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Kinetic properties of flavocytochrome b_2 from *Hansenula polymorpha*

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Flavocytochrome b_2 (fcb2) (EC 1.1.2.3) is a 4 × 58 kD homotetrameric FMN and heme containing enzyme localized in the intermembrane space of yeast mitochondria. During the catalysis, FMN oxidizes *L*-lactate to pyruvate. Subsequently, the reduced FMN transfers electrons to the heme cofactor, which subsequently reduces cytochrome *c*. The kinetic properties and thermostability of the enzyme depend on its source, and these factors may be important in the construction of biosensors for *L*-lactate based on fcb2. In this work, we have investigated the kinetic properties of fcb2 from the thermotolerant methanotrophic strain *Hansenula polymorpha*. Like in our previous studies of *Saccharomyces cerevisiae* fcb2 [1], *H. polymorpha* fcb2 exhibited biphasic Lineweaver–Burk plots at fixed *L*-lactate concentrations using varied ferricyanide concentrations. It reflects the acceptance of electrons from both the reduced FMN and heme after the slower intramolecular FMN-to-heme electron transfer. At the infinite *L*-lactate concentration, the maximal rate of ferricyanide reduction is close to 120 s^{-1}. The maximal rate of cytochrome *c* reduction is close to the second (slower) phase of ferricyanide reduction, because cytochrome *c* accepts electrons only from heme. The reaction product pyruvate inhibits *H. polymorpha* fcb2 in a mixed way at the millimolar concentration, and decreases the maximal rates of both fast and slow phases of ferricyanide reduction.

Keywords: flavocytochrome b_2, steady-state kinetics, cytochrome *c*, redox reactions

Abbreviations: fcb2, flavocytochrome b_2; cytc, cytochrome *c*; FMN, flavin adenine mononucleotide; k_{cat}, the catalytic constant (maximal turnover rate) in enzymatic steady-state reactions; k_{cat}/K_m, the bimolecular reaction rate constant in enzymatic steady-state reactions; K_i, the inhibition constant

INTRODUCTION

Flavocytochrome b_2 (fcb2, EC 1.1.2.3) is a *L*-lactate dehydrogenase found in the intermembrane space of yeast mitochondria. The enzyme catalyzes the oxidation of *L*-lactate to pyruvate at the expense of cytochrome *c* thus allowing the yeast to respire on *L*-lactate ([2] and references therein). Each of the four of its identical 58 kD subunits consists of the *N*-terminal ~100 amino acid domain with bound protoheme IX, *C*-terminal ~400 amino acid flavodehydrogenase domain with a non-covalently bound FMN and a short hinge region that connects these two domains. During the catalysis, *L*-lactate transfers two redox equivalents to FMN with the formation of pyruvate with the maximal rates on the two-electron basis of ~200 s^{-1} (*Saccharomyces cerevisiae* fcb2 [3]) or ~500 s^{-1} (*Hansenula anomala* fcb2 [4, 5]) at pH 7.0 and 25 °C. Further, the electron is transferred from reduced FMN to heme with a rate of 1500 s^{-1} (on the one-electron basis) [6]. After the reoxidation of heme by cytochrome *c,* the second electron is transferred from FMN semiquinone ($FMN^{\cdot-}$) with a rate of 120 s^{-1} (*S. cerevisiae* fcb2 [3]) or ~500 s^{-1} (*H. anomala* fcb2 [1]). One may note that cytochrome *c*, the physiological electron acceptor of fcb2 may accept the electrons from heme only [7]. Thus, the intramolecular electron transfer between the reduced forms of FMN and heme may limit the overall rate of catalysis, i. e. cytochrome *c* reduction [2]. On the other hand, the nonphysiological electron acceptors such as ferricyanide or quinones may accept electrons both from the reduced heme and the reduced forms of FMN [8]. At millimolar concentrations, the reaction product, pyruvate,

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inhibits the reactions of fcb2 in a dual way: i) it competes with *L*-lactate for the binding in the FMN domain [3, 9], and ii) it rises the redox potential of the $FMN^{-}/FMNH^{-}$ couple, thus slowing down the intramolecular FMN-to-heme electron transfer [10].

