Role of the Interdomain Hinge of Flavocytochrome b_2 in Intra- and Inter-Protein Electron Transfer†

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ABSTRACT: The two distinct domains of flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) are connected by a hinge peptide. Kinetics experiments [White, P., Manson, F. D. C., Brunt, C. E., Chapman, S. K., & Reid, GA. (1993) *Biochem. J. 291*, 89–94] have illustrated the importance for efficient interdomain electron transfer of maintaining the structural integrity of the hinge. To probe the role of the hinge in a more subtle manner, we have constructed a mutant enzyme, HΔ3, which has a three amino acid deletion in the hinge region. Intra- and inter-protein electron transfer within HΔ3 flavocytochrome b_2 and the HΔ3:cytochrome *c* redox complex was investigated by steady-state and stopped-flow kinetics analysis. The HΔ3 mutant enzyme remains a good L-lactate dehydrogenase, as is evident from steady-state experiments with ferricyanide as electron acceptor (40% less active than wild-type enzyme) and stopped-flow experiments monitoring flavin reduction (15% less active than wild-type enzyme). The global effect of the deletion is to lower the enzyme's effectiveness as a cytochrome *c* reductase. This property of the HΔ3 enzyme is manifested at two electron-transfer steps on the catalytic cycle of flavocytochrome b_2. First, the rate of heme reduction has fallen 5-fold in HΔ3 compared with the wild-type enzyme (from 445 to 91 s^{-1}), due to poor interdomain electron transfer from flavin to heme. Second, the rate of cyclochrome *c* reduction in the steady-state has fallen 5-fold (from 207 to 39 s^{-1}), indicating that b_2 heme to cytochrome *c* electron transfer has also been disrupted. These data, along with the measured kinetic isotope effects, indicate that cytochrome *c* reduction has become the rate-limiting step in the catalytic cycle for the HΔ3 enzyme. Further evidence for the importance of the hinge in inter-protein electron transfer is obtained from second-order rate constants for cytochrome *c* reduction by prereduced flavocytochrome b_2: the rate constant for HΔ3 is an order of magnitude less than the corresponding value for the wild-type enzyme, with values of 4×10^6 and 4.7×10^7 M^{-1} s^{-1}, respectively. From our data, we conclude that the hinge plays an important role in facilitating both intra- and inter-protein electron transfer.

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a homotetramer of subunit molecular weight 57 500 (Jacq & Lederer, 1974). The enzyme 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 crystal structure of flavocytochrome b_2 has been solved to 0.24-nm resolution (Xia & Mathews, 1990) and shows that each subunit consists of two distinct domains: an N-terminal domain containing protoheme IX and a C-terminal domain containing flavin mononucleotide. The two domains are connected by a single segment of polypeptide chain which constitutes the interdomain hinge (Figure 1). Crystallographic (Xia & Mathews, 1990) and NMR data (Labeyrie *et al.*, 1988) support the idea that this segment of polypeptide functions as a hinge, by indicating that the cytochrome domain is mobile relative to the flavin domain.

The primary structure of flavocytochrome b_2 from another yeast species, *Hansenula anomala*, has been determined (Black *et al.*, 1989a), and although there is an overall 60% identity between the amino acid sequences of the *Saccharomyces* and *Hansenula* enzymes, there are striking differences in the primary structure and net charge of the hinge region. The idea that these marked differences may account, at least in part, for the known kinetic differences between the two flavocytochromes b_2 has been tested by White *et al.* (1993). They investigated the role of the hinge in intramolecular electron transfer between the two domains of flavocytochrome b_2 by the construction of an interspecies hybrid enzyme comprising the bulk of the *Saccharomyces* enzyme but with the hinge region from the *Hansenula* enzyme. This "hinge-swap" enzyme, in which 29 residues from the *Saccharomyces* flavocytochrome b_2 had been replaced by 23 residues from the shorter and more acidic interdomain region from the *Hansenula* enzyme, was still a good L-lactate dehydrogenase, but it was a poor cytochrome *c* reductase compared to the wild-type *S. cerevisiae* flavocytochrome b_2. Data showed that the rate of b_2 heme reduction was at least 300-fold lower in the hinge-swap enzyme compared to that of the wild-type enzyme, implying that the structural integrity of the hinge region is crucial in mediating electron transfer between the flavin- and heme-containing domains of flavocytochrome b_2 (White *et al.*, 1993).

