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# Comparative Kinetic Study of D-Glucose Oxidation by Ruthenium(III) Compounds Catalyzed by FAD-Dependent Glucose Oxidase and PQQ-Dependent Glucose Dehydrogenase

**E. V. Ivanova[1]*, A. Yu. Ershov[2], V. Laurinavičius[3], R. Meskus[3], and A. D. Ryabov[1]**

*[1]School of Chemistry, Lomonosov Moscow State University, Moscow 119899, Russia; fax: (095) 254-2179; E-mail: ivanka@enzyme.chem.msu.ru*
*[2]Department of Chemistry, St. Petersburg State University, St. Petersburg 198904, Russia*
*[3]Institute of Biochemistry, ul. Mokslininku 12, Vilnius 2600, Lithuania*

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**Abstract**—The comparative kinetic study of two glucose oxidizing enzymes, FAD-dependent glucose oxidase and PQQ-dependent glucose dehydrogenase, is presented in the artificial electron transfer mediator system based on ruthenium(III) compounds. It is demonstrated that FAD-dependent glucose oxidase and PQQ-dependent glucose dehydrogenase follow Michaelis kinetics in the D-glucose/ruthenium(III) system. PQQ-dependent glucose dehydrogenase is more active than FAD-dependent glucose oxidase in the process of D-glucose oxidation by ruthenium(III) compounds, this being due to the different catalytic mechanisms of these enzymes.

*Key words*: D-glucose, ruthenium compounds, ruthenacycles, PQQ-dependent