Fcb2-based electroanalytical systems were used in the determination of *L*-lactate levels in blood samples, dairy products, and wine [11]. The use of fcb2 in these analytical systems warrants its high stability. Therefore it is important to compare and assess the catalytic properties and thermostability of fcb2 from the classical and novel sources. The aim of this paper is to characterize the kinetic properties of fcb2 from the thermostable methanotrophic yeast *H. polymorpha*. It may comprise the initial stage in its further studies related to its practical application.

EXPERIMENTAL

Enzyme purification

The recombinant cells of *Hansenula polymorpha* strain "tr1" (*gcr1 catX/prAOX_CYB2*) were cultivated at 30 °C in 1% glucose, 0.2% *L*-lactate, and 0.75% yeast extract. The freshly grown cells were collected by centrifugation at 3000 rpm, washed twice with water, and stored till usage. The purification started with the cell disintegration with glass beads in a phosphate buffer (pH 7.8) containing 1.0 mM EDTA and 1.0 mM PMSF. After the cell lysis and the centrifugation of the cell-free extract (3000 rpm, 15 min), the supernatant was collected and further chromatographically purified using the DEAE-Toyopearl 650 M (TSK-Gel, Japan) column. The concentration of fcb2 in this preparation was estimated according to the absorption difference at 556 nm of oxidized and reduced (10 mM *L*-lactate) enzyme forms ($\Delta\varepsilon_{556}$ = 21.8 $mM^{-1} cm^{-1}$) [2].

MATERIALS AND METHODS

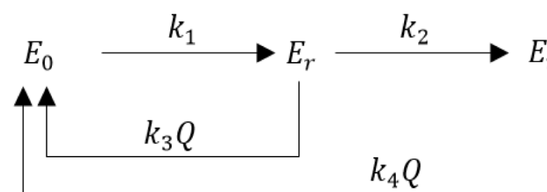
Cytochrome *c* from equine heart, sodium *L*-lactate, sodium pyruvate and potassium ferricyanide were obtained from Sigma-Aldrich and were used as received. All the spectrophotometric measurements were performed using a PerkinElmer Lambda 25 spectrophotometer at 25 °C in the 0.1 M K-phosphate buffer (pH 7.0) containing 1.0 mM EDTA. The concentrations of *H. polymorpha* fcb2 in the steady-state assays were typically 5–20 nM. The reaction rates were assessed using varied *L*-lactate (1.5–10 mM) and ferricyanide (0.0312–2.0 mM) or cytochrome *c* (10–100 μM) concentrations. In these cases, the decrease in the absorbance of ferricyanide ($\Delta\varepsilon_{400}$ = 1.0 $mM^{-1} cm^{-1}$), or an increase in the absorbance of cytochrome *c* ($\Delta\varepsilon_{550}$ = 20 $mM^{-1} cm^{-1}$) was monitored. The kinetic parameters k_{cat} (maximal enzyme turnover number per FMN cofactor), and its k_{cat}/K_m (the apparent bimolecular reaction rate constant) correspond to the inverse intercept and slope in Lineweaver–Burk plots ([E]/*v* vs 1/[Q], respectively, where *v* is the rate of the reaction, [E] is the concentration of the enzyme and [Q] is the concentration of the electron acceptor). They were calculated using SigmaPlot 12 and Wolfram Mathematica 10. The inhibition of the reactions of fcb2 by pyruvate was assayed using its concentrations of 1.0–5.0 mM under different concentrations of *L*-lactate and the electron acceptors, ferricyanide or cytc.

