The role of the C-terminal tail of flavocytochrome b_2

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A flavocytochrome b_2 (L-lactate dehydrogenase) mutant was constructed in which the C-terminal tail (23 amino acid residues) had been deleted (Gly-489 → Stop). This tail appears to form many intersubunit contacts in the tetrameric wild-type protein, and it was expected that its removal might lead to the formation of monomeric flavocytochrome b_2 . The isolated tail-deleted mutant enzyme (TD- b_2), however, was found to be tetrameric (M_r 220 000). TD- b_2 shows K_m and k_{cat} values (at 25 °C and pH 7.5) of 0.96 ± 0.06 mM and 165 ± 6 s⁻¹ respectively compared with 0.49 ± 0.04 mM and 200 ± 10 s⁻¹ for the wild-type enzyme. The kinetic isotope effect with [2-³H]lactate as substrate seen for TD- b_2 , with ferricyanide as electron acceptor, was essentially the same as that observed for the wild-type enzyme. TD- b_2 exhibited loss of activity during turnover in a biphasic process. The rate of the faster of the two phases was dependent on L-lactate concentration and at saturating concentrations showed a first-order deactivation rate constant, $k_{f(deact.)}$, of 0.029 s⁻¹ (at 25 °C and pH 7.5). The slower phase, however, was independent of L-lactate concentration and gave a first-order deactivation rate constant, $k_{s(deact.)}$, of 0.01 s⁻¹ (at 25 °C and pH 7.5). This slower phase was found to correlate with dissociation of FMN, which is one of the prosthetic groups of the enzyme. Thus fully deactivated TD- b_2 , which was also tetrameric, was found to be completely devoid of FMN. Much of the original activity of TD- b_2 could be recovered by re-incorporation of FMN. Thus the C-terminal tail of flavocytochrome b_2 appears to be required for the structural integrity of the enzyme around the flavin active site even though the two are well separated in space.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase, EC 1.1.2.3) from baker's yeast (*Saccharomyces cerevisiae*) is a tetramer of identical subunits with M_r 57 500 [1]. Each subunit contains FMN, which is non-covalently bound, and protohaem IX prosthetic groups in two functionally distinct domains [2]. The enzyme is a soluble component of the mitochondrial intermembrane space [3], where it catalyses the oxidation of L-lactate to pyruvate [2]. In the proposed mechanism for L-lactate oxidation, hydrogen abstraction at C-2 is the major rate-limiting step [4]. Studies with [2-³H]lactate as substrate with ferricyanide as electron acceptor are consistent with this proposal, showing a deuterium isotope effect of between 4 and 5 [5]. The crystal structure of flavocytochrome b_2 has been solved to 0.24 nm resolution [6,7] and reveals that each subunit of the protein consists of three readily identified regions: a haem-containing domain, a flavin-containing domain and an extended C-terminal tail. The observation that this tail wraps around each of the other three subunits in the holoenzyme (see Fig. 1) would be consistent with the idea that the primary role of the C-terminal tail is to hold the subunits together as a tetramer. One experimental approach to test this idea was to investigate the properties of the enzyme lacking the tail. In the present paper we describe the generation of a C-terminal-tail-deleted mutant of flavocytochrome b_2 (referred to below as TD- b_2) by site-directed mutagenesis and report the kinetic and physical properties of this mutant enzyme.

MATERIALS AND METHODS

Strains, media, growth and DNA manipulation

The gene encoding tail-deleted flavocytochrome b_2 (TD- b_2) was generated by the double-primer method of Zoller & Smith [8], with single-stranded plasmid pGR401 [9] as template. The following oligonucleotide, synthesized on an Applied Biosystems model 380B DNA synthesizer, was used for mutagenesis: GAACAGTTTGAGTACCA. The nucleotide sequence of the entire TD- b_2 gene was determined and found to contain no secondary mutations. The TD- b_2 gene was excised from plasmid pGR401 as a 1.8 kb *EcoRI*–*HindIII* restriction fragment and inserted into the polylinker region of the plasmid pDS6 [10]. *Escherichia coli* strain HB101 was grown in L-broth and transformed by the $CaCl_2$ method [11]. Transformants were selected by ability to grow in the presence of ampicillin (100 µg/ml) and by pink coloration due to the presence of the haem-containing TD- b_2 .

