B

	k^H (s^{-1})		K_m^H (mM)		k^H/K_m^H($\times 10^5$ M^{-1} s^{-1})		[^{1}H]Lac inhibition (mM)		
enzyme	[^{1}H]Lac	[^{2}H]Lac	[^{1}H]Lac	[^{2}H]Lac	[^{1}H]Lac	[^{2}H]Lac	[S_{opt}][d]	K_i	KIE
wild type[a]	445 ± 50[f]	71 ± 5	0.53 ± 0.05	0.68 ± 0.05	8.4 ± 1.9	1.0 ± 0.2	9.6 ± 0.7	174 ± 8	6.3 ± 1.2
HΔ3[b]	91 ± 3	32 ± 1	0.38 ± 0.07	0.80 ± 0.13	2.4 ± 0.6	0.4 ± 0.1	6.2 ± 0.4[e]	132 ± 8[e]	2.8 ± 0.2
HΔ6[g]	29 ± 1	15 ± 1	0.04 ± 0.01	0.10 ± 0.02	7.3 ± 2.7	1.5 ± 0.5			1.9 ± 0.2
HΔ9[g]	9 ± 1	8 ± 1	0.07 ± 0.02	0.21 ± 0.06	1.3 ± 0.7	0.4 ± 0.2			1.1 ± 0.2

[a] Pre-steady-state kinetic parameters and deuterium KIE values for reduction of FMN and heme in wild-type and hinge-deletion flavocytochromes b_2. The parameters for flavin reduction are shown in (A) and the parameters for heme reduction in (B). All experiments were performed at 25 °C in 10 mM Tris-HCl buffer (pH 7.5, $I = 0.10$ M). Stopped-flow data were analyzed as described in Materials and Methods. Values of k^F and k^H are expressed as the number of prosthetic groups reduced per second. The k_{cat} values reported correspond to the rapid phase of biphasic traces, unless otherwise indicated. [b] Miles *et al*. (1992). [c] Sharp *et al*. (1994). [d] [S_{opt}], optimal [substrate] for the fastest observed rate. [e] This work. [f] The actual rate constant for fully reduced FMN → heme electron transfer is 1500 ± 500 s^{-1}; the value quoted in the table is a consequence of the traces from heme reduction by L-lactate being fitted to a biphasic exponential equation (Chapman *et al*., 1994). [g] Rapid phase of triphasic fit.

The rate of FMN reduction by L-lactate is not significantly altered by the hinge deletions; the rate constants, k^F, for the three mutant enzymes are within 15% of the value for wild-type enzyme (Table 2A). Values of K_m^F for L-lactate show a steady decrease from wild-type through successive truncations of the hinge region to HΔ9. The deuterium KIE values for all of the hinge-deletion enzymes, within experimental error, are the same as the value for the wild-type enzyme.

The effect of the hinge-deletion mutations on heme reduction is more striking than that for FMN reduction. The rate constant for heme reduction by L-lactate, k^H, decreases in value from that of the wild-type enzyme as the length of the hinge region is truncated (Table 2B). The values of k^H are 5-, 16-, and 50-fold lower for HΔ3, HΔ6, and HΔ9, respectively, than the corresponding value for the wild-type enzyme. This shows that, as the hinge region is progressively truncated, the degree of impairment of the rate of heme reduction increases. Thus, maintenance of the structural integrity of the hinge region is crucial for effective interdomain (FMN → heme) electron transfer in flavocytochrome b_2.

The K_m^H value of the HΔ3 enzyme for L-lactate is slightly lower than the value for the wild-type enzyme. However, the HΔ6 and HΔ9 enzymes have K_m^H values that are about 10-fold lower than that for wild-type flavocytochrome b_2. The deuterium KIE values for all three hinge-deletion enzymes are significantly lower than the value for wild-type flavocytochrome b_2, implying that, for these mutants, αH abstraction from C-2 of L-lactate contributes much less to rate limitation than for the wild-type enzyme, and in the case of the HΔ9 enzyme it hardly contributes at all. Interestingly, the HΔ6 and HΔ9 enzymes, which exhibit strong substrate inhibition in the steady-state reduction of cytochrome *c*, exhibit no inhibition during pre-steady-state heme reduction. Unlike the heme reduction behavior of some of the hinge-deletion enzymes, no substrate inhibition occurred over the equivalent concentration ranges for FMN reduction (data not shown), which is a situation similar to that observed for the steady-state reduction of ferricyanide.