RESULTS AND DISCUSSION

It has been established that stationary kinetics of electron acceptor (ferricyanide, quinones) reduction by fcb2 from *H. anomala* shows biphasicity with the appearance of the breaks in the Lineweaver–Burk plots [12]. It was assumed that the maximal rate of the fast phase (high k_{cat}, low k_{cat}/K_m) corresponds to the oxidation of reduced FMN or its semiquinone, and the maximal rate of the slow phase (low k_{cat}, high k_{cat}/K_m) corresponds to the oxidation of the reduced heme. This phenomenon is explained by the fact that the rate of FMN reduction is higher than the rate of heme reduction in pre steady-state kinetics [2, 8]. It is also in line with the previous data which show that the maximal rate of cytochrome *c* reduction is lower than the maximal rate of reduction of low m. w. oxidants like ferricyanide that may oxidize both reduced heme and FMN [8].

The results of this study show that the reduction of ferricyanide by a currently poorly characterized fcb2 from *H. polymorpha* follows the same regularities as observed in the kinetics of *H. anomala* enzyme [12], namely, the biphasic character of Lineweaver–Burk plots (Fig. 1). It shows that the reaction follows the Scheme, where $k_1 > k_2$, and $k_3 < k_4$. In this case, the reaction rate is expressed by the Equation:

$$\frac{[E_0]}{V} = \frac{1}{k_1} + \frac{1}{k_2 + k_3[Q]} + \frac{k_2}{(k_2 + k_3[Q])k_4[Q]}.$$



Scheme. The simplified scheme of the fcb2 catalyzed reaction at fixed *L*-lactate concentration, where E_0 is oxidized enzyme; E_r is enzyme with reduced FMN; E_r* is enzyme with reduced heme; *Q* is electron acceptor

At varied *L*-lactate concentrations and low ferricyanide concentrations, we observe a series of parallel but biphasic plots in Lineweaver–Burk coordinates (Fig. 1). The k_{cat}/K_m for ferricyanide, corresponding to k_3 in the Scheme and calculated according to the Equation (fast phase), is equal to $8.0 \pm 1.0 \times 10^4\ M^{-1} s^{-1}$, and the value of k_4 (slow phase) is equal

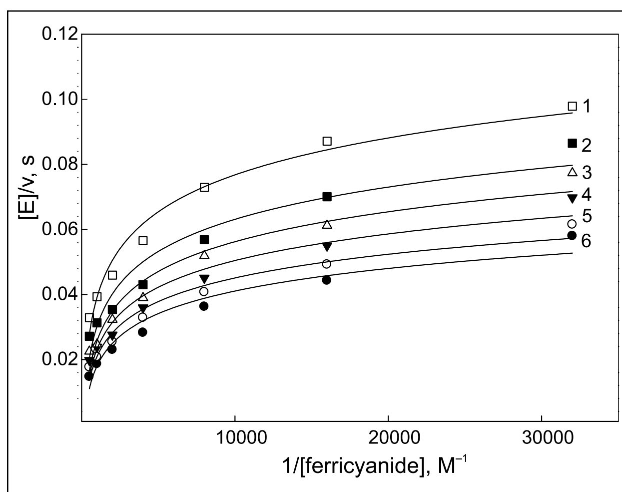


Fig. 1. The rates of fcb2-catalyzed reduction of ferricyanide at constant concentrations of *L*-lactate: 1.5 mM (1), 2.0 mM (2), 3.0 mM (3), 4.44 mM (4), 6.67 mM (5), 10 mM (6). Concentration of ferricyanide was varied from 1.0 mM to 0.0312 mM

to $1.5 \pm 0.2 \times 10^6$ M^{-1} s^{-1}. Extrapolated to infinite *L*-lactate concentrations, the maximal rate of the fast phase of ferricyanide reduction was equal to 120 ± 10 s^{-1}, and that of the slow phase was equal to 49 ± 7.0 s^{-1}. The calculations according to the dependence of the maximal rate of the fast phase on *L*-lactate concentration show that k_{cat}/K_m for *L*-lactate is equal to $7.5 \pm 0.9 \times 10^4$ M^{-1} s^{-1} (on the single-electron basis). In parallel, we studied the reduction of cytochrome *c* by *H. polymorpha* fcb2. The reaction patterns followed the simple 'ping-pong' Scheme yielding a series of parallel 1/[cytc] vs [E]/*v* plots at varied *L*-lactate concentrations (Fig. 2). In this case, the maximal rates of cytochrome *c* reduction at fixed *L*-lactate concentrations were close to the maximal rates observed in the second (slow) phase of ferricyanide reduction (Fig. 3). At the infinite cytochrome *c* and *L*-lactate concentrations, the k_{cat} for the reaction was equal to 59 ± 6.0 s^{-1}. This value most probably corresponds to the rate of intramolecular $FMN^{\cdot-}$-to-heme electron transfer. These trends are similar to the data of *S. cerevisiae* fcb2 or *H. anomala* fcb2 reactions ([2], and references therein), although the *H. polymorpha* enzyme is slower several times.

