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Steady-state kinetic analysis of substrate pair cycling between two enzymes: application to a mediated electron transport between the cytoplasmic membrane and the periplasmic nitrite reductase of *Paracoccus denitrificans*

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Abstract

An extended kinetic model is presented for the process catalysed by two enzymes mutually connected by the cycling of two reversibly interconvertible chemically relative species. Expressions are derived for the steady-state velocity, limiting velocity (V) and the half-saturation concentration of the cycling substrate ($A_{0.5}$). It is shown that the velocity depends on the total concentration of cycling substrate hyperbolically if both enzymes have equal activities. Based on these theoretical considerations, an experimental comparison was made between pseudoazurin and cytochrome c_{550} as physiological electron transfer mediators for nitrite reduction in an in vitro reconstituted part of the respiratory chain of *Paracoccus denitrificans*. Pseudoazurin exhibited 1.7-fold higher V and 14-fold higher $A_{0.5}$ than cytochrome c_{550} under the experimental conditions used (20 mM Tris chloride, pH 7.3, 30 °C).

Keywords: Enzymatic cycling; Half-saturation concentration; Redox mediator; Cytochrome *c*; *Pseudoazurin*; Nitrite reduction

1. Introduction

In the most simple enzymatic cycling system, two chemically relative species A_1 and A_2 are interconverted by two enzymes E_1 and E_2, so that the substrate of E_1 is the product of E_2 and vice versa, as shown in Eqs. (1) and (2):

$$A_1 + S_1 \xrightarrow{E_1} A_2 + P_1 \quad (1)$$

$$A_2 + S_2 \xrightarrow{E_2} A_1 + P_2 \quad (2)$$

The principle of substrate cycling has been employed to develop sensitive assays for the determination of very small quantities of metabolites [1]. Here, the assay mixture typically includes A_1 and A_2 at the concentrations well bellow the respective Michaelis constants, while the other substrates (S_1 and S_2) are present in excess. A theoretical treatment of this particular case has shown that the steady state velocity is directly proportional to the sum $A = [A_1] + [A_2]$, which

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forms a basis for the construction of a calibration curve [2].

The condition of sub-saturating values of $[A_1]$ and $[A_2]$ may not be met by the naturally occurring substrate cycling systems. One example is the dissimilatory reduction of nitrite, accomplished by a soluble cytochrome cd_1-type nitrite reductase in the periplasmic space of *Paracoccus denitrificans* cells. It has been conclusively demonstrated by reconstitution experiments that the enzyme does not receive electrons from the cytoplasmic membrane directly, but requires the participation of an additional electron transport protein, either cytochrome c_{550} [3] or pseudoazurin [4]. However, these studies have not involved a comparison of these proteins with regard to their ability to mediate electron transport.

When working on nitrite reduction in *P. denitrificans* we realised that the above recyclisation scheme could be in principle applicable also on periplasmic redox reactions by identifying A_1 with the oxidised and A_2 with the reduced form of the mediator protein. We therefore undertook a more complete theoretical analysis covering the entire range of substrate concentrations, with a hope to obtain a conceptual framework for the intended kinetic experiments.

2. Experimental

2.1. Material

Preparation of periplasmic and membrane fractions and isolation of nitrite reductase and redox mediator proteins followed the previously described procedures [4,5].

2.2. Nitrite reductase assay

Eppendorf tubes were used to measure the rates of nitrite reduction. They were gassed with argon before being sealed with caps. The reaction was initiated by addition of nitrite. Samples (50 μl) were removed at 3-min intervals for 15 min and placed in the tubes containing 1 ml of sulfanilic acid–HCl reagent for nitrite determination. The rate was calculated by linear regression in Excel as the slope of the time dependence of nitrite amount and expressed in the units nmol nitrite reduced s^{-1}.

2.3. Analytical methods

Nitrite was determined spectrophotometrically after conversion to a chromophoric azo derivative [6]. The concentrations of stock solutions of cytochrome c_{550}, pseudoazurin and nitrite reductase were determined spectrophotometrically using the following absorbance coefficients: 26.8 mM^{-1} cm^{-1} (550 nm, reduced form) [7], 1.36 mM^{-1} cm^{-1} (590 nm, oxidised form) [8] and 284 mM^{-1} cm^{-1} (407 nm, oxidised dimeric form) [9]. Protein content was determined by the Lowry method with bovine serum albumin as the standard.