Enzyme preparation and characterization

Wild-type and mutant flavocytochromes b_2 were isolated from *E. coli* as previously described [10]. When fully purified the wild-type enzyme showed a single band at M_r 57 500 on SDS/polyacrylamide-gel electrophoresis, whereas the tail-deleted mutant enzyme showed a single band at M_r 55 000, as expected. TD- b_2 had an electronic absorption spectrum in the visible region that was superimposable on that of the wild-type enzyme. The M_r of TD- b_2 in the native state was determined by gel

Abbreviation used: TD- b_2 , C-terminal-tail-deleted mutant of flavocytochrome b_2 .

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Fig. 1. Three-dimensional structure of flavocytochrome b_2 as determined by Mathews & Xia [7]

The C-terminal tails and FMN prosthetic groups of the four subunits are highlighted in view (a). A side view (b) is also shown. The four subunits are numbered 1 to 4 in view (a). The shaded portions seen in subunits 2 and 4 represent the two haem-containing domains, which are disordered in the X-ray crystal structure.

Fig. 2. Determination of the M_r of the TD- b_2 by gel filtration on a Sepharose S300 column (150 cm $\times$ 3 cm)

The markers were wild-type flavocytochrome b_2 (M_r 230000), β -amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 66000), carbonic anhydrase (M_r 29000), myoglobin (M_r 17500) and cytochrome c (M_r 12500). The calculated M_r of TD- b_2 was 220000, confirming it as a tetramer. Deactivated (FMN-deficient) TD- b_2 was also eluted with an apparent M_r of 220000.

filtration on a Sepharose S300 column (150 cm $\times$ 3 cm) calibrated with the following markers (Sigma Chemical Co.): β -amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), bovine serum albumin (M_r 66000), carbonic anhydrase (M_r 29000), myoglobin (M_r 17500) and cytochrome c (M_r 12500) (see Fig. 2). Wild-type flavocytochrome b_2 (M_r 130000) was also used as a marker.

Bound flavin was detected by using the sulphite method of Lederer [12]. Identical solutions of oxidized TD- b_2 (8 μ M) in Tris/HCl buffer, pH 7.5, were placed in the

reference and observation cells of a Perkin-Elmer λ 9 spectrophotometer. To the reference cell was added 10 μ l of 0.3 M- Na_2SO_3 (which bleaches the flavin absorbance) and to the observation cell 10 μ l of buffer. The resulting difference spectrum corresponds to bound flavin and is illustrated in Fig. 3.

Flavin re-incorporation

Under turnover conditions TD- b_2 was found to lose flavin, resulting in fully deactivated enzyme (see the Results section). Re-incorporation of flavin into the flavin-deficient enzyme was carried out according to the following procedure. Deactivated TD- b_2 was partially denatured in 20 ml of solution A (150 mM-phosphate buffer, pH 7.0, containing 4 M-urea, 100 mM-2-mercaptoethanol, 100 mM-lactate and 1 mM-EDTA) to yield a final protein concentration of 8 μ M. A 10-fold excess of FMN (Sigma Chemical Co.) was added, and renaturation was facilitated by dialysis of the TD- b_2 /FMN mixture firstly against solution A in the absence of urea and then against solution A in the absence of both urea and 2-mercaptoethanol. Each dialysis was carried out for 20 h at 4 $^\circ\text{C}$ under N_2 . After the final dialysis the reconstituted protein showed similar kinetics to the original TD- b_2 . The procedure for the wild-type protein was as above except that flavin was first removed from the protein by denaturation as previously described [10].

Kinetic analysis

Kinetic experiments involving enzymic oxidation of L-lactate were carried out under steady-state conditions at 25 ± 0.1 $^\circ\text{C}$ in 10 mM-Tris/HCl buffer, pH 7.5 and 1.0.10 (NaCl), with ferricyanide (1 mM) as electron acceptor as previously described [9,10]. Flavocytochrome b_2 concentrations were determined spectrophotometrically by

using published molar absorption coefficients [9]. Kinetic isotope effects were measured by using $[2\text{-}^2\text{H}]\text{lactate}$ prepared and purified as previously described [5]. Isotopic purity of $[2\text{-}^2\text{H}]\text{lactate}$ was confirmed by ^1H n.m.r. [13].