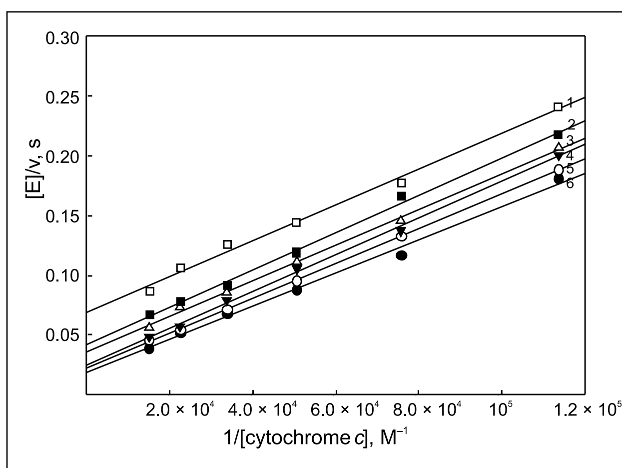


Fig. 2. The rates of fcb2-catalyzed reduction of cytochrome *c* at constant concentrations of *L*-lactate: 1.5 mM (1), 2.0 mM (2), 3.0 mM (3), 4.44 mM (4), 6.67 mM (5), 10 mM (6). Concentration of cytochrome *c* was varied from 100 to 10 μM

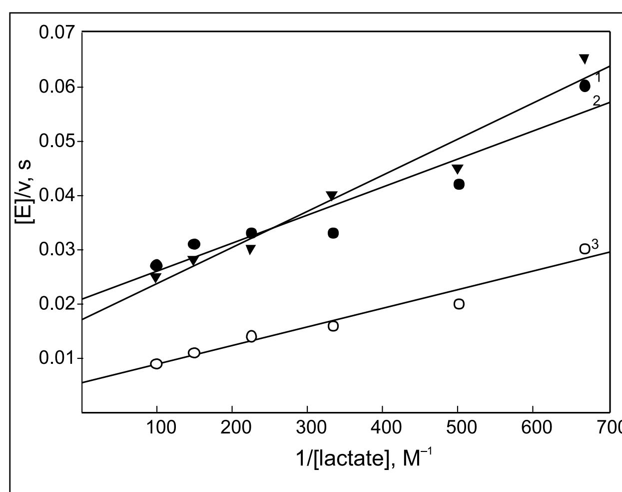


Fig. 3. The maximal rates of reactions: the reduction of cytochrome *c* (1), the "slow phase" of ferricyanide reduction (2), and the "fast phase" of ferricyanide reduction (3)

Pyruvate acts as an inhibitor of fcb2 both through competition with *L*-lactate for the binding to the oxidized enzyme form, and through its binding to $FMN^{\cdot-}$ semiquinone which slows down the electron transfer between $FMN^{\cdot-}$ and heme [9, 13]. Thus, it should act as a mixed-type inhibitor. In the case of *H. polymorpha* fcb2, under the constant ferricyanide concentration and varied *L*-lactate concentration, pyruvate increased the slopes in Lineweaver–Burk plots (Fig. 4), with a concomitant decrease in V_{max}. K_i, calculated from V_{max}

