The importance of the interdomain hinge in intramolecular electron transfer in flavocytochrome \( b_2 \)

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The two distinct domains of flavocytochrome \( b_2 \) (\( \alpha \)-lactate:cytochrome \( c \) oxidoreductase) are connected by a typical hinge peptide. The amino acid sequence of this interdomain hinge is dramatically different in flavocytochromes \( b_2 \) from *Saccharomyces cerevisiae* and *Hansenula anomala*. This difference in the hinge is believed to contribute to the difference in kinetic properties between the two enzymes. To probe the importance of the hinge, an interspecies hybrid enzyme has been constructed comprising the bulk of the *S. cerevisiae* enzyme but containing the *H. anomala* flavocytochrome \( b_2 \) hinge. The kinetic properties of this ″hinge-swap″ enzyme have been investigated by steady-state and stopped-flow methods. The hinge-swap enzyme remains a good lactate dehydrogenase as is evident from steady-state experiments with ferricyanide as acceptor (only 3-fold less active than wild-type enzyme) and stopped-flow experiments monitoring flavin reduction (2.5-fold slower than in wild-type enzyme). The major effect of the hinge-swap mutation is to lower dramatically the enzyme’s effectiveness as a cytochrome \( c \) reductase; \( k_{\text{cat}} \), for cytochrome \( c \) reduction falls by more than 100-fold, from 207 ± 10 \( \text{s}^{-1} \) (25 °C, pH 7.5) in the wild-type enzyme to 1.62 ± 0.41 \( \text{s}^{-1} \) in the mutant enzyme. This fall in cytochrome \( c \) reductase activity results from poor interdomain electron transfer between the FMN and haem groups. This can be demonstrated by the fact that the \( k_{\text{cat}} \) for haem reduction in the hinge-swap enzyme (measured by the stopped-flow method) has a value of 1.61 ± 0.42 \( \text{s}^{-1} \), identical with the value for cytochrome \( c \) reduction and some 300-fold lower than the value for the wild-type enzyme. From these and other kinetic parameters, including kinetic isotope effects with \([2-^2\text{H}]\)lactate, we conclude that the hinge plays a crucial role in allowing efficient electron transfer between the two domains of flavocytochrome \( b_2 \).

INTRODUCTION

Flavocytochrome \( b_2 \) (\( \alpha \)-lactate:cytochrome \( c \) oxidoreductase, EC 1.1.2.3) from baker’s yeast (*Saccharomyces cerevisiae*) is a tetramer of identical subunits each with \( M_r \) 57 500 [1]. The enzyme is a soluble component of the mitochondrial intermembrane space [2], where it catalyses the oxidation of \( \alpha \)-lactate to pyruvate and transfers electrons to cytochrome \( c \) [3]. The crystal structure of *Saccharomyces* flavocytochrome \( b_2 \) has been solved to 0.24 nm resolution [4] and reveals that each subunit consists of two distinct domains: an N-terminal haem-containing, or cytochrome, domain and a C-terminal FMN-containing domain. The two domains are connected by a single segment of polypeptide chain which constitutes the interdomain hinge (Figure 1a). That this segment of polypeptide functions as a hinge is supported by crystallographic [4] and n.m.r. data [5], all of which indicate that the cytochrome domain is relatively mobile. The primary structure of flavocytochrome \( b_2 \) from another yeast, *Hansenula anomala*, has been determined [6]. Although there is an overall 60% identity between the amino acid sequences of the *Hansenula* and *Saccharomyces* enzymes, there are striking differences in the primary structure and net charge of the hinge segment. We have suggested that these marked differences may account, at least in part, for the known kinetic differences between the flavocytochromes \( b_2 \) from the two yeasts [6]. To test this idea, and to probe the role of the hinge more broadly, we have constructed an interspecies hybrid enzyme consisting of the bulk of the *Saccharomyces* enzyme but containing the hinge section from the *Hansenula* enzyme (Figure 1b).