Fig. 3. Spectra determined by using the sulphite method (described in the Materials and methods section) for (a) active TD- b_2 and (b) deactivated TD- b_2

TD- b_2 concentration was $8\text{ }\mu\text{M}$ and $10\text{ }\mu\text{l}$ of $0.3\text{ M-Na}_2\text{SO}_3$ was added. It is clear that the deactivated TD- b_2 has no bound flavin.

The dissociation of FMN during turnover was monitored as an increase in fluorescence at 520 nm on a Perkin-Elmer LS-5 luminescence spectrometer with an emission bandwidth of 2.5 nm and with the excitation wavelength set at 450 nm with an excitation bandwidth of 10 nm . The intensity of the fluorescence as a function of FMN concentration is known to be linear in the concentration range 0.1 nM – $10\text{ }\mu\text{M}$ [14], and consequently all experiments were carried out with the enzyme concentrations between 0.1 and $0.01\text{ }\mu\text{M}$. The ferricyanide concentration was 8 mM and the L-lactate concentration was varied between 1 and 10 mM .

RESULTS

Determination of M_r

The subunit M_r of TD- b_2 was 55000 according to SDS/polyacrylamide-gel electrophoresis, as expected for flavocytochrome b_2 lacking 23 amino acid residues. The native M_r of TD- b_2 , determined by gel filtration on a calibrated Sepharose S300 column, was found to be 220000 (see Fig. 2). This indicates that TD- b_2 in the native state is tetrameric. TD- b_2 remained tetrameric in the active and inactive (flavin-deficient) states. The behaviour of TD- b_2 was very similar to that of the wild-type enzyme, and this can also be seen in Fig. 2.

Kinetics of L-lactate oxidation

The steady-state rate of oxidation of L-lactate by TD- b_2 was found to decrease long before L-lactate or ferricyanide was depleted. However, initial rates at several L-lactate concentrations were determined and did give rise to typical saturation behaviour, yielding the K_m and k_{cat} values listed in Table 1. These values can be compared with those found for the wild-type enzyme, which are also listed in Table 1. The decrease in rate of reaction seen with TD- b_2 proceeded in a biphasic manner, leading eventually to complete loss of enzyme activity. This biphasic process was independent of ferricyanide concentration, and was also observed if cytochrome c was used as an alternative electron acceptor (results not shown). TD- b_2 was perfectly stable for long periods [months at $4\text{ }^\circ\text{C}$ in the reduced state as an $(\text{NH}_4)_2\text{SO}_4$ precipitate, and hours at $25\text{ }^\circ\text{C}$ in solution at $\text{pH } 7.5$ in either reduced or oxidized state]; rapid deactivation was only observed under turnover conditions. The faster of the two phases was found to be dependent on L-lactate

Table 1. Kinetic properties of wild-type and tail-deleted (TD- b_2) flavocytochromes b_2

All values, unless otherwise stated, were determined at $25\text{ }^\circ\text{C}$, $\text{pH } 7.5$ and $1/0.10$, with ferricyanide (1 mM) as terminal electron acceptor. Values of $k_{f(\text{deact.})}$ and $k_{s(\text{deact.})}$ were determined at saturating L-lactate concentrations. The kinetic isotope effect was determined with $[2\text{-}^2\text{H}]\text{lactate}$. The value of $k_{diss.}$ was determined by monitoring the increase in fluorescence (caused by dissociation of FMN during turnover) with time; ferricyanide concentration was 8 mM in this case.

	Wild-type enzyme	TD- b_2 enzyme	Reconstituted wild-type enzyme	Reconstituted TD- b_2 enzyme
k_{cat} (s^{-1})	200 ± 10	165 ± 6	98 ± 3	97 ± 8
K_m (mM)	0.49 ± 0.04	0.96 ± 0.06	0.65 ± 0.05	1.76 ± 0.26
$k_{f(\text{deact.})}$ (s^{-1})	—	0.029 ± 0.002	—	0.028 ± 0.006
$k_{s(\text{deact.})}$ (s^{-1})	—	0.010 ± 0.001	—	0.008 ± 0.001
$k_{diss.}$ (s^{-1})	—	0.012 ± 0.002	—	—
Kinetic isotope effect	4.2 ± 0.2	3.6 ± 0.4	—	—