To more subtly probe the role of the hinge region in intra-protein and possibly inter-protein electron transfer within the flavocytochrome b_2:cytochrome *c* redox complex, we have constructed a mutant flavocytochrome b_2, HΔ3, which has a three amino acid deletion of residues 98–100 (APG) in the sequence (Figure 2). These residues were selected for deletion

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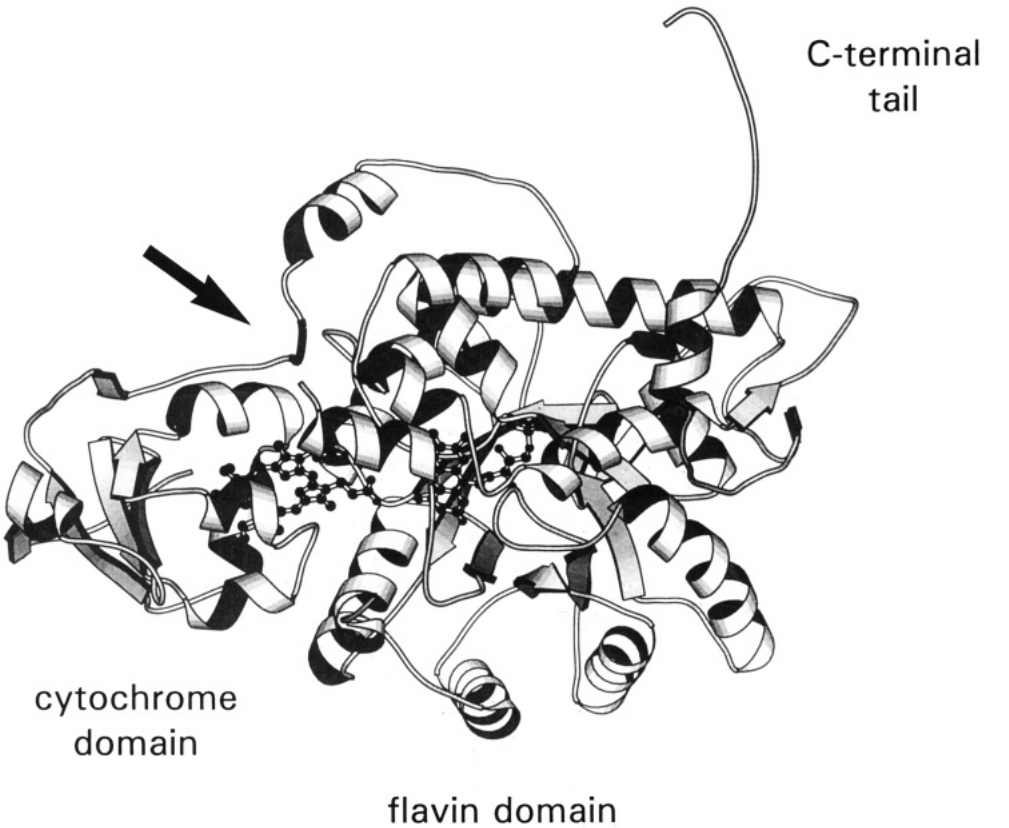

FIGURE 1: Graphic representation of a single wild-type flavocytochrome b_2 subunit, generated using the *MOLSCRIPT* molecular graphics program (Kraulis, 1991). α helices and β sheets are shown as ribbons, the remaining α carbon backbone is shown as a wire, and ball and stick atoms represent the prosthetic groups. The arrow indicates the position of the hinge region, and the position of the three residues deleted in the HΔ3 mutant is highlighted in black.

^{89}**P P E L V C P P Y *A P G* E T K** ^{103} → **P P E L V C P P Y E T K**

wild-type flavocytochrome b_2 HΔ3 flavocytochrome b_2

FIGURE 2: Construction of HΔ3 flavocytochrome b_2. The amino acid sequence of the interdomain hinge is shown. Amino acid residues APG (residues 98–100) are deleted in the mutant.

because, as is apparent from the crystal structure, they lie at the interdomain boundary and may thus be considered to lie at the center of the hinge region. Furthermore, this deletion is expected to have no effect on net charge.