This hybrid enzyme is referred to as the ″hinge-swap″ flavocytochrome \( b_2 \).

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

Standard methods for growth of *Escherichia coli*, plasmid purification, DNA manipulation and transformation were performed as described in Sambrook et al. [7].

Modification of the flavocytochrome \( b_2 \) genes

The plasmid pGR401 contains the entire *S. cerevisiae* flavocytochrome \( b_2 \)-coding sequence on a 1.8 kb *EcoRI–HindIII* fragment [8]. The *H. anomala* flavocytochrome \( b_2 \) gene was subcloned from λFG1 [6] as a 5.7 kb *BamHI* fragment into plasmid pTZ19r [9]. The resulting recombinant plasmid, pMB3, was used for site-directed mutagenesis by the method of Kunkel [10] (using oligonucleotides synthesized by the OSWEL DNA service, University of Edinburgh) to introduce cleavage sites for *EcoRI* and *HindIII* at the start and end respectively of the *H. anomala* flavocytochrome \( b_2 \) sequence encoding the mature protein. The oligonucleotide used to introduce the *EcoRI* site was also designed to introduce an ATG initiation codon immediately between the *EcoRI* site and the codon for Asp-1 of the mature protein. To facilitate transfer of coding sequence between plasmids, we also removed the *HindIII* site within the coding sequence of *H. anomala* flavocytochrome \( b_2 \) by introducing a silent mutation (using oligonucleotide 982D, gctgtggaaccttttgtg).

Abbreviation used: KIE, kinetic isotope effect.

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For construction of hybrid proteins, restriction-enzyme-cleavage sites were introduced at equivalent positions flanking the interdomain-hinge-coding region in both sequences (Figure 2a). An XbaI site occurs naturally in the S. cerevisiae sequence but the Apal sites and the XbaI site in the H. anomala sequence were introduced by site-directed mutagenesis (Figure 2b) using the following oligonucleotides: 661G, catctgggctcttaggtgg (to insert an Apal site in the H. anomala coding sequence); 662G, gaaacaccctctagcataatg (to insert an XbaI site in the H. anomala coding sequence); 660G, aaatgtggccctctcaat (to insert an Apal site in the S. cerevisiae coding sequence). Introduction of the Apal sites did not alter the amino acid sequences, but introduction of the XbaI site into the H. anomala sequence necessitated replacement of Ser-112 by Asp, the amino acid residue found at the equivalent position in the S. cerevisiae enzyme. DNA sequences of the mutated flavocytochrome \( b_2 \) sequences were determined using a series of suitably designed primers in conjunction with the Sequenase kit (US Biochemicals). Two secondary mutations were found in the H. anomala coding sequence but in each case the encoded amino acid sequence was unaltered. The plasmids containing the modified S. cerevisiae and H. anomala coding sequences were named pFM200 and pFM102 respectively.

**Construction and expression of the hinge-swap flavocytochrome \( b_2 \)**

Plasmid pFM200 was cleaved with Apal and XbaI and the large fragment was isolated. The small Apal-XbaI fragment, encoding the hinge region, was isolated from plasmid pFM102 and ligated to the large fragment from pFM200. The structure of recombinants was verified by restriction enzyme analysis and the hybrid coding sequence from one recombinant was subcloned on a 1.8 kb EcoRI-HindIII fragment into the expression vector pDS6 as previously described [11].

**Enzymes**

Wild-type and hinge-swap flavocytochromes \( b_2 \) expressed in E. coli were isolated from cells, which had been stored at \(-20^\circ C\), using a previously reported purification procedure [11]. Purified enzyme preparations were stored under nitrogen at \(4^\circ C\) as precipitates in 70% \((NH_4)_2SO_4\).