The heme reduction traces of HΔ6 and HΔ9 flavocytochromes b_2 fit more accurately to triphasic rather than biphasic exponential equations, as illustrated in Figure 4B,C for the HΔ9 enzyme. The first phase comprises about 20% of the total amplitude of the trace over most of the L-lactate concentrations investigated and is the initial rate of heme reduction for both enzymes. The other phases, of which there are at least two, are too slow to be kinetically relevant in the catalytic cycle and represent a number of electron redistribution steps between subunits (see Discussion), although they are fast enough to perturb the equilibrium between flavin and heme. This may explain why the amplitude of the first phase is relatively small. In the case of both enzymes, an increase in the flavocytochrome b_2 concentration had no effect upon the rate constants for heme reduction (data not shown), implying that none of the phases observed are due to interprotein electron transfer.

Heme Redox Midpoint Potentials of Hinge-Deletion Flavocytochromes b_2. We considered the possibility that the construction of deletion mutations in the hinge region of flavocytochrome b_2 might affect the redox potentials of the prosthetic groups. Clearly, there can have been very little or no effect on the FMN oxidized/reduced midpoint potential for the three hinge-deletion enzymes, as the microscopic rate constants for FMN reduction are not significantly different from those of wild-type flavocytochrome b_2. However, it appears possible that a large change in the heme midpoint potential could have contributed to the decrease in k^H, the rate constant for heme reduction, in some or all of the hinge-deletion enzymes. To check this, the heme midpoint potentials for the three hinge-deletion flavocytochromes b_2 were measured (Table 3 and Figure 5). Both the HΔ3 and HΔ6 enzymes have the same heme potential within experimental error as the wild-type enzyme. However, HΔ9 has a slightly lower heme potential, by about 15 mV, than the value for wild-type flavocytochrome b_2 (Miles, 1992).

DISCUSSION

Effect of the Hinge Deletions on the Catalysis of L-*Lactate Dehydrogenation by Flavocytochrome b_2.* The catalytic cycle for flavocytochrome b_2 is shown diagrammatically in Figure 1. Step 1, flavin reduction by lactate, is virtually