MATERIALS AND METHODS

DNA Manipulation, Strains, and Growth. Site-directed mutagenesis was performed by the Kunkel method of non-phenotypic selection (Kunkel, 1985) using the oligonucleotide 326N (GTCCTCCTTATGAAACTAAGGA) (Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland, U.K.). Standard methods for growth of *Escherichia coli*, plasmid purification, DNA manipulation, and transformation were performed as described in Sambrook *et al.* (1991). *E. coli* strain TG1 was used for expression of wild-type and mutant flavocytochromes b_2.

Enzymes. Wild-type and HΔ3 flavocytochromes b_2 expressed in *E. coli* were isolated from cells which had been stored at −20 °C, using a previously reported purification procedure (Black *et al.*, 1989b). Purified enzyme preparations were stored under a nitrogen atmosphere at 4 °C as precipitates in 70% $(NH_4)_2SO_4$. Enzyme concentrations were calculated by using previously published extinction coefficients (Pajot & Groudinsky, 1970).

Kinetics Analysis. All kinetics experiments were carried out at 25 ± 0.1 °C in Tris/HCl at pH 7.5 and I 0.10 M. The buffer concentration was 10 mM in HCl with I adjusted to 0.10 M by addition of NaCl.

Steady-state kinetics measurements involving the enzymatic oxidation of L-lactate were performed using a Beckman DU62 spectrophotometer with either horse heart cytochrome c (type VI, Sigma) or ferricyanide (potassium salt, BDH Chemicals) as electron acceptor, as previously described (Miles *et al.*, 1992).

Stopped-flow measurements involving single turnover of L-lactate were carried out with an Applied Photophysics SF.17 MV stopped-flow spectrofluorimeter. Flavocytochrome b_2 was prepared for stopped-flow kinetics by dissolving the 70% $(NH_4)_2SO_4$ protein precipitate in a minimal amount of Tris buffer; this was passed through a G25 Sephadex column equilibrated and eluted with Tris buffer, to remove salts and lactate. The eluted enzyme was fully oxidized. Flavin reduction was monitored at 438.3 nm (a heme isosbestic point), and heme reduction was monitored at either 423 or 557 nm (the results were identical at both wavelengths). Collection and analysis of data were as previously described (Miles *et al.*, 1992). K_m and k_{cat} parameters were determined using nonlinear regression analysis.

Kinetic isotope effect analysis was performed with L-[2-^{2}H]lactate. Purification (Pompon *et al.*, 1980) and measurements of kinetic isotope effects (KIEs),[1] using this substrate, were as previously described (Miles *et al.*, 1992).

[1] Abbreviations: FMN, flavin mononucleotide; 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*.

Table 1: Steady-State Kinetic Parameters and Deuterium Kinetic Isotope Effects for Wild-Type and HΔ3 Flavocytochromes b_2[a]

		k_{cat} (s^{-1})		K_m (mM)		k_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1})			
enzyme	electron acceptor	[^{1}H]Lac	[^{2}H]Lac	[^{1}H]Lac	[^{2}H]Lac	[^{1}H]Lac	[^{2}H]Lac	KIE	reference
wild type	ferri	400 ± 10	86 ± 5	0.49 ± 0.05	0.76 ± 0.06	8.2	1.1	4.7 ± 0.4	Miles *et al.* (1992)
HΔ3	ferri	257 ± 10	70 ± 2	0.72 ● 0.07	0.94 ± 0.11	3.6	0.75	3.7 ± 0.4	this work
wild type	cyt *c*	207 ± 10	70 ± 10	0.24 ± 0.04	0.48 ± 0.10	8.6	1.5	3.0 ± 0.6	Miles *et al.* (1992)
HΔ3	cyt *c*	39 ± 1	20 ± 1	0.25 ± 0.03	0.61 ± 0.07	1.6	0.33	2.0 ± 0.1	this work

[a] All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (I 0.10). Concentrations of acceptors used with the HΔ3 enzyme were as follows: [cyctochrome *c*], 35 μM (84% saturating); [ferricyanide], 2 mM (93% saturating). 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 substrates reduced per second).