**Kinetic analysis**

All kinetic experiments were carried out at 25±0.1°C in Tris/HCl at pH 7.5, 0.10 mol/l. The buffer concentration was 10 mM in HCl with \(I\) adjusted to 0.10 mol/l by addition of NaCl.

For stopped-flow measurements were carried out with an Applied Photophysics SF.17MV stopped-flow spectrophurometer. Flavin reduction was monitored at 438.3 nm (a haem isoosbestic point) and haem reduction at either 557 or 423 nm (results were identical at both wavelengths). Collection and analysis of data were as previously described [12].

Kinetic isotope effects (KIEs) were measured using L-[2-\(2\text{H}\)]-lactate as previously described [12].

**Measurement of redox potential**

The midpoint potential of the haem groups in the wild-type and hinge-swap enzymes were determined spectrophotometrically using a previously published redox potentiometry method [13]. The mediators, N-ethylphenazonium sulphate, N-methylphaznonium sulphate, 2,3,5,6-tetramethylphenylenediamine and 2-hydroxy-1,4-naphthoquinone, were used as previously described [13]. Enzyme was reduced by titrating with sodium dithionite under anaerobic conditions and oxidized by titrating with potassium ferricyanide as reported elsewhere [14].

**RESULTS**

**Steady-state kinetic analysis**

Results from steady-state kinetic measurements on the hinge-swap enzyme using L-[2-\(2\text{H}\)]-lactate and L-[2-\(2\text{H}\)]-lactate as substrates and with ferricyanide and cytochrome \( c \) as electron acceptors are presented in Table 1, where they are compared with...
Importance of the hinge in flavocytochrome $b_2$

(a) DNA sequences encoding flavocytochrome $b_2$ from $S$. cerevisiae and $H$. anomala were modified to have the same general structure as represented by the restriction map above and the corresponding structure below (N-terminus at the left). (b) Sequence modifications introduced by site-directed mutagenesis at either end of the hinge-coding sequence are shown along with the DNA sequences around the introduced restriction sites and the amino acid sequence of the entire hinge regions. Site-directed modifications are indicated by arrows and the restriction sites used for construction of the hinge-swap enzyme are underlined.

![Restriction Map](image)

**Figure 2** Construction of the hinge-swap flavocytochrome $b_2$

Table 1 Steady-state kinetic parameters and $^2$H KIEs for wild-type and hinge-swap flavocytochromes $b_2$.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Electron acceptor</th>
<th>$k_{cat}$ (s$^{-1}$) $[^{1}H]$Lac</th>
<th>$K_m$ (mM) $[^{1}H]$Lac</th>
<th>$[^{1}H]/[^{2}H]$</th>
<th>$10^{-3}K_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>KIE</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Ferri</td>
<td>400 ± 10</td>
<td>86 ± 5</td>
<td>0.49 ± 0.05</td>
<td>0.76 ± 0.08</td>
<td>8.3</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Hinge-swap</td>
<td>Ferri</td>
<td>126 ± 6</td>
<td>61 ± 3</td>
<td>0.16 ± 0.02</td>
<td>0.60 ± 0.10</td>
<td>7.9</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Cyt. c</td>
<td>207 ± 10</td>
<td>70 ± 10</td>
<td>0.24 ± 0.04</td>
<td>0.48 ± 0.10</td>
<td>8.6</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Hinge-swap</td>
<td>Cyt. c</td>
<td>152 ± 41</td>
<td>97 ± 0.29</td>
<td>0.002 ± 0.001</td>
<td>0.005 ± 0.003</td>
<td>8.1</td>
<td>1.7 ± 1.3</td>
</tr>
</tbody>
</table>

Previously reported values for the wild-type enzyme. It should be noted first that the hinge-swap enzyme is still a good lactate dehydrogenase as judged by the results with ferricyanide as electron acceptor (only a 3-fold decrease in the $k_{cat}$ value in this case). However, it is apparent that there is a number of more significant differences between the kinetic properties of the wild-type and hinge-swap enzymes. The most striking of these differences is the very large (over 100-fold) decrease in the $k_{cat}$ value for the hinge-swap enzyme when cytochrome $c$ is used as the electron acceptor. The fact that $k_{cat}$ values differ depending on whether ferricyanide or cytochrome $c$ is used as electron acceptor indicates that electron flow to these acceptors has been affected in different ways by the hinge-swap mutation, with electron flow to cytochrome $c$ being severely impaired. Thus the hinge-swap enzyme, although it remains a good lactate dehydrogenase, is a very poor cytochrome $c$ reductase.