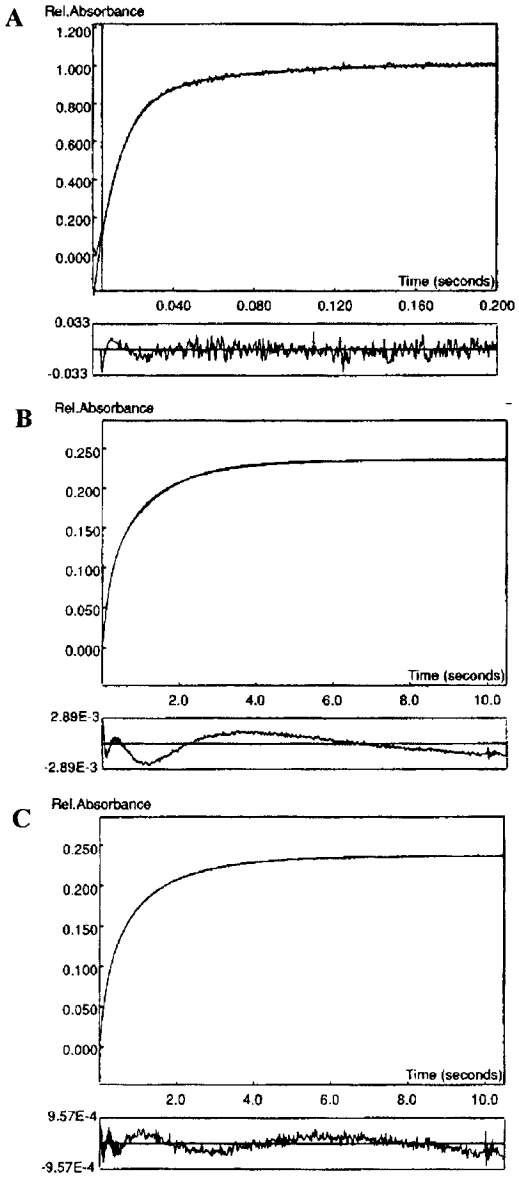

FIGURE 4: Heme reduction of HΔ3 and HΔ9 flavocytochromes b_2. Absorbance changes at 423 nm with time by monitoring single turnover of flavocytochrome b_2 heme, upon reduction by L-lactate. The experiments were performed at 25 °C in 10 mM Tris-HCl buffer (pH 7.5, I = 0.10 M). Panel A is a trace of HΔ3 flavocytochrome b_2 heme reduction, where [HΔ3 flavocytochrome b_2] = 10 μM and [L-lactate] = 10 mM. Panels B and C are traces of HΔ9 flavocytochrome b_2 heme reduction, where [HΔ9 flavocytochrome b_2] = 2.5 μM and [L-lactate] = 1 mM. Traces A and B are fit to a biphasic exponential equation, $y = A\exp(-k_a t) + B\exp(-k_b t)$, where, A and B are the amplitudes of the first and second phases, and $-k_a$ and $-k_b$ are the rate constants for the first and second phases (s^{-1}). Trace C is fitted to a triphasic exponential equation, $y = A\exp(-k_a t) + B\exp(-k_b t) + C\exp(-k_c t)$, where C and k_c are the amplitude and rate constant of the third phase, respectively. For (A), $A = 1.10 \pm 0.01$, $B = 0.19 \pm 0.01$, $k_a = 85.8 \pm 1.6\ s^{-1}$, and $k_b = 14.9 \pm 1.3\ s^{-1}$. For (B), $A = (9.13 \pm 0.04) \times 10^{-2}$, $B = (1.47 \pm 0.05) \times 10^{-2}$, $k_a = 5.38 \pm 0.05\ s^{-1}$, and $k_b = 0.81 \pm 0.01\ s^{-1}$. For (C), $A = (5.13 \pm 0.05) \times 10^{-2}$, $B = (9.46 \pm 0.06) \times 10^{-2}$, $C = (9.79 \pm 0.09) \times 10^{-2}$, $k_a = 9.45 \pm 0.09\ s^{-1}$, $k_b = 2.11 \pm 0.02\ s^{-1}$, and $k_c = 0.63 \pm 0.01\ s^{-1}$. The residuals of the data fits to the experimental traces are illustrated below each trace and show the reliability of fitting a trace to a particular equation. In the case of the HΔ3 enzyme, the heme reduction trace fits very well to a biphasic exponential equation (A). However, for the HΔ9 enzyme, from an examination of the residuals, a much better fit is obtained for a triphasic (C) rather than a biphasic (B) exponential equation: the magnitude of the deviation from the experimental fit is 3-fold smaller for the latter than for the former.

Table 3: Heme Midpoint Redox Potentials of Wild-Type, b_2-Core, and Hinge-Deletion Flavocytochromes b_2[a]

enzyme	heme redox potential (mV)
wild type[b]	-17 ± 3
b_2-core[c]	-31 ± 2
HΔ3[d]	-12 ± 5
HΔ6	-24 ± 5
HΔ9	-36 ± 5

[a] Heme midpoint redox potentials of wild-type, b_2-core, and hinge-deletion flavocytochromes b_2. All experiments were performed in 10 mM Tris-HCl buffer (pH 7.5, I = 0.10 M) at room temperature, as described in Materials and Methods. The midpoint potentials were determined with a platinum electrode, relative to the Ag^+/AgCl cell, and were corrected for SHE by the addition of 196 mV to the determined potential. [b] Miles (1992). [c] Independently expressed and purified cytochrome b_2 domain of flavocytochrome b_2, b_2-core (Brunt *et al*., 1992; Sharp *et al*., 1994). [d] Sharp *et al*. (1994).

