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ФЕНОМЕНОЛОГИЧЕСКАЯ МОДЕЛЬ СОПРЯЖЕННОГО ТРАНСПОРТИРОВАНИЯ ИОНОВ K^+, Na^+ И Cl^- ЧЕРЕЗ ПЛАЗМАТИЧЕСКУЮ МЕМБРАНУ КЛЕТКИ

З. Федорович, Э. Личковский

Львовский национальный медицинский университет имени Даниила Галицкого
ул. Пекарская, 69, Львов 79010, Украина
e-mail: zoryana.ivanytska@gmail.com

Разработана модель плотности потоков ионов калия, натрия, хлора, ионного баланса и трансмембранного потенциала через плазматическую мембрану зародышевой клетки с позиции линейной термодинамики необратимых процессов. Модель описывает распределение ионов калия, натрия, хлора во внутриклеточной и внеклеточной средах. В модели учитывается влияние трансмембранного потенциала на динамику плотности потоков ионов и концентраций ионов. В численном эксперименте исследованы зависимости плотности потоков ионов от их концентраций. Показано, что решение модели качественно согласуется с экспериментальными исследованиями.

Ключевые слова: математическая модель, ионный баланс клетки, плотности потоков ионов, ион-транспортные системы.

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KINETIC CHARACTERISTICS OF PYROPHOSPHATASE OF THE SULFATE-REDUCING BACTERIA FROM HUMAN INTESTINE

I. Kushkevych

Institute of Animal Biology of NAAS of Ukraine
38, Vasyl Stus St., Lviv 79034, Ukraine
e-mail: ivan.kushkevych@gmail.com

Kinetic characteristics of pyrophosphatase in cell-free extracts of sulfate-reducing bacteria (*Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9) isolated from human intestine were studied. The enzyme activity, initial (instantaneous) reaction rate (V_0), maximum amount of the product of reaction (P_{max}), the reaction time (half saturation period, τ), and maximum rate of the pyrophosphatase reaction (V_{max}) in both strains *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were defined. Michaelis constants (K_m) of the studied enzyme reaction (2.53±0.27 mM for *D. piger* Vib-7 and 2.60±0.21 mM for *Desulfomicrobium* sp. Rod-9) were calculated.

Keywords: pyrophosphatase activity, kinetic analysis, sulfate-reducing bacteria, intestinal microbiocenosis, inflammatory bowel diseases.

The initial steps in the dissimilatory reduction of sulfate in the sulfate-reducing bacteria can be summarized by the following equations [1, 7]:

$$ATP + SO_4^{2-} \leftrightarrow APS + PP_i; \quad PP_i + H_2O \rightarrow 2P_i; \quad APS + 2\bar{e} \leftrightarrow AMS + SO_3^{2-}.$$

Pyrophosphatase (or inorganic pyrophosphatase) is an enzyme that catalyzes the conversion of one molecule of pyrophosphate to two phosphate ions [3, 7]. Inorganic pyrophosphate (PP_i) is produced in various reversible nucleoside 5'-triphosphatedependent reactions, which presumably are pulled in the biosynthetic direction *in vivo* by enzymatic hydrolysis of PP, catalyzed by inorganic pyrophosphatase (EC 3.6.1.1; hereafter referred to as PPase). In addition, PP_i, is formed, like ATP, by photophosphorylation, oxidative phosphorylation, and glycolysis [11].

In literature, there are a lot of data about pyrophosphatase of the sulfate-reducing bacteria isolated from environment [1, 3, 7, 11, 14]. However, the data on activity and the kinetic properties of this enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. has not been reported yet.

The aim of our work was to study pyrophosphatase activity in cell-free extract of intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 and to carry out the kinetic analysis of enzymatic reaction.

Materials and Methods

Objects of the study were sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and identified [9, 10].