Fig. 4. Dependence of the deactivation rate constants for TD- b_2 on the concentration of L-lactate

The biphasic nature of TD- b_2 deactivation gave rise to first-order rate constants for a fast phase, $k_{f(\text{deact.})}$, and a slow phase, $k_{s(\text{deact.})}$. Note that whereas $k_{f(\text{deact.})}$ shows a marked dependence on L-lactate concentration $k_{s(\text{deact.})}$ is essentially L-lactate-independent. $k_{\text{diss.}}$ was determined by spectrofluorimetric measurements and corresponds to $k_{s(\text{deact.})}$, indicating it is this phase in which FMN is lost. ●, $k_{f(\text{deact.})}$; ○, $k_{s(\text{deact.})}$; ■, $k_{\text{diss.}}$. Inset: double-reciprocal plot demonstrating the linear relationship of $1/k_{f(\text{deact.})}$ against $1/[\text{L-lactate}]$. All data shown were determined at 25 ± 0.1 °C, in Tris/HCl buffer, pH 7.5 and 1.0 M (NaCl).

concentration, whereas the slow phase was L-lactate-concentration-independent (Fig. 4). The first-order deactivation rate constants at saturating L-lactate concentrations for the fast ($k_{f(\text{deact.})}$) and slow ($k_{s(\text{deact.})}$) phases are listed in Table 1. A plot of $1/k_{f(\text{deact.})}$ against $1/[\text{L-lactate}]$ yielded a straight line (Fig. 4 inset), from which an estimate of the apparent dissociation constant, K_d , for L-lactate could be made. This was found to be 0.8 ± 0.1 mM, in close agreement with the K_m value listed in Table 1. Kinetic parameters were also obtained for FMN-reconstituted TD- b_2 (which again showed a biphasic deactivation process), and are listed in Table 1.

Kinetic isotope effect with [2- ^3H]lactate

With ferricyanide as electron acceptor, the deuterium isotope effect seen for the TD- b_2 enzyme was the same, within experimental error, as that for the wild-type enzyme. Values are shown in Table 1. This indicates that hydrogen abstraction at C-2 of L-lactate remains the rate-limiting step in TD- b_2 .

FMN dissociation and re-incorporation

The deactivation of TD- b_2 during turnover was found to lead eventually to complete loss of the FMN prosthetic group. Before turnover a typical spectrum for bound FMN could be observed by using the sulphite method described in the Materials and methods section. After deactivation this bound FMN spectrum was found to be completely absent (Fig. 3), indicating loss of FMN. Absence of FMN was also shown by an inability to extract it under denaturing conditions as previously described [10]. The rate of dissociation of FMN was determined directly by monitoring the increase in fluor-

escence with time during enzyme turnover (the fluorescence intensity of bound FMN is quenched by the protein). These measurements gave a first-order rate constant for FMN dissociation, $k_{\text{diss.}}$ of 0.012 ± 0.002 s $^{-1}$, which was independent of L-lactate concentration (Fig. 4). The value of $k_{\text{diss.}}$ and its independence of L-lactate concentration correlate (within experimental error) with the slow phase of deactivation already mentioned (Table 1), indicating that it is the slow phase only in which FMN is lost. Confirmation that activity loss was due to flavin absence was obtained by restoring up to 60 % of the activity by re-incorporation of flavin. The reconstituted protein showed similar kinetic behaviour to the original TD- b_2 (see Table 1).

DISCUSSION

The three-dimensional structure of a subunit of flavocytochrome b_2 shows very clearly an extended C-terminal tail that projects from the main body of the molecule. In the tetramer the tail of the subunit wraps around each of the other three, making many intersubunit contacts (Fig. 1) [7]. Therefore the most obvious role one might suggest for this C-terminal tail is that it holds the four subunits together. To test this and hopefully to generate monomeric enzyme (in order to simplify kinetic analysis), a stop codon was introduced into the flavocytochrome b_2 gene at a position corresponding to amino acid residue 489. This mutation resulted in production of a mutant enzyme (TD- b_2) lacking 23 amino acid residues at the C-terminal portion of the molecule, which showed, as expected, an M_r of 55 000 according to SDS/polyacrylamide-gel electrophoresis. However, TD- b_2 exhibited an M_r (under non-denaturing conditions) of 220 000, corresponding to a tetramer (Fig. 2).