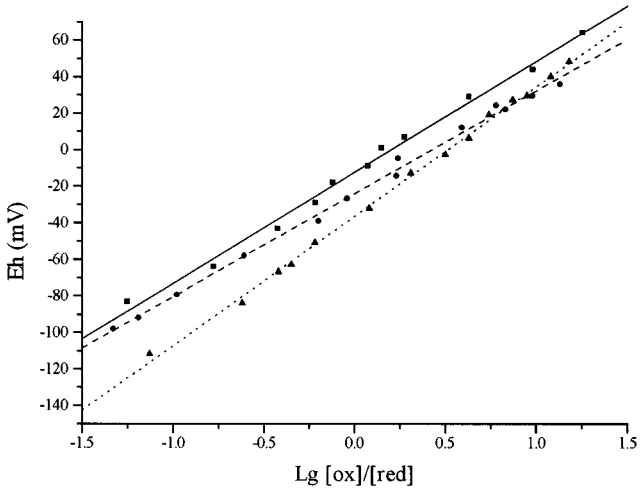

FIGURE 5: Nernst plots illustrating the heme midpoint potentials of hinge-deletion flavocytochromes b_2. The experiments were performed under anaerobic conditions, at 25 °C, in 10 mM Tris-HCl buffer (pH 7.5, I = 0.10 M). The titration was monitored at 557 nm, where the absorbance due to the mediators was negligible. Sodium dithionite was used as reductant and ferricyanide as the oxidant. The midpoint potentials are reported in Table 3. The slopes of the fits were all close to +59 mV, with values of 61 ± 2, 56 ± 1, and 71 ± 1 mV for the HΔ3 (solid line), HΔ6 (dashed line), and HΔ9 (dotted line) enzymes, respectively, indicating that a one-electron reduction step was occurring (n = 1).

unaffected in all three hinge-deletion flavocytochromes b_2; the rate constants for the pre-steady-state reduction of FMN by L-lactate are within 15% of the value for the wild-type enzyme. Intriguingly, HΔ9 is the first mutant flavocytochrome b_2 enzyme to be characterized that has a higher catalytic efficiency (k_{cat}/K_m) for L-lactate dehydrogenation than the wild-type enzyme. The deuterium KIE values with L-[2-^{2}H]lactate are the same for the hinge-deletion enzymes, compared to that for wild-type flavocytochrome b_2. Thus, the nature of the rate-limiting step for L-lactate dehydrogenation, hydrogen abstraction from C-2 of L-lactate, has not been altered by the hinge-deletion mutations. These data imply that the structural integrity of the interdomain hinge region is not of great importance for efficient FMN reduction by L-lactate, and all of the hinge-deletion flavocytochromes b_2 remain good L-lactate dehydrogenases compared to the wild-type enzyme. This conclusion is supported by the steady-state kinetic measurements using ferricyanide as the electron acceptor.

Effect of the Hinge-Deletion Mutations on the Rate of Interdomain Electron Transfer. As illustrated in Figure 6,

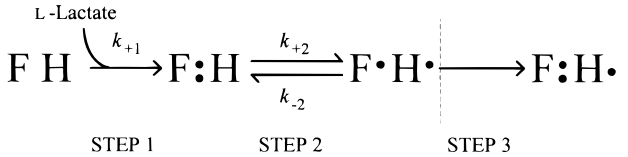

FIGURE 6: Electron-transfer steps occurring in a single flavocytochrome b_2 subunit during full reduction of the enzyme by L-lactate: step 1, reduction of FMN; step 2, FMN → heme electron transfer; step 3, entry of the third electron per subunit to generate fully reduced enzyme. Key: F, FMN; H, heme; black dots denote electrons.

the rapid first phase of heme reduction by L-lactate is a two-step process, where two electrons from L-lactate enter the flavocytochrome b_2 subunit at the level of the flavin and are redistributed between flavin and heme. The slower, second phase (step 3 in Figure 6) is dominated by the entry of the third electron per subunit due to inter-subunit electron transfer and is catalytically irrelevant during the turnover of flavocytochrome b_2. This step arises from the fact that full reduction of flavocytochrome b_2, in the absence of electron acceptors, requires three electrons per protomer, i.e., six lactate molecules per tetramer (Pompon *et al*., 1980; Miles *et al*., 1992). Following the reaction of a flavocytochrome b_2 tetramer with four lactate molecules, one can envisage electron transfer to heme resulting in the formation of flavin semiquinone in each subunit. Further reaction with lactate will then depend on inter-subunit electron transfer between flavin groups: two flavin semiquinones within a tetramer can disproportionate to yield one fully reduced and one fully oxidized FMN, which can then react with an additional lactate molecule. Reaction with a sixth lactate molecule can proceed similarly, and possibly simultaneously, so that the fully reduced enzyme is produced. This process is kinetically complex and leads to multiphasic absorbance vs time traces.