Obtaining cell-free extracts. Cells were harvested at the beginning of the stationary phase, suspended at +4°C in buffer containing 50 mM Tris·HCl (pH 8.0), and passed through a Manton-Gaulin press at 9,000 psi. The extracts were centrifuged at +4°C for 10 min at 15,000*g*; the pellet was then used as sedimentary fraction, and the supernatant obtained was termed the soluble fraction [4]. This extract was subjected to further centrifugation at 180,000*g* for 1 h to eliminate the membrane fraction. A clear supernatant, containing the soluble fraction, was then used as cell-free extract. Protein concentration in the cell-free extracts was determined by the Lowry method [12].

Assays for pyrophosphatase activity. Enzyme assays were performed by using cell-free extracts, soluble and sedimentary fraction. The enzyme was assayed by Akagi and Campbell as described in paper [1]. Pyrophosphatase activity was measured by determining the amount of inorganic phosphate liberated from sodium pyrophosphate after 10 min of incubation at +30°C. The reaction mixture contained: $MgCl_2$, 5 μmoles; sodium pyrophosphate, 5 μmoles; Tris buffer (pH 8.0), 50 μmoles; and enzyme in a final volume of 0.7 ml. At the end of the incubation period, the reaction was stopped by the addition of 1.0 ml of 5 N H_2SO_4. Any turbidity formed was removed by centrifugation prior to reading the tubes in a spectrophotometer. The level of inorganic phosphorus (P_i) was measured spectrophotometrically in the obtained supernatant without protein. The amount of reaction products were determined by Rathbun and Betlach (1969) [13]. One unit of enzyme is defined as that amount

which produces 1 μmoles of inorganic phosphate under the conditions specified. Specific enzyme activity was expressed as U×mg^{-1} protein. The activity of the enzyme in the cell-free extracts of both strains under the effect of different temperature and pH in the incubation medium were measured.

Kinetic analysis. Kinetic analysis of the enzyme reaction was performed in a standard incubation medium (as it was described above) with modified physical and chemical characteristics of the respective parameters (the incubation time, substrate concentration, temperature and pH). The kinetic parameters characterizing the pyrophosphatase reaction are the initial (instantaneous) reaction rate (V_0), maximum rate of the reaction (V_{max}), maximum amount of the reaction product (P_{max}) and characteristic reaction time (time half saturation) τ were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing pyrophosphatase reactions are Michaelis constant (K_m) and maximum reaction rate of substrate composition were determined by Lineweaver-Burk plot [6].

Statistical analysis. Kinetic and statistical calculations of the results were carried out using the Microsoft Excel and Origin computer programs. The research results were treated by the methods of variation statistics using Student *t*-test. The equation of the straight line that the best approximates the experimental data was calculated by the method of least squares. The absolute value of the correlation coefficient *r* was from 0.90 to 0.98. The significance of the calculated parameters of line was tested by the Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ [2].

Results and Discussion

Activity of pyrophosphatase was measured in different fractions (cell-free extract, soluble, and sedimentary) obtained from bacterial cells (Table 1). The highest specific activity of the enzyme was 24.27±2.47 and 8.16±0.82 U×mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively, in cell-free extract. Much lower activity of pyrophosphatase for both bacterial strains was determined in soluble fraction compared to the cell-free extracts. Its values designated 5.32±0.56 U×mg^{-1} protein for *D. piger* Vib-7 and 1.54±0.15 U×mg^{-1} protein for *Desulfomicrobium* sp. Rod-9. There was not observed enzyme activity in sedimentary fraction. Perhaps, the lower level of enzyme activity in soluble fraction indicates that phosphate was not fully released in the fraction and may still be in a bound state with some compounds.