3. Results and discussion

3.1. Derivation of the steady-state velocity equation

In order to simplify the derivation, we assume the reactions (1) and (2) catalysed by enzymes E_1 and E_2 proceed irreversibly. Therefore, the kinetic equations are:

$$v = V_1[A_1]/(K_{m,1} + [A_1]) \quad (3)$$

$$v = V_2[A_2]/(K_{m,2} + [A_2]) \quad (4)$$

where $K_{m,i}$ and V_i $(i = 1,2)$ stand for the Michaelis constants and limiting velocities, respectively. Note that the velocity symbol v is the same in both equations, which reflects attainment of a steady-state. If only small fractions of A_1 and A_2 are involved in attachment to E_1 and E_2, the total substrate concentration A can be written as

$$A = [A_1] + [A_2] \quad (5)$$

Eqs. (3) and (4) allow $[A_1]$ and $[A_2]$ to be expressed in terms of the steady-state velocity v, and insertion of the expressions into Eq. (5) leads to

$$A = \frac{K_{m,1}v}{V_1 - v} + \frac{K_{m,2}v}{V_2 - v} \quad (6)$$

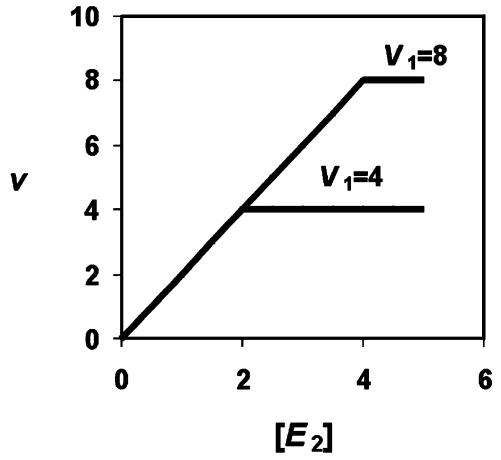

Fig. 1. The cycling rate v at substrate saturation as a function of the concentration of the enzyme E_2. The curves were computed according to Eq. (10) for the indicated values of V_1, assuming that $k_{cat,2}=2$.

or, after a little rearrangement, to

$$(K_{m,1}+K_{m,2}+A)v^2-\{K_{m,1}V_2+K_{m,1}V_1+(V_1+V_2)A\}v+V_1V_2A=0 \quad (7)$$

On solving the quadratic Eq. (7) and neglecting the second, physically meaningless root, one may obtain the following explicit expression for v as a function of A:

$$v=\frac{2V_1V_2A}{V_2(K_{m,1}+A)+V_1(K_{m,2}+A)+\sqrt{D}} \quad (8)$$

where

$$D=V_2^2(K_{m,1}+A)^2+V_1^2(K_{m,2}+A)^2-2V_1V_2\{A^2+(K_{m,1}+K_{m,2})A-K_{m,1}K_{m,2}\}$$

3.2. Limiting cases

Two limiting cases of our general solution can be considered.

1. At sufficiently low A, Eq. (8) reduces to a linear relationship between v and A

$$v=\frac{V_1V_2A}{V_2K_{m,1}+V_1K_{m,2}} \quad (9)$$

which is equivalent to that given by Passonneau and Lowry [2].

2. Conversely, if A is very large, v reaches a limiting value

$$V=\frac{2V_1V_2}{V_1+V_2+|V_1-V_2|} \quad (10)$$

The last expression relates V to the constituent enzyme activities V_1 and V_2. By increasing the concentration of one enzyme, say E_2, V will vary linearly (as $V_2=k_{cat,2}\times[E_2]$) until it becomes equal to the constant activity of the enzyme E_1. This is illustrated in Fig. 1 for two fixed values of V_1.