The reported rate constant for heme reduction of wild-type flavocytochrome b_2 by L-lactate is 445 ± 50 s^{-1} (Table 2), measured from a biphasic exponential fit to the heme reduction trace (Miles *et al*., 1992). However, as demonstrated by Chapman *et al*. (1994), fitting of the data to a biphasic exponential equation is an approximation. Step 2 in Figure 6 (fully reduced FMN → heme electron transfer) is faster than the preceding step 1 (FMN reduction by L-lactate); therefore, the rate constant for heme reduction by L-lactate will be limited by the rate of formation of reduced FMN. If these factors are taken into account and the data are fitted to the model described by Chapman *et al*. (1994), a rate constant of 1500 ± 500 s^{-1} is obtained for fully reduced FMN → heme electron transfer (step 2 in Figures 1 and 6). This value is the same within experimental error as the direct measurement of fully reduced FMN → heme electron transfer by using laser flash photolysis (1900 ± 100 s^{-1}; Hazzard *et al*., 1994). In the case of the hinge-deletion enzymes, however, fitting of the traces to biphasic or triphasic exponential equations is valid, as FMN reduction is very fast compared to the subsequent heme reduction rate, and no appreciable time lag occurs before sufficient reduced FMN is formed prior to heme reduction. Thus, in the hinge-deletion flavocytochromes b_2, the rate of fully reduced FMN → heme electron transfer is *actually* 16-, 50-, and 170-fold lower for the HΔ3, HΔ6, and HΔ9 enzymes, respectively, compared to that of wild-type flavocytochrome b_2. These results show that, as the interdomain hinge region is sequentially truncated, the rate constant for heme reduction (Figures 1 and 6, step 2) decreases in magnitude, implying that the maintenance of the structural integrity of the hinge region is crucial for effective interdomain communication.

Nature of the Rate-Limiting Steps in the Catalytic Cycle of Hinge-Deletion Flavocytochromes b_2. For the hinge-deletion enzymes, HΔ6 and HΔ9, the rate constants for heme and cytochrome *c* reduction are the same within experimental error (30 and 8 s^{-1}, respectively), implying that, for these enzymes, interdomain electron transfer from FMN → heme is the rate-limiting step. In the case of the HΔ3 enzyme, the rate constant for interdomain electron transfer is 91 s^{-1}, but the steady-state rate constant for cytochrome *c* reduction is 39 s^{-1}. Thus, interdomain electron transfer is not totally rate limiting in the HΔ3 enzyme, and some other electron-transfer step must contribute to rate limitation. Pseudo-first-order reduction of horse heart cytochrome *c* by prereduced HΔ3 flavocytochrome b_2 under pre-steady-state conditions yields a second-order rate constant of 4.3 × 10^6 M^{-1} s^{-1} (Sharp *et al*., 1994). This value was determined by fitting a plot of k_{obs} against HΔ3 concentration to linear regression analysis. Over the concentration range investigated, the plot did not deviate from a straight line fit, indicating that the system was not saturating, and k_{obs} values up to 100 s^{-1} were measured. This implies that, contrary to previous ideas (Sharp *et al*., 1994), electron transfer from b_2 heme to cytochrome *c* cannot be rate limiting in the steady-state reduction of cytochrome *c*. Step 4 in Figure 1 shows interdomain electron transfer from the semiquinone form of FMN to oxidized heme. In light of the preceding evidence, it is reasonable to propose that, in the HΔ3 enzyme, FMN semiquinone → heme electron transfer has a large contribution to rate limitation, and one could tentatively suggest that the rate of this step is about 30 s^{-1}.