Table 1

Pyrophosphatase activity in different fractions obtained from bacterial cells

Sulfate-reducing bacteria	Specific activity of pyrophosphatase (U×mg^{-1} protein)		
	Cell-free extract	Individual fractions	
		Soluble	Sedimentary
Desulfovibrio piger Vib-7	24.27±2.47	5.32±0.56	0
Desulfomicrobium sp. Rod-9	8.16±0.82***	1.54±0.15***	0

Comment: The assays were carried out at a protein concentration of 48.12 mg/ml (for *D. piger* Vib-7) and 43.75 mg/ml (for *Desulfomicrobium* sp. Rod-9). Enzyme activity was determined after 10 min incubation. Statistical significance of the values M±m, n=3; ***P<0.001, compared to *D. piger* Vib-7 strain.

The effect of temperature and pH of the incubation medium on the pyrophosphatase activity in the cell-free extracts of the sulfate-reducing bacteria was studied (fig. 1). The enzyme activity exhibited typical bell-shaped curves as a function of temperature and pH. The maximum specific activity for both bacterial strains was determined at +35ºC.

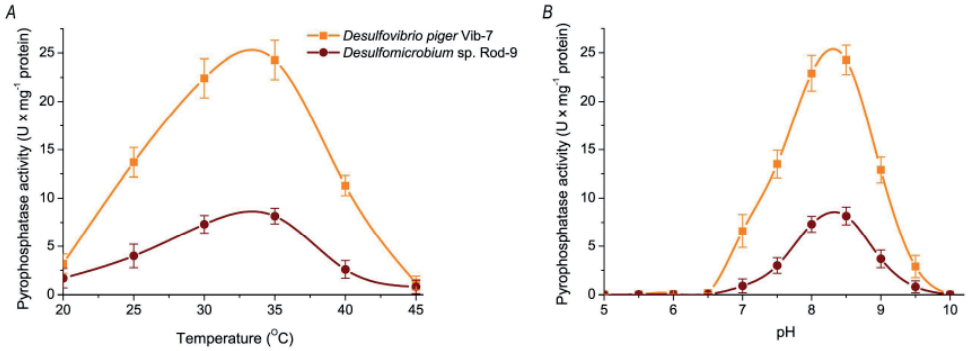

Fig. 1. Effect of temperature (*A*) and pH (*B*) on pyrophosphatase activity in the cell-free extracts

An increase or decrease in temperature of incubation leads to a decrease of the activity of studied enzyme in the cell-free bacterial extracts. The highest enzyme activity of pyrophosphatase was determined in the cell-free extracts of both strains at pH 8.0…8.5.

To study the characteristics and mechanism of pyrophosphatase reaction, the initial (instantaneous) reaction rate (V_0), maximum rate of the reaction (V_{max}), maximum amount of reaction product (P_{max}) and reaction time (τ) were defined. Dynamics of inorganic phosphate accumulation in the cell-free extracts was studied for investigation of the kinetic parameters of pyrophosphatase (fig. 2).

Experimental data showed that the kinetic curves of pyrophosphatase activity have tendency to saturation (fig. 2, *A*). Analysis of the results allows to reach the conclusion that the kinetics of enzyme activity in the studied bacteria was consistent to the zero-order reaction in the range of 0–3 min (the graph of the dependence of product formation on the incubation time was almost linear in this interval of time). Therefore the duration of the incubation of extracts was 3 min in subsequent experiments.