The pre-steady-state reduction of cytochrome *c* (type VI, Sigma) by fully prereduced flavocytochrome b_2 was monitored with the stopped-flow apparatus. The reaction was followed at 416.5 nm, which is an isosbestic point for flavocytochrome b_2. Flavocytochrome b_2 was prepared for such experiments as described above except that the enzyme, which eluted from the G25 column in the fully oxidized state, was then fully reduced by addition of excess L-lactate (>10 mM). Experiments were performed under aerobic conditions, as autooxidation of flavocytochrome b_2 did not occur to any significant extent over the time scale of the experiment (2–3 h). Cytochrome *c* was fully oxidized by addition of a few crystals of ferricyanide, and the excess oxidant was removed by passing the mixture through a Sephadex G-25 column, equilibrated and eluted with Tris buffer.

To ensure that the reduction of cytochrome *c* occurred under pseudo-first-order conditions, flavocytochrome b_2 was always present in excess. Reduction was carried out over a range of flavocytochrome b_2 concentrations (2–15 μM). Cytochrome *c* concentration was 0.75 μM. The traces were fitted to single-exponential nonlinear regression analysis. At least six runs were performed at each flavocytochrome b_2 concentration. Second-order rate constants were determined by plotting the k_{obs} for electron transfer (rate of cytochrome *c* reduction) against flavocytochrome b_2 concentration and fitting the data to a linear regression analysis.

Measurement of Redox Potential. The midpoint potential of the heme group in HΔ3 was determined spectrophotometrically by using a previously published redox potentiometry method (Dutton, 1978). The mediators, *N*-ethylphenazonium ethyl sulfate, *N*-methylphenazonium methyl sulfate, 2,3,5,6-tetramethylphenylenediamine, 2-hydroxy-1,4-naphthoquinone, and flavin mononucleotide were used as previously described (Dutton, 1978). The enzyme concentration was 10 μM.

Reduction was achieved by titrating with sodium dithionite under anaerobic conditions, and oxidation, by titrating with potassium ferricyanide (Brunt *et al.*, 1992). Changes in heme absorbance at 557 nm were measured with changing electrode potential. The system was buffered with the same Tris buffer as for kinetics 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 HΔ3 Flavocytochrome b_2. Table 1 presents the results of steady-state kinetics measurements on HΔ3 flavocytochrome b_2 using L-[2-^{1}H]-lactate and L-[2-^{2}H]lactate as substrates and with ferricyanide and cytochrome *c* as electron acceptors; these results are compared with previously reported values for the wild-type enzyme. From the data, it is clear that the HΔ3 enzyme is still a good L-lactate dehydrogenase as judged by the k_{cat} with ferricyanide as electron acceptor (only a 40% decrease). However, there are significant differences between the kinetic properties of wild-type and HΔ3 flavocytochromes b_2. The most striking of these is the 5-fold decrease in k_{cat} for HΔ3 when cytochrome *c* was used as the electron acceptor. The fact that k_{cat} values differ depending on whether ferricyanide or cytochrome *c* is used as electron acceptor implies (Miles *et al.*, 1992) that electron flow to these acceptors has been affected in different ways by the hinge deletion, with electron flow to cytochrome *c* being impaired to a greater extent. Thus, the HΔ3 enzyme remains a good L-lactate dehydrogenase, but is a poor cytochrome *c* reductase.

In the wild-type enzyme, proton abstraction at C-2 of lactate is the rate-limiting step. From the data presented in Table 1, it can be seen that the deuterium kinetic isotope effect values for the HΔ3 enzyme are lower than those for the wild-type enzyme with both electron acceptors. This indicates that electron-transfer reactions following C-2 proton abstraction contribute to overall rate limitation in the HΔ3 enzyme to a greater extent than in wild-type flavocytochrome b_2.

The value of the K_m for L-lactate observed with HΔ3 when ferricyanide is used as the electron acceptor is slightly higher than that for the wild-type enzyme. However, with cytochrome *c* as the electron acceptor, the apparent K_m for L-lactate is identical for HΔ3 and wild-type enzymes. The net effect of these changes in the kinetic parameters on the catalytic efficiency (k_{cat}/K_m) between HΔ3 and wild-type enzymes is a 2-fold decrease when ferricyanide is used as electron acceptor and a more significant 5-fold decrease when cytochrome *c* is used as electron acceptor.