The preceding conclusions are supported by the deuterium KIE values reported in Tables 1 and 2. The wild-type enzyme has the same KIE value for L-lactate → heme electron transfer, within experimental error, as that for FMN reduction by L-lactate, implying that hydrogen abstraction from C-2 of L-lactate is still rate limiting (this is not surprising in view of the fact that the rate of fully reduced FMN → heme electron transfer is faster than the rate constant for FMN reduction by L-lactate). However, the KIE values for heme reduction by L-lactate in the hinge-deletion enzymes are significantly lower than their corresponding KIE values for FMN reduction by L-lactate (Table 2A) and appear to follow a trend: as the length of the hinge region is progressively truncated, the magnitude of the KIE value decreases. In the case of the HΔ6 and HΔ9 enzymes, where the KIE values are insignificant (<2), hydrogen abstraction from C-2 of L-lactate no longer contributes to the rate limitation of heme reduction. This implies that the deletion of at least six residues from the hinge region causes FMN → heme electron transfer to become totally rate limiting. This conclusion is supported by the fact that, for the HΔ6 enzyme, the KIE value for steady-state cytochrome *c* reduction by L-lactate (Table 1B) is the same within experimental error as that for pre-steady-state heme reduction. For the HΔ3 enzyme, the KIE value for heme reduction by L-lactate is 2.8, and although this is much lower than the corresponding KIE value of 7.3 for FMN reduction, it is still significant, implying that hydrogen abstraction from C-2 of L-lactate still contributes to the rate limitation of heme reduction in the HΔ3 enzyme, but much less so than in wild-type flavocytochrome b_2 (Sharp *et al*., 1994). Also, the KIE value for steady-state cytochrome *c* reduction is lower than

that for pre-steady-state heme reduction in the HΔ3 enzyme, implying that an electron-transfer step subsequent to fully reduced FMN → heme electron transfer contributes to rate limitation. In summary, for the hinge-deletion enzymes, the KIE values are consistent with the rate constant data for FMN, heme, and cytochrome *c* reduction, since both the rate constants and the KIE values erode in a similar fashion along the electron-transfer pathway.

Effect of the Hinge-Deletion Mutations on the Heme Group Midpoint Potentials. As already discussed, the most probable cause of the decreased rate of interdomain electron transfer in hinge-deletion flavocytochromes b_2 is that the truncated hinge region has reduced flexibility, resulting in impaired recognition between the flavin and cytochrome domains. The possibility that these decreases in rate constants could have been caused by a change in the thermodynamic driving force for electron transfer, due to a large shift in the heme group midpoint potential, has been eliminated. The heme potentials of the HΔ3 and HΔ6 enzymes are the same within experimental error as that found with the wild-type enzyme. The HΔ9 enzyme has a heme potential that is about 15 mV more negative than that of the wild-type enzyme, but is the same, within experimental error, as that for the independently expressed and purified cytochrome b_2 domain of flavocytochrome b_2, commonly referred to as b_2-core (Brunt *et al*., 1992). However, this change in the heme potential for the HΔ9 enzyme is too small to alter the equilibrium constant or the driving force of the FMN → heme electron-transfer reaction to such an extent that it would account for the large decrease observed in the interdomain electron-transfer rate constant. It is interesting that both b_2-core and the HΔ9 enzyme have the same heme potential. The pK_a values of the heme propionates in b_2-core have been determined (Brunt *et al*., 1992) and are close in value to that of free heme propionic acid groups (Moore & Pettigrew, 1990), implying that the propionate groups of b_2-core must be exposed to solvent. The exposure of the heme edge to solvent in b_2-core probably accounts for the chain in redox potential of the heme group compared to the wild-type enzyme. Thus, it is plausible to suggest that the heme edge is more exposed to solvent in the HΔ9 enzyme than in wild-type, HΔ3, or HΔ6 flavocytochrome b_2. This idea is consistent with the proposal that the large 9-amino acid deletion in the hinge region of the HΔ9 enzyme has impaired interdomain communication by "pulling" the cytochrome domain away from the flavin domain, hindering effective contact and exposing the heme group.