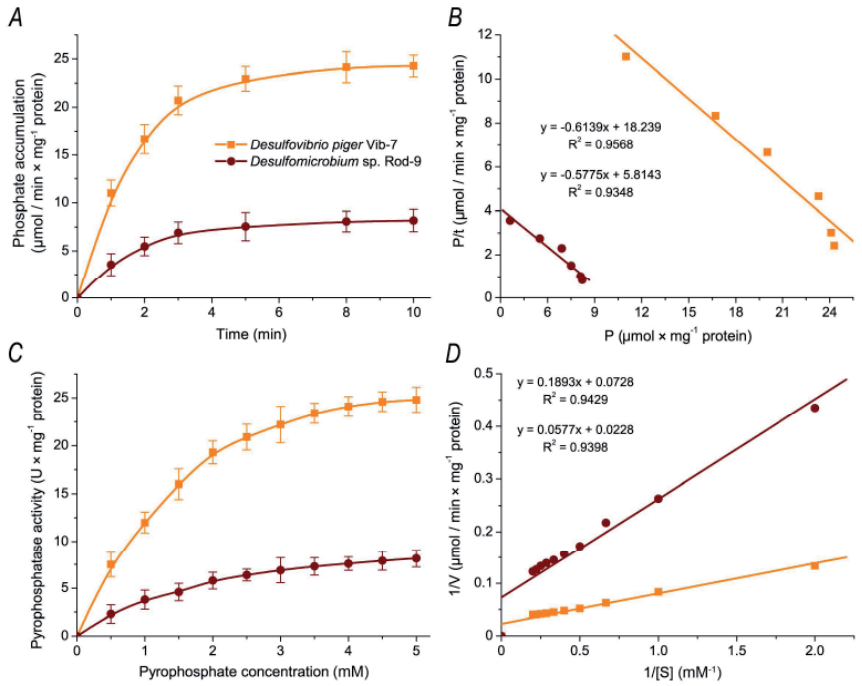

Fig. 2. Kinetic parameters of pyrophosphatase activity in cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9: ***A*** – dynamics of product accumulation (M±m, n=3); ***B*** – linearization of curves of product accumulation in {P/t; P} coordinates (n=3; R^2>0.9; F<0.02); ***C*** – the effect of different substrate concentrations on pyrophosphatase activity (M±m, n=3); ***D*** – linearization of concentration curves, which are shown in fig. 2***C***, in the Lineweaver-Burk plot, where V is rate of pyrophosphatase reaction and S is substrate concentration (n=3; R^2>0.93; F<0.005).

Amount of product of pyrophosphatase reaction was higher (in three times) in the *D. piger* Vib-7 compared to the *Desulfomicrobium* sp. Rod-9 in the entire range of time factor. The basic kinetic properties of the reaction in the cell-free extracts of the sulfate-reducing bacteria were calculated by linearization of the data in the {P/t; P} coordinates (fig. 2*B*, table 2).

Table 2

Kinetic parameters of the phosphate accumulation in the cell-free extracts of bacterial strains

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
V_0 (μmol/min×mg^{-1} protein)	18.24±1.92	5.81±0.52***
P_{max} (μmol×mg^{-1} protein)	29.71±3.17	10.07±1.12***
τ (min)	1.62±0.15	1.73±0.18

Comment: V_0 is initial (instantaneous) reaction rate; P_{max} is maximum amount (plateau) of the product of reaction; τ is the reaction time (half saturation period). Statistical significance of the values M±m, n=3; ***P<0.001, compared to the *Desulfovibrio piger* Vib-7 strain.

The kinetic parameters of pyrophosphatase in cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different. Values of initial (instantaneous) reaction

rate (V_0) for pyrophosphatase activity in both bacterial strains was calculated by the maximum amount of the product reaction (P_{max}). Initial rate for pyrophosphatase reaction was higher in the *D. piger* Vib-7 (18.24±1.92 μmol/min×mg^{-1} protein) compared to *Desulfomicrobium* sp. Rod-9 (5.81±0.52 μmol/min×mg^{-1} protein). Based on these data, there is assume that the *D. piger* Vib-7 can consume pyrophosphate faster (approximately three times) in their cells than *Desulfomicrobium* sp. Rod-9. Moreover, this hypothetical assumption can be also confirmed by obtained data on the product accumulation in enzymatic reaction, its maximum values designated 29.71±3.17 μmol×mg^{-1} protein for *D. piger* Vib-7 and 10.07±1.12 μmol×mg^{-1} protein for *Desulfomicrobium* sp. Rod-9. However, the reaction time (half saturation period) for pyrophosphate reduction was almost similar in both bacterial strains. The intracellular PP, concentration depends mainly on the activity of PPase in the cells. Hence factors which affect the intracellular location or total activity of PPase influence secondarily all reactions in which PP, has a role as a substrate or regulator. Changes in the state of PPase thus have a wide effect on the general metabolism [11].