Stopped-Flow Kinetic Parameters for L-Lactate Oxidation. Reduction of the FMN and heme prosthetic groups of HΔ3 flavocytochrome b_2 by L-[2-^{1}H]lactate and L-[2-^{2}H]lactate was monitored directly using stopped-flow spectrophotometry. The kinetic parameters are summarized in Table 2. The effect of the hinge deletion on the rate of FMN reduction is insignificant (k_{cat} is lowered by 15% in HΔ3 flavocytochrome b_2 compared to that of the wild-type enzyme). The rate of heme reduction, however, is 5-fold lower in HΔ3 compared to that for the wild-type enzyme (Table 2). These results imply that introducing the deletion mutation in the hinge region of flavocytochrome b_2 has only a very slight effect on FMN reduction by lactate, but must have a greater effect on electron transfer from FMN to heme. This conclusion is supported by the KIE values reported in Table 2.

Redox Potential. We considered the possibility that the deletion mutation in the hinge region might have affected the redox potentials of the prosthetic groups. Clearly, there can have been little or no effect on the flavin potential, as the k_{cat} values for flavin reduction are not significantly different between HΔ3 and the wild-type enzyme. However, it seemed possible that a large change in the heme potential could have contributed to the decrease in the k_{cat} value for heme reduction

Table 2: Stopped-Flow Kinetic Parameters and Deuterium Kinetic Isotope Effects for Reduction of FMN and Heme in Wild-Type and HΔ3 Flavocytochromes b_2[a]

enzyme	prosthetic group reduction	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	KIE	reference
wild type	FMN	604 ± 60	75 ± 5	0.84 ± 0.20	1.33 ± 0.28	8.1 ± 1.4	Miles *et al.* (1992)
HΔ3	FMN	518 ± 17	71 ± 3	0.60 ± 0.08	1.46 ± 0.21	7.3 ± 0.6	this work
wild type	heme	445 ± 50	71 ± 5	0.53 ± 0.05	0.68 ± 0.05	6.3 ± 1.2	Miles *et al.* (1992)
HΔ3	heme	91 ± 3	32 ± 1	0.38 ± 0.07	0.80 ± 0.13	2.8 ± 0.2	this work

[a] All experiments were carried out at 25 °C in Tris/HCl buffer, I 0.10. Stopped-flow data were analyzed as in the Materials and Methods section. Values of k_{cat} are expressed as number of prosthetic groups reduced per second. Where biphasic kinetics were observed, the values reported correspond to those of the rapid phase as previously described (Miles *et al.*, 1992).

in the HΔ3 enzyme. To check this, we measured the heme redox potential (at pH 7.5) for the HΔ3 enzyme and found that the value was the same within experimental error as that previously determined for wild-type flavocytochrome b_2: wild type = −17 ± 3 mV (Miles, 1992; White *et al.*, 1993); HΔ3 = −14 ± 3 mV.

Stopped-Flow Kinetic Parameters for Cytochrome c Reduction. Reduction of cytochrome *c* by wild-type and HΔ3 flavocytochromes b_2 was monitored directly using stopped-flow spectrophotometry as described in the Materials and Methods section. The second-order rate constant for cytochrome *c* reduction by HΔ3 was an order of magnitude lower than the corresponding value for the wild-type enzyme. These data reflect the rate of electron transfer from flavocytochrome b_2 heme to cytochrome *c* within the flavocytochrome b_2: cytochrome *c* complex [(4.0 ± 0.3) × 10^6 and (4.7 ± 1.0) × 10^7 M^{-1} s^{-1} for wild-type and HΔ3 flavocytochromes b_2, respectively]. Clearly, b_2 heme to cytochrome *c* heme electron transfer has been disrupted in the HΔ3 enzyme, implying that the hinge region has an important role in maintaining the integrity of the complex.