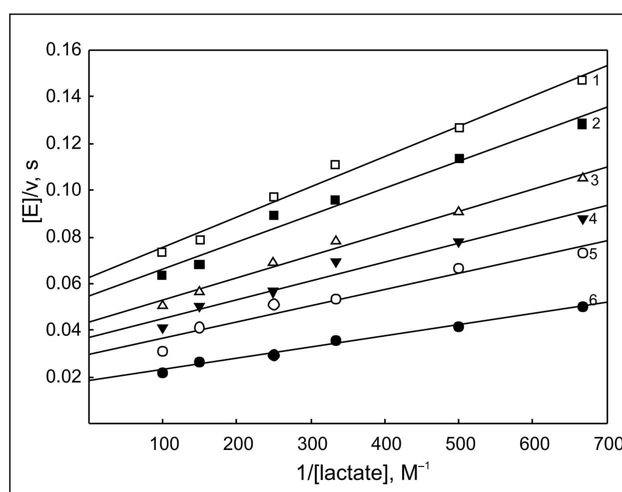


Fig. 4. Inhibition of the fcb2 enzymatic reaction by the reaction product pyruvate under constant concentration of ferricyanide (1.0 mM) and varied concentrations of L-lactate (1.5–10 mM). The concentration of pyruvate is 5.0 mM (1), 4.0 mM (2), 3.0 mM (3), 2.0 mM (4), 1.0 mM (5), and pyruvate is absent (6)

is equal to 2.4 ± 0.3 mM, and K_i, calculated from the slopes, is equal to 2.8 ± 0.3 mM. It shows that pyruvate competes with *L*-lactate for the binding to the oxidized enzyme form, and also that it impedes the other catalytic steps. Under constant concentration of *L*-lactate (10 mM) and the varied concentration of ferricyanide, pyruvate decreases the V_{max} and increases the apparent biphasicity in the Lineweaver–Burk plots (Fig. 5). Its inhibition constants (K_i), calculated according to the changes in V_{max} of slow and fast phases, are 3.3 ± 0.3 and 1.5 ± 0.2 mM, respectively. When cytochrome *c* is used as an electron acceptor, pyruvate uncompetitively inhibits its reduction with K_i = 2.7 ± 0.3 mM which is close to the K_i for the slow phase of ferricyanide reduction (Fig. 6). Because the reduction rate of cytochrome *c* by fcb2 of other sources is limited by the rate of intramolecular FMN^{-}-to-heme electron transfer [6, 7], our data show that pyruvate may also slow down this step in the catalytic cycle of *H. polymorpha* fcb2. In conclusion, this data show that the patterns of inhibition of *H. polymorpha* fcb2-catalyzed reactions by the reaction product, pyruvate, are similar to those observed in the reactions of fcb2 of *S. cerevisiae* and *H. anomala* ([9, 13] and our unpublished data), thus reflecting the affinity of pyruvate to the various redox states of fcb2 at the millimolar range.

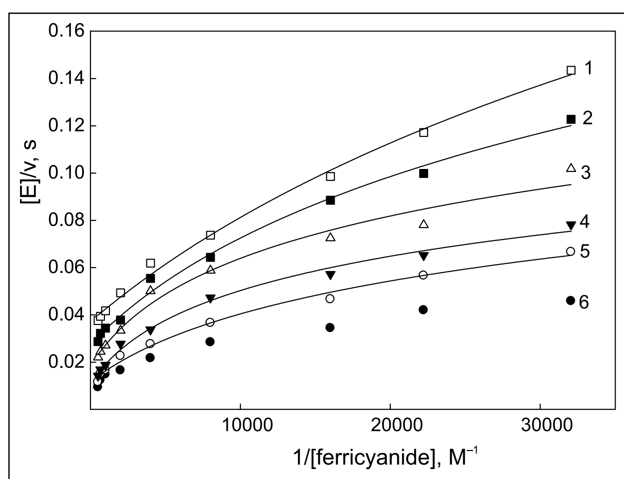


Fig. 5. Inhibition of the fcb2 enzymatic reaction by the reaction product pyruvate under constant concentration of *L*-lactate (10 mM) and varied concentrations of ferricyanide (0.0312–1.0 mM). The concentration of pyruvate is 5.0 mM (1), 4.0 mM (2), 3.0 mM (3), 2.0 mM (4), 1.0 mM (5), and pyruvate is absent (6)