3.3. Assessment of the shape of the $v(A)$ dependence

Eq. (8) predicts that the $v(A)$ relationship will be generally non-hyperbolic. The deviation from the hyperbolic law can best be detected as a non-

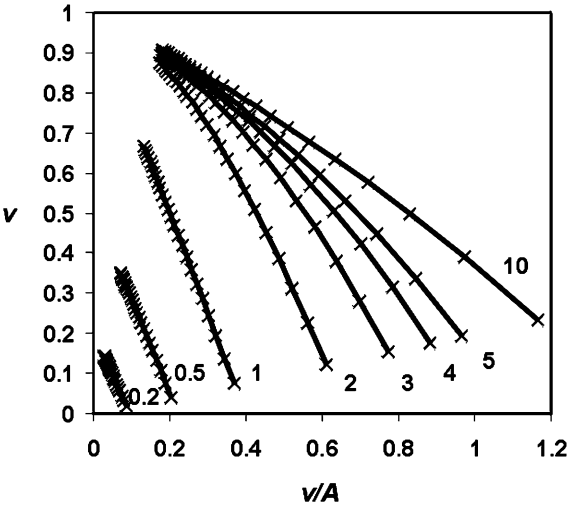

Fig. 2. Theoretical dependencies of the cycling rate v on the substrate concentration A in Eadie–Hofstee coordinates $\{v/A; v\}$. The computation was based on Eq. (8), with $K_{m,1}=0.5$, $K_{m,2}=2$, $V_1=1$ and $A=0.2, 0.4,..., 5.0$. The different curves refer to different values of V_2, which are indicated in the figure.

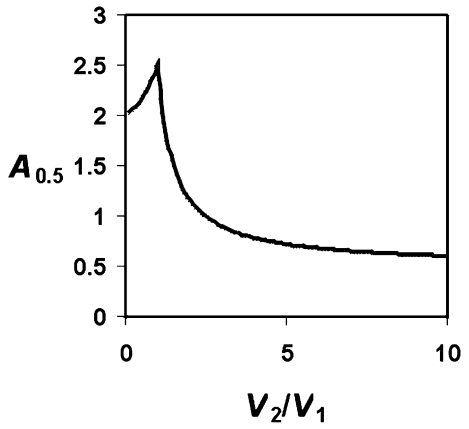

Fig. 3. Relationship of the half-saturation concentration $A_{0.5}$ to the activity ratio V_2/V_1. $A_{0.5}$ was calculated from Eq. (11) assuming $K_{m,1}=0.5$ and $K_{m,2}=2$.

linearity in an Eadie–Hofstee-type plot (Fig. 2). As follows algebraically from Eq. (6) by setting $V_1=V_2$, and visually from Fig. 2, true hyperbolic kinetics arise if both enzymes have equal activities. All the commonly used means of estimating the kinetic parameters of the hyperbolic law are then applicable and expected to provide the sum of both Michaelis constants ($K_{m,1}+K_{m,2}$). The saturation behaviour at V_1 differing from V_2 can also be formulated in a quantitative way. Since the considered two-enzyme system is saturable by the cycling substrate at any combinations of enzyme activities, one may define the half-saturation concentration $A_{0.5}$ as the total substrate concentration required to achieve 0.5 V under a particular condition of V_1 and V_2. Inserting $A=A_{0.5}$ and $v=V/2$ into Eq. (6) and combining it with Eq. (10), we can express $A_{0.5}$ as

$$A_{0.5}=\frac{K_{m,1}V_2}{V_1+|V_1-V_2|}+\frac{K_{m,2}V_1}{V_2+|V_1-V_2|} \qquad (11)$$

From Eq. (11) and its graphical presentation in Fig. 3 it can be deduced that $A_{0.5}\to K_{m,1}$ if $V_2/V_1\to\infty$, $A_{0.5}\to K_{m,2}$ if $V_2/V_1\to 0$, and $A_{0.5}=K_{m,1}+K_{m,2}$ if $V_2/V_1=1$. Thus, the actual value of $A_{0.5}$ lies in the interval between min($K_{m,1}$, $K_{m,2}$) and $K_{m,1}+K_{m,2}$.