DISCUSSION

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 2). 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. This proposal is supported by a number of observations, involving crystallography and NMR spectroscopy. In the three-dimensional structure of *S. cerevisiae* flavocytochrome b_2, two crystallographically distinct types of subunit are seen 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 would be expected for a protein as large as the flavocytochrome b_2 tetramer; the observed line widths for 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; S. K. Chapman, C. E. Brunt, M. Cox, and G. Moore, unpublished results). Thus, a large body of structural information exists highlighting the importance of the hinge in interdomain interactions. Recently, we have published data concerning the kinetic properties of a mutant flavocytochrome b_2, designed to further investigate the role of the hinge in interdomain communication (White *et al.*, 1993). This enzyme was termed hinge-swap, as it comprised the bulk of the *S. cerevisiae* enzyme, but with the native hinge region replaced by the equivalent region of the *H. anomala* enzyme. The most striking difference between the wild-type and hinge-swap enzymes was the 300-fold decrease in the k_{cat} value for heme reduction by lactate, implying that this mutation had severely impaired interdomain communication. These data support the conclusions from structural studies and stress the importance of maintaining the structural integrity of the hinge region for efficient interdomain communication.

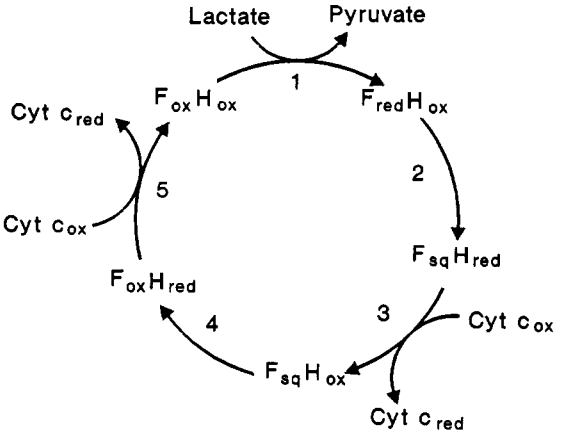

FIGURE 3: Catalytic cycle of flavocytochrome b_2. The redox states of cytochrome *c* and the flavocytochrome b_2 flavin (F) and heme (H) are indicated by the subscripts ox and red for the oxidized and reduced forms, respectively. The flavin semiquinone is shown as F_{sq}. (1) Oxidation of lactate to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

To further our understanding of the role of the hinge region in intra- and inter-protein communication, we have constructed a mutant flavocytochrome b_2 with a three amino acid deletion in the hinge region. This enzyme, HΔ3, has some interesting differences in its electron-transfer properties when compared with wild-type and hinge-swap flavocytochromes b_2.

The catalytic cycle for flavocytochrome b_2 is shown diagrammatically in Figure 3. The first step, flavin reduction by lactate, is virtually unaffected in HΔ3; k_{cat} is only 15% lower than that for the wild-type enzyme, and the other measurable kinetic parameters for this step are very similar between the two enzymes. In the case of the hinge-swap enzyme (White *et al.*, 1993) a similar situation was observed,

where k_{cat} for FMN reduction was only 2-fold less than that for the wild-type enzyme. These data imply that the hinge is not of great importance in FMN reduction by lactate, and thus HΔ3 remains a good L-lactate dehydrogenase. This conclusion is supported by the steady-state measurements with ferricyanide as electron acceptor (Table 1).

The steps following FMN reduction are very different in wild-type, hinge-swap (White *et al.*, 1993), and HΔ3 enzymes. The second step in the catalytic cycle (Figure 3) is intraprotein electron transfer from fully reduced flavin to oxidized heme. The k_{cat} values for heme reduction by L-lactate in the HΔ3 and hinge-swap enzymes are 5-fold and 300-fold lower, respectively, than in the wild-type enzyme. These results show that, in the case of the HΔ3 enzyme, there has been a significant impairment of electron transfer from FMN to heme. Thus, the three amino acid deletion in the hinge region has disrupted interdomain communication, but to a much lesser extent than the hinge-swap mutation. A likely explanation for this is that the truncation of the hinge region in HΔ3 and the hinge-swap enzymes (3 and 6 amino acids shorter, respectively, than the wild-type enzyme) has led to restriction of hinge flexibility, thereby preventing efficient recognition between the two domains. The much more pronounced effect observed for the hinge-swap enzyme compared to the HΔ3 enzyme is probably due to the more drastic structural change caused by the hinge-swap compared to the deletion mutation.