The kinetic analysis of pyrophosphatase activity depending on the substrate concentration was carried out. According to the obtained results, increasing of sulfite concentrations from 0.5 to 5.0 mM causes a monotonic rise of the studied enzyme activity and the activity was maintained on unchanged level (plateau) under substrate concentrations over 5.0 mM (fig. 2*C*). Curves of the dependence {1/V; 1/[S]} were distinguished by the tangent slope and intersect the vertical axis in one point (fig. 2*D*). The basic kinetic parameters of pyrophosphatase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were identified. The kinetic parameters indicate that the maximum rate (V_{max}) of product accumulation in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was significantly different to each other. The maximum rate of the enzyme reaction was designated 43.86±4.24 μmol/min×mg^{-1} protein for *D. piger* Vib-7 and 13.74±1.32 μmol/min×mg^{-1} protein for *Desulfomicrobium* sp. Rod-9 strain. However, Michaelis constants (K_m) for enzyme reaction in both bacterial strains were similar: 2.53±0.27 and 2.60±0.21 mM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively (Table 3).

Table 3

Kinetic parameters of pyrophosphatase activity depending on pyrophosphate concentration in the incubation medium

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
V_{max} (μmol/min×mg^{-1} protein)	43.86±4.24	13.74±1.32***
K_m (mM)	2.53±0.27	2.60±0.21

Comment: V_{max} is maximum rate of the enzyme reaction; K_m is Michaelis constant which was determined by the substrate. Statistical significance of the values M±m, n=3; ***P<0.001, compared to the *Desulfovibrio piger* Vib-7 strain.

Inorganic pyrophosphatase was purified from *Desulfovibrio desulfuricans* and described by J.M. Akagi and L.L. Campbell [1]. The authors have shown that enzymatic activity in sonically disrupted *D desulfuricans* cells was 5.5 U×mg^{-1} protein. The pH optimum (8.0…8.5) for pyrophosphatase activity in *D. desulfuricans* was also consistent to our results for both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains**.** This pH optimum corresponds to conditions which are present in the human large intestine from where the bacterial strains were isolated. Perhaps, such conditions provide their intensive development in the gut. The K_s (K_m), for the pyrophosphatase of *D. desulfuricans* was found to be in the region of 1.9×10^{-3} M that is also similar to the obtained results. Moreover, the pyrophosphatase activity in *D. piger* Vib-7 was similar to the enzyme activity in *D. desulfuricans* previously described by D.A. Ware and J.R. Postgate [14]. In their paper, the enzyme activity was 19 U/mg of protein in cell-free extract from *D. desulfuricans.*

Pyrophosphate, serves as a source of energy for several reactions in the sulfate-reducing bacteria. It also regulates many enzymes without actually participating in the reactions. In addition, recent discoveries have suggested that PPase might have important roles not only in the regulation of macromolecular synthesis and growth, but also in evolutionary events by affecting the accuracy by which DNA molecules are copied during chromosome duplication [11].

Inorganic pyrophosphatases as a substrate, which has three mechanisms was described by Kajander T. et al. (2013). Soluble inorganic pyrophosphatases catalyse an essential reaction, the hydrolysis of pyrophosphate to inorganic phosphate. In addition, an evolutionarily ancient family of membraneintegral pyrophosphatases couple of this hydrolysis to Na^+ and/or H^+ pumping, and so recycle some of the free energy from the pyrophosphate. The structures of the H^+-pumping mung bean PPase and the Na^+-pumping *Thermotoga maritima* PPase revealed an entirely novel membrane protein containing 16 transmembrane helices. The hydrolytic centre, well above the membrane, is linked by a charged “coupling funnel” to the ionic gate. By comparing the active sites, fluoride inhibition data and the various models for ion transport, Kajander et al. (2013) have concluded that membrane-integral PPases probably use binding of pyrophosphate to drive pumping [5].