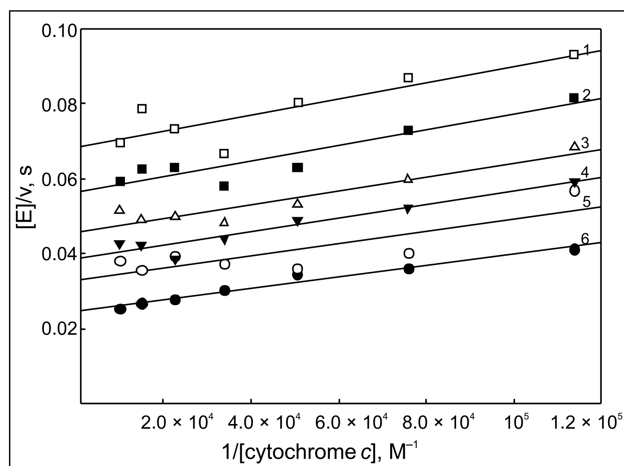


Fig. 6. Inhibition of the fcb2 enzymatic reaction by the reaction product pyruvate under constant concentration of *L*-lactate (10 mM) and varied concentrations of cytochrome *c* (10–100 µM). The concentration of pyruvate is 5.0 mM (1), 4.0 mM (2), 3.0 mM (3), 2.0 mM (4), 1.0 mM (5), and pyruvate is absent (6)

CONCLUSIONS

Our data show that the turnover number of fcb2 from the thermotolerant yeast *H. polymorpha* is several times slower than that of analogous enzymes from *H. anomala* and *S. cerevisiae*. However, the *H. polymorpha* enzyme exhibits kinetic properties similar to those of enzymes from other sources, i. e. its rate of FMN^{-}-to-heme electron transfer is slower than the rate of FMN reduction by *L*-lactate, and it is inhibited by millimolar concentrations of pyruvate in a mixed way.

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KINETINĖS FLAVOCITOCHROMO b_2 IŠ *Hansenula polymorpha* SAVYBĖS

Santrauka

Flavocitochromas b_2 (fcb2) (EC 1.1.2.3) yra 4 × 58 kD homotetramerinis FMN ir hemą turintis fermentas, lokalizuotas mielių mitochondrijų tarpmembraniniame tarpe. Katalizės metu FMN oksiduoja *L*-laktatą iki piruvato. Po to redukuotas FMN perneša elektronus hemo kofaktoriui, kuris savo ruožtu redukuoja citochromą *c*. Fermento kinetinės savybės ir termostabilumas priklauso nuo jo šaltinio, šie veiksniai gali būti svarbūs kuriant *L*-laktato biosensorius, pagrįstus fcb2. Šiame darbe tyrėme kinetines fcb2 iš termotolerantiškų metanotrofiškų mielių *Hansenula polymorpha* savybes. Kaip ir ankstesniuose tyrimuose su *Saccharomyces cerevisiae*, taip ir *H. polymorpha* atveju fcb2 pasižymi bifaziškumu Lineweaver-Burk koordinatėse esant fiksuotai *L*-laktato koncentracijai ir varijuojant fericianido koncentraciją. Tai atspindi elektronų tiek iš redukuoto FMN, tiek iš hemo paėmimą po lėtesnės vidumolekulinės elektronų pernašos iš FMN į hemą. Esant begalinėms *L*-laktato koncentracijoms maksimalus fericianido redukcijos greitis yra artimas 120 s^{-1}. Maksimalus citochromo *c* redukcijos greitis artimas antrajai (lėtesnei) fericianido redukcijos fazei, kadangi citochromas *c* priima elektronus tik iš hemo. Reakcijos produktas piruvatas inhibuoja *H. polymorpha* fcb2 mišriu būdu esant milimolinėms koncentracijoms ir mažina maksimalius greitosios ir lėtosios fericianido redukcijos fazių greičius.