It has been previously shown that proton abstraction at C-2 of lactate is the major rate-limiting step in the wild-type enzyme [12,15]. From Table 1 it can be seen that the deuterium KIE values for the hinge-swap enzyme are lower than those for the wild-type enzyme with both electron acceptors. This suggests that electron-transfer reactions subsequent to C-2 proton abstraction contribute to overall rate limitation in the hinge-swap enzyme.
The value of $K_m$ for L-lactate seen with the hinge-swap enzyme is similar to that seen for the wild-type enzyme when ferricyanide is used as electron acceptor. However, with cytochrome $c$ as electron acceptor the apparent $K_m$ for L-lactate in the hinge-swap enzyme is dramatically decreased (Table 1). Clearly this $K_m$ value is not a reflection of the $K_m$ for L-lactate since its value is affected by steps after L-lactate binding, e.g. electron transfer to cytochrome $c$. It is interesting to note that, with cytochrome $c$ as electron acceptor, the decrease in the $K_m$ value for L-lactate seen with the hinge-swap enzyme is similar in magnitude to the decrease in $k_{cat}$ (both decreased by around 100-fold with respect to the wild-type enzyme) so that the value of $k_{cat}/K_m$ is in fact not greatly altered.

### Stopped-flow kinetic analysis

Reduction of the FMN and haem groups of hinge-swap flavocytochrome $b_2$ by L-[2-1H]lactate and L-[2-2H]lactate was monitored directly using stopped-flow spectrophotometry. Kinetic parameters are summarized in Table 2. FMN reduction was monophasic at all lactate concentrations, whereas haem reduction was biphasic. This is in marked contrast with the results of previous work on wild-type enzyme in which biphasic kinetics were observed for FMN reduction, at least at high lactate concentrations [12,15–17]. For wild-type enzyme, it is known that the rapid phase corresponds to initial reduction of FMN and haem (with the slow phase being kinetically irrelevant in catalytic turnover of the enzyme) [12,15–17]. The effect of the hinge-swap mutation on the rate of FMN reduction is small, with $k_{cat}$ for the mutant enzyme being only 2- to 3-fold lower than that for the wild-type enzyme. However, there are dramatic effects on the rate of haem reduction, with $k_{cat}$ for the hinge-swap enzyme being over 300-fold less than that for the wild-type enzyme (Table 2). These results indicate that introducing the hinge-swap mutation has only a slight effect on electron transfer from L-lactate to FMN but must have a major effect on electron transfer from FMN to haem. This conclusion is supported by the KIE values reported in Table 2.

### Redox potentials

There was the possibility that the hinge-swap mutation might have affected the redox potentials of the prosthetic groups. Clearly there can have been little effect on the flavin potential since the $k_{cat}$ values for flavin reduction are not greatly altered by the mutation. However, it was possible that a large change in haem potential could have contributed to the dramatic lowering of the $k_{cat}$ value for haem reduction in the hinge-swap enzyme. To check this, we measured the haem redox potentials for the wild-type and hinge-swap enzymes and found that these values are the same within experimental error: wild-type = $-20 \pm 2$ mV; hinge-swap = $-23 \pm 3$ mV.