In summary, we have concluded that the pyrophosphatase activity, initial (instantaneous) and maximum reaction rate, maximum amount of the product of enzyme reaction were significantly higher in the *D. piger* Vib-7 cells compared to the *Desulfomicrobium* sp. Rod-9 strain. The maximum pyrophosphatase activity for both strains was determined at +35ºC and at pH 8.0…8.5 that corresponds to conditions in the human large intestine from where the bacterial strains were isolated. According to the obtained results, the studies of the kinetic characteristics of pyrophosphatase in process sulfate reduction in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 can be perspective for clarification of the etiological role of these bacteria in the development of the bowel diseases.

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КІНЕТИЧНІ ХАРАКТЕРИСТИКИ ПІРОФОСФАТАЗИ СУЛЬФАТВІДНОВЛЮВАЛЬНИХ БАКТЕРІЙ КИШЕЧНИКА ЛЮДИНИ

І. Кушкевич

Інститут біології тварин НААН України
вул. В. Стуса, 38, Львів 79034, Україна
e-mail: ivan.kushkevych@gmail.com

Досліджено кінетичні властивості пірофосфатази у безклітинних екстрактах сульфатвідновлювальних бактерій (*Desulfovibrio piger* Vib-7 і *Desulfomicrobium* sp. Rod-9), ізольованих з кишечника людини. Визначено активність ферменту, початкову (миттєву) швидкість реакції (V_0), максимальну кількість продукту реакції (P_{max}), час реакції (період напіврозпаду (насиченість), τ) і максимальну швидкість пірофосфатазної реакції (V_{max}) в обох штамів *D. piger* Vib-7 і *Desulfomicrobium* sp. Rod-9. Розраховано константи Міхаеліса (K_m) досліджуваної ферментативної реакції (2,53±0,27 мМ для *D. piger* Vib-7 і 2,60±0,21 мМ для *Desulfomicrobium* sp. Rod-9).

Ключові слова: активність пірофосфатази, кінетичний аналіз, сульфатвідновлювальні бактерії, кишкові мікробіоценози, запальні захворювання кишечника.

КИНЕТИЧЕСКИЕ ХАРАКТЕРИСТИКИ ПИРОФОСФАТАЗЫ СУЛЬФАТВОССТАНАВЛИВАЮЩИХ БАКТЕРИЙ КИШЕЧНИКА ЧЕЛОВЕКА

И. Кушкевич

Институт биологии животных НААН Украины
ул. В. Стуса, 38, Львов 79034, Украина
e-mail: ivan.kushkevych@gmail.com

Исследовано кинетические свойства пирофосфатазы в бесклеточных экстрактах сульфатвосстанавливающих бактерий (*Desulfovibrio piger* Vib-7 и *Desulfomicrobium* sp. Rod-9), изолированных из кишечника человека. Определено активность фермента, начальною (мгновенною) скорость реакции (V_0), максимальное количество продукта реакции (P_{max}), время реакции (период полураспада (насыщенность), τ) и максимальную скорость пирофосфатазнои реакции (V_{max}) в обоих штаммов *D. piger* Vib-7 и *Desulfomicrobium* sp. Rod-9. Определено константы Михаэлиса (K_m) исследуемой ферментативной реакции (2,53±0,27 мМ для *D. piger* Vib-7 и 2,60±0,21 мМ для *Desulfomicrobium* sp. Rod-9) .

Ключевые слова: активность пирофосфатазы, кинетический анализ, сульфатвосстанавливающие бактерии, кишечные микробиоценозы, воспалительные заболевания кишечника.