The final steps in the catalytic cycle involve reduction of cytochrome *c*, the physiological electron acceptor (Figure 3). In the case of the hinge-swap enzyme, k_{cat} for the steady-state reduction of cytochrome *c* was the same within experimental error as the k_{cat} for b_2 heme reduction (1.6 s^{-1}), implying that cytochrome *c* reduction does not contribute to rate limitation in the catalytic cycle and that the slowest step in the catalytic cycle must be FMN to heme electron transfer (White *et al.*, 1993). In contrast, the HΔ3 enzyme has a k_{cat} for pre-steady-state b_2 heme reduction of 91 s^{-1}, but the k_{cat} for steady-state cytochrome *c* reduction is slower at 39 s^{-1}. Direct evidence for impaired electron transfer between the hemes of the HΔ3 enzyme:cytochrome *c* complex was obtained from the measurement of the second-order rate constants for cytochrome *c* reduction under stopped-flow conditions. The rate constant for the HΔ3 enzyme was an order of magnitude less than the corresponding value for wild-type flavocytochrome b_2. Thus, we can conclude that the three amino acid deletion in the hinge region has resulted in impaired electron transfer from mutant flavocytochrome b_2 to cytochrome *c*, as well as disruption of interdomain communication.

The above conclusions are supported by the deuterium KIE values reported in Tables 1 and 2. For FMN reduction the KIE is the same within experimental error for HΔ3 and wild-type enzymes. This confirms that proton abstraction at C-2 of lactate is still rate-limiting for FMN reduction. However, for heme reduction there is a more significant lowering of the KIE value from 6.3 in the wild type to 2.8 in HΔ3. This indicates that proton abstraction at C-2 of lactate still contributes to rate-limitation of heme reduction in the HΔ3 enzyme, but much less so than in wild type. For cytochrome *c* reduction in the steady-state, the KIE is 2.0 for HΔ3 compared to 3.0 for the wild-type enzyme, implying that, for the mutant, proton abstraction at C-2 of lactate is less rate-limiting for cytochrome *c* reduction than for wild type. In summary, this is consistent with the k_{cat} data for FMN, b_2 heme, and cytochrome *c* reduction, since both the k_{cat} and the KIE values erode in a similar fashion along this electron-transfer pathway for HΔ3.

As already mentioned, the most probable reason for the effect of the hinge deletion mutation on intra- and inter-protein electron transfer is that for the former, the shorter hinge region has reduced flexibility and impaired recognition between the cytochrome and flavin domains and for the latter, the cytochrome *c* binding site has been disrupted. We have excluded the possibility that the heme redox potential was altered by the HΔ3 mutation, as the measured values for the wild-type and HΔ3 enzymes are identical within experimental error.

Previously published experiments investigating cytochrome *c* binding to both *S. cerevisiae* and *H. anomala* flavocytochrome b_2 have concentrated on determining the stoichiometry and the dissociation constant, K_d, for cytochrome *c* binding (Baudras & Spyridakis, 1971; Baudras *et al.*, 1971; Baudras, 1971; Prats, 1977; Tegoni *et al.*, 1983, 1990) and localizing the binding area to either the flavin or the cytochrome domains (Thomas *et al.*, 1983; Albani, 1985) but not to any specific region on flavocytochrome b_2. In this paper, we have shown that maintaining the structural integrity of the hinge region is important for efficient cytochrome *c* reduction, and thus it seems reasonable to propose that the hinge may form part of a recognition site for cytochrome *c*. This idea is supported by recent molecular modeling studies of the flavocytochrome b_2:cytochrome *c* complex, which was based on the known crystal structures of the two proteins and energy minimization techniques (Tegoni *et al.*, 1993). The model predicts that, within the complex, a significant number of inter-protein interactions occur between the hinge region and cytochrome *c* which could be important determinants for cytochrome *c* binding.

CONCLUSIONS

From our studies on HΔ3 flavocytochrome b_2, we draw the following conclusions: (i) The interdomain hinge has little influence on the lactate dehydrogenase function of the enzyme. (ii) The hinge is crucial in mediating electron transfer between the flavin- and heme-containing domains of the enzyme (intra-protein electron transfer). (iii) The three amino acid deletion results in b_2 heme to cytochrome *c* heme electron transfer becoming the slowest step in the catalytic cycle (inter-protein electron transfer). (iv) The hinge region forms at least part of the binding site for cytochrome *c* on flavocytochrome b_2. (v) The three amino acid deletion has little or no effect on the redox potential of the heme group.

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