3.4. Analysis of experimental data

The applicability of the above theoretical findings will be demonstrated by an analysis of a real experimental system involving a proteinous redox mediator (cytochrome c_{550} or pseudoazurin) and enzymes catalysing its reduction (membrane vesicles) and oxidation (nitrite reductase). A direct spectrophotometric determination of K_m for individual reactions from the changes in the concentrations of the oxidised or reduced form of the mediator poses a problem in that it requires low mediator concentrations at which the changes in absorbance are very small so that the initial velocities cannot be obtained at all or are too inaccurate. We intended to remove this obstacle by using a cycling arrangement of the assay that would allow us to measure nitrite consumption in the steady state. To this end, nitrite reductase amount had firstly to be adjusted to the mediator reductase activity of membrane vesicles by a titration procedure according to Fig. 1. Experiments examining the effects of mediator concentration then produced results summarised in Fig. 4. It is clear that the experimental data fits well to the hyperbolic law, in agreement with the theoretical expectation. The obtained values of limiting velocities suggest that pseudoazurin is somewhat more effective than cytochrome c_{550} in carrying out electron transport at saturating concentrations. However, as manifested by a distinctly lower value of $A_{0.5}$, cytochrome c_{550} binds more tightly to the enzymes than does pseudoazurin. In one-enzyme kinetics, the apparent second-order rate constant, k_{cat}/K_m, is the parameter of choice to express the relative rates of competing enzymatic reactions and hence enzymatic selectivities. If an analogous formalism were also adopted for the two-enzyme system studied here, cytochrome c_{550} would appear to be preferred over pseudoazurin as a redox mediator by a factor of 8.4.

Richter et al. [10] have recently performed a thorough kinetic analysis of redox reactions catalysed by nitrite reductase from *Paracoccus pantotrophus*, a close relative of *P. denitrificans*. Both this study and the present study concur in that the enzyme has a higher molecular activity with pseudoazurin relative to cytochrome c_{550}. However, the

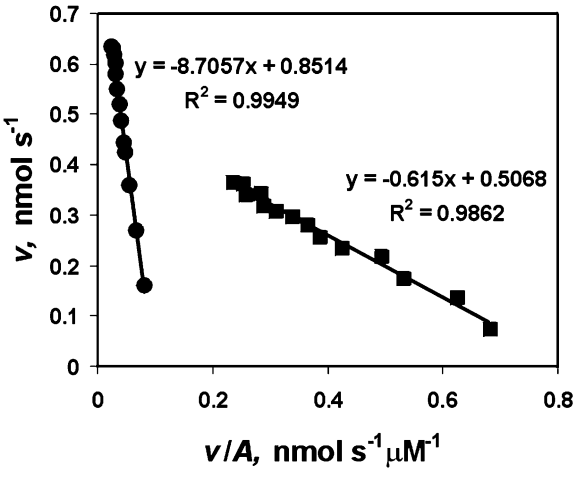

Fig. 4. Cytochrome c_{550} (squares) or pseudoazurin (circles)-mediated reduction of nitrite by succinate in the mixture of membranes and nitrite reductase from *P. denitrificans*. The samples contained, in a total volume of 0.5 ml, 20 mM Tris chloride, pH 7.3, 30 °C, 10 mM sodium succinate, 0.76 mg of membrane protein, 40.5 or 48.5 pmol of nitrite reductase and various concentrations of the mediator protein. The rate of nitrite consumption was measured immediately upon addition of 10 μl of 50 mM NO_2^- as outlined in the Experimental section. The results are presented in a form of the Eadie–Hofstee plot. Displayed are the equations for the regression lines and the squares of the correlation coefficients. The slopes of the lines equal to $-A_{0.5}$ and the intercepts on the ordinate give V.

K_m value for the reduced cytochrome c_{550} was found to exceed the $A_{0.5}$ value in Fig. 4 by over an order of magnitude. Possible reason for this discrepancy include: (i) intrinsic interspecies differences, (ii) differences in ionic strength, which adversely influences interactions between the electron-transfer components [4,9], (iii) specific effects of the involved membrane vesicles (binding of the soluble proteins, removal of the accumulating nitric oxide by virtue of the membrane-bound nitric oxide reductase). Clearly, validity of (iii) would mean that kinetic data obtained with recyclisation systems are more relevant to the actual situation in intact cells than data form one-enzyme studies.

4. Conclusions

The main result obtained in this work consists in clarifying the kinetic behaviour of a two-enzyme system at increasing concentration of the recycling substrate and finding the exact physical meaning of the half-saturation concentration. It is demonstrated that this quantity can be easily measured under the specific condition of equal activities of both enzymes, which leads to a hyperbolic law familiar in classical enzymology. When obtained in this manner, the half-saturation concentration reaches its upper limit and is equal to the sum of both Michaelis constants. Use of cycling assays enables simple comparative studies of various substrates and is especially recommendable if the kinetic studies of individual enzymes are hampered by low Michaelis constants.

Acknowledgments

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