### DISCUSSION

The two domains of *S. cerevisiae* flavocytochrome $b_2$ are connected by a typical hinge sequence that contains several proline, glycine and charged residues. The most likely function of this hinge is to allow the cytochrome domain to move with respect to the flavin domain. This suggestion is supported by both crystallographic and n.m.r. experiments. 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 case, substrate is absent from the active site and the cytochrome domain is resolved. In the other, where pyruvate is found at the active site, no electron density is observed for the cytochrome domain, suggesting that it is positionally disordered [4]. In solution, n.m.r. 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 linewidths indicate considerable flexibility of this domain [5, and our unpublished work]. There is thus a significant body of evidence to indicate that the hinge is important in interdomain interactions. It is interesting therefore that the hinge sequence is dramatically different in flavocytochromes $b_2$ from *S. cerevisiae* and *H. anamala*; the hinge in the *H. anamala* enzyme is six residues shorter and considerably more acidic than in the *S. cerevisiae* enzyme [6]. We believe that these differences contribute to the well-known differences in the kinetic properties of the flavocytochromes $b_2$ from these two yeasts [18]. To test this idea and to probe the importance of the hinge more broadly, site-directed mutagenesis has been used to generate an interspecies hybrid which retains the cytochrome and flavin domains of *S. cerevisiae* flavocytochrome $b_2$ but has the hinge segment replaced by the equivalent segment from the *H. anamala* enzyme. The resulting hinge-swap flavocytochrome $b_2$ has some very interesting differences in its electron-transfer properties when compared with the original enzyme.

The catalytic cycle for flavocytochrome $b_2$ is shown in Scheme 1. The first step, electron transfer from lactate to flavin, is only slightly affected by the hinge-swap mutation. $K_m$ and $k_{cat}$ values for FMN reduction are only a little over 2-fold lower in the mutant enzyme than in the wild-type enzyme (Table 2). This indicates that the hinge is not of great importance in FMN reduction by lactate and thus the hinge-swap enzyme retains a good lactate dehydrogenase. This conclusion is supported by the steady-state measurements with ferricyanide as electron acceptor (Table 1). Steps after FMN reduction are very different.

### Table 2 Stopped-flow kinetic parameters and 3H KIEs for reduction of FMN and haem in the wild-type and hinge-swap flavocytochromes $b_2$

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 10. Stopped-flow data were analysed as described in the Materials and methods section. Values of $k_{cat}$ are expressed as number of prosthetic groups reduced/s. Where biphasic kinetics were observed, the values reported correspond to those of the rapid phase as previously described [12]. Abbreviations are as follows: $[^1]H$[Lac], L-[2-1H]lactate; $[^2]H$[Lac], L-[2-2H]lactate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Prosthetic group reduction</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_m$ (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>FMN</td>
<td>604 ± 60</td>
<td>75 ± 5</td>
<td>0.84 ± 0.20</td>
</tr>
<tr>
<td>Hinge-swap</td>
<td>FMN</td>
<td>240 ± 12</td>
<td>38 ± 2</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Haem</td>
<td>445 ± 50</td>
<td>71 ± 5</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Hinge-swap</td>
<td>Haem</td>
<td>1.61 ± 0.42</td>
<td>1.00 ± 0.11</td>
<td>0.003 ± 0.001</td>
</tr>
</tbody>
</table>
in the mutant and wild-type enzymes. The $k_{\text{cat}}$ for haem reduction in the hinge-swap enzyme is some 300-fold lower than in the wild-type enzyme. This dramatic result indicates that there has been a major impairment of electron transfer from FMN to haem (step 2 in Scheme 1). Clearly the hinge-swap mutation has disrupted interdomain communication. A possible explanation for this is that the hinge in the mutant enzyme, which is six residues shorter than in the wild-type enzyme, might be restricting hinge bending and thus preventing efficient recognition between the two domains, as indicated schematically in Figure 1(b).

The final steps in the catalytic cycle involve reduction of the physiological electron acceptor, cytochrome $c$ (Scheme 1). Electrons can be transferred to cytochrome $c$ only from the flavocytochrome $b_5$ haem [19,20]. It is therefore not surprising that the rate of cytochrome $c$ reduction is greatly affected by the hinge-swap mutation (over 100-fold slower than in the wild-type enzyme). In fact, in the hinge-swap enzyme the $k_{\text{cat}}$ value for reduction of cytochrome $c$ is identical, within experimental error, to the $k_{\text{cat}}$ for $b_5$-haem reduction. This means that, in the mutant enzyme, cytochrome $c$ reduction does not contribute to rate limitation in the catalytic cycle. Considered together, these results indicate that FMN $\rightarrow$ haem electron transfer is the slowest step in the catalytic cycle of the hinge-swap flavocytochrome $b_5$. This conclusion is supported by the deuterium KIE values reported in Table 2. For FMN reduction there is only a slight erosion of the KIE; 8.1 for the wild-type and 6.3 for the mutant enzyme. This confirms that proton abstraction at C-2 of lactate is still rate-limiting for FMN reduction. However, for haem reduction there is a more significant lowering of the KIE value from 6.3 in the wild-type to 1.6 in the mutant enzyme. This indicates that, in contrast with the wild-type enzyme, there is little or no rate limitation to haem reduction arising from proton abstraction at C-2 of lactate in the hinge-swap flavocytochrome $b_5$. Also, since cytochrome $c$ can only accept electrons from the haem group, it is not surprising that the KIE for cytochrome $c$ reduction measured in the steady state is identical with the value for $b_5$-haem reduction measured by stopped-flow spectrophotometry.

The fact that FMN $\rightarrow$ haem electron transfer is the slowest step in the catalytic cycle of the hinge-swap enzyme allows us to explain another difference between this mutant enzyme and the wild-type flavocytochrome $b_5$. In the wild-type enzyme, flavin reduction monitored by stopped-flow spectrophotometry is biphasic. A number of workers have previously described the origin of this biphasic behaviour which arises because each flavocytochrome $b_5$ protomer requires three electrons for full reduction (i.e. each tetramer requires 12 electrons from six lactate molecules for full reduction) [12,15–17]. These workers have shown that the rapid phase corresponds to the initial reduction of flavin and haem groups and the slow phase (which is kinetically irrelevant during catalytic turnover of the enzyme) corresponds to the entry of the third electron which is permitted because of interprotomer electron transfer [12,15–17]. For interprotomer electron transfer to occur from one flavin group to another within the tetramer, the flavin must be in the semiquinone state, i.e. one of the electrons must have been passed on to the haem group and FMN $\rightarrow$ haem electron transfer must be faster than interprotomer electron transfer. In the hinge-swap enzyme, the reduction of the flavin is monophasic. This is because FMN $\rightarrow$ haem electron transfer is now slower than interprotomer electron transfer would be. Thus on the time scale for FMN reduction, only one phase is observed.

As already mentioned, the most likely reason for the effect of the hinge-swap mutation on the rate of FMN $\rightarrow$ haem electron transfer is that the shorter hinge has reduced flexibility and impaired recognition between the cytochrome and flavin domains. There is also the possibility that the mutation might have affected the redox potential of the haem groups. We have excluded this possibility by showing that the measured values of the haem redox potentials for the wild-type and hinge-swap enzymes are identical within experimental error.

Conclusions

From our studies on the hinge-swap flavocytochrome $b_5$, we draw the following conclusions: (i) the interdomain hinge has little influence on the lactate dehydrogenase function of the enzyme but is important for its role as a cytochrome $c$ reductase; (ii) the hinge is crucial in mediating electron transfer between the flavin- and haem-containing domains of the enzyme; (iii) the hinge-swap mutation results in FMN $\rightarrow$ haem electron transfer becoming the slowest step in the catalytic cycle; (iv) the hinge-swap mutation has little or no effect on the redox potential of the haem group.

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REFERENCES

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