Comparison with Other Mutant Flavocytochromes b_2. We have previously reported the characterization of two other mutant enzymes that have disrupted interdomain electron transfer. As already stated, the crystal structure of flavocytochrome b_2 shows two crystallographically distinct subunits in the asymmetric unit. In subunit 1, both domains are clearly visible in the electron density map, and tyrosine 143 forms an interdomain hydrogen bond with a heme propionate group (the only direct interdomain hydrogen bond). In subunit 2, however, there is a molecule of pyruvate located in the active site with its carboxylate group hydrogen bonded to Y143, and in this case, electron density for the cytochrome domain is not apparent due to positional disorder (Xia & Mathews, 1990). Thus, it appears that Y143 is uniquely positioned to play an important role in controlling electron transfer from substrate to FMN and from FMN to heme. The mutant Y143F flavocytochrome b_2 was constructed and characterized to test these proposals. The pre-steady-state experiments of Miles *et al*. (1992) clearly support the proposal that Y143 is important for FMN → heme electron transfer, as the rate constant for interdomain electron transfer is 21 s^{-1} in the Y143F enzyme, which is 50-fold lower than the value for wild-type flavocytochrome b_2. Additionally, this mutation had little effect upon the L-lactate dehydrogenase function of the enzyme (Miles *et al*., 1992; Rouvière-Fourmy *et al*., 1994). The crystal structure of the Y143F flavocytochrome b_2−sulfite complex has been solved to 3 Å resolution (Tegoni & Cambillau, 1994b) and is essentially isostructural with both the native pyruvate-liganded enzyme (Mathews & Xia, 1990) and the recombinant flavocytochrome b_2−sulfite complex (Tegoni & Cambillau, 1994a). A difference map of the wild-type and mutant structures shows a peak of negative electron density attributed to the loss of a hydroxide group at position 143. There are no other significant conformational changes in the Y143F mutant compared to wild-type flavocytochrome b_2. Clearly, the effect of the Y143F mutation on the rate of interdomain electron transfer cannot be due to gross structural changes, but must be attributed solely to the loss of a single interdomain hydrogen bond.

Hinge-swap flavocytochrome b_2 is an interspecies hybrid enzyme comprising the bulk of the *S. cerevisiae* enzyme, but with the hinge region from the *H. anomala* enzyme; it has 29 amino acid residues of the *S. cerevisiae* flavocytochrome b_2 replaced by 23 residues from the shorter and more acidic interdomain hinge region of the *H. anomala* enzyme (White *et al*., 1993). This is a more drastic mutation than any of the hinge deletions described in this paper. The rate constant for interdomain electron transfer in hinge-swap flavocytochrome b_2 is about 1.6 s^{-1}, which is 1000-fold lower than the value for the wild-type enzyme. The rate constant for FMN reduction, however, was only 50% lower than that of the wild-type enzyme (White *et al*., 1993). Considering the more drastic mutation imposed, it is not surprising that the effect of the hinge swap on the rate of interdomain electron transfer is more severe than that caused by the hinge deletions.

In the flavocytochrome b_2 crystal structure, the edge to edge distance between the prosthetic groups in subunit 1, from N(5) of FMN to C-2A of the heme, is 9.7 Å, the prosthetic groups are approximately coplanar, and the intervening medium comprises several water molecules (Xia & Mathews, 1990). The potential difference between fully reduced FMN and oxidized heme is on the order of 60 mV (Tollin & Walker, 1991). If one considers these parameters, then the theoretical rate of interdomain electron transfer between these prosthetic groups should be much greater than 1500 s^{-1} (Marcus & Suttin, 1985). Clearly, in the case of flavocytochrome b_2, some other factor must be rate limiting in this electron-transfer step, as the observed rate is 1500 s^{-1}. In light of the kinetic and crystallographic experiments on Y143F flavocytochrome b_2, it is not surprising that deletion mutations within the hinge region of flavocytochrome b_2 decrease the rate of interdomain electron transfer. A likely explanation that supports the conclusions drawn from the hinge-deletion, hinge-swap, and Y143F mutations is that conformational gating is rate limiting in the interdomain electron-transfer reaction and not the electron-transfer event itself (Hoffman & Ratner, 1987). In the hinge-deletion and hinge-swap enzymes, correct juxtapositioning of the cytochrome domain with respect to the flavin domain may

be impaired, hindering the formation of an optimal conformation for efficient electron transfer. This could be due to truncation of the hinge resulting in decreased flexibility of this region, impairing optimal conformational exploration.

ACKNOWLEDGMENT

We thank Dr. G. W. Pettigrew for help in determining redox potentials, and we are indebted to Prof. F. S. Mathews and Drs. F. Lederer, M. Tegoni, and C. Cambillau for helpful discussions.

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BI950457Z