Kinetic studies indicated that deletion of the C-terminal tail had significantly affected the stability of the enzyme under turnover conditions. Investigations of L-lactate oxidation by TD- b_2 revealed that this mutant enzyme became totally deactivated during turnover. This deactivation did not occur as a result of dissociation of TD- b_2 into monomers during turnover: the enzyme remained tetrameric. Further studies revealed that the fully deactivated TD- b_2 totally lacked FMN and that a substantial amount (60 %) of the original activity could be restored by flavin re-incorporation. The kinetics of the deactivation process were investigated in an attempt to elucidate the mechanism by which the non-covalently bound flavin had been lost from TD- b_2 .

The rate of deactivation proceeded in a biphasic manner. The faster of the two phases gave a first-order rate constant, $k_{f(\text{deact.})}$, that was dependent on L-lactate concentration (Fig. 4). Increasing the L-lactate concentration decreased the value of $k_{f(\text{deact.})}$ until a limiting value was reached (Table 1). From the double-reciprocal plot (Fig. 4 inset) an apparent K_d for L-lactate of 0.8 ± 0.1 mM was determined, which is very close to the K_m value of 0.96 ± 0.06 mM. The similarity of these values is consistent with an intimate association between deactivation during the fast phase and L-lactate binding. A first-order rate constant, $k_{s(\text{deact.})}$, was determined for the slower phase of the biphasic deactivation and was essentially independent of L-lactate concentration. This slower phase was found to correlate (within experimental error) with the rate of dissociation of FMN from the enzyme as determined by fluorescence measurements

(Table 1), with the rate of FMN dissociation also being independent of L-lactate concentration. A possible explanation for the biphasic deactivation process is that there is an initial, substrate-dependent, fast phase that leads to a partially deactivated conformationally altered enzyme. This conformational alteration is sufficient to allow dissociation of FMN, which occurs in the slow phase and is independent of substrate concentration. This process might be represented as shown in the equation below, where E_C is the conformationally altered enzyme and E_I is the fully inactive (FMN-deficient) enzyme, and k_f and k_s are the rate constants for the fast and slow phases respectively:

After FMN has been lost the enzyme is completely inactive. TD- b_2 that had been reactivated by flavin re-incorporation underwent flavin loss again by a biphasic process, resulting in $k_{f(\text{deact.})}$ and $k_{s(\text{deact.})}$ values identical with the original values, within experimental error (see Table 1). From these results we suggest that deletion of the C-terminal tail allows an alteration in the active-site conformation to occur during turnover that reduces the ability of the enzyme to bind FMN sufficiently tightly to prevent its loss.

Although TD- b_2 loses activity during turnover, it was possible to use initial-rate data from steady-state experiments to determine K_m and k_{cat} values for L-lactate. The difference in the values of K_m and k_{cat} between TD- b_2 and wild-type enzymes (Table 1) are of an order that would be consistent with a general destabilization of the enzyme resulting from a conformational difference. The possibility that such a change could affect the rate-limiting step, which is known to be hydrogen abstraction at C-2 of L-lactate [5], was considered. To explore this, the kinetic isotope effect seen with [2- ^3H]lactate was investigated and found to be essentially the same, within experimental error, for both TD- b_2 and wild-type enzymes. Thus, although K_m and k_{cat} values are affected by tail deletion, this would not appear to be due to any change in the rate-limiting step.

From our studies on TD- b_2 we draw the following conclusions: (i) although it appears to be involved in many intersubunit contacts, the C-terminal tail is not essential for holding the subunits together as a tetramer;

(ii) the C-terminal tail acts as a conformational 'anchor' influencing the structure of the protein in regions distant from it, particularly the FMN active site; (iii) TD- b_2 is deactivated during turnover in a biphasic process of which the slow phase corresponds to FMN dissociation; (iv) any alteration in the structural integrity of the flavin active site, caused by tail deletion, does not result in any significant change in the rate-determining step for L-lactate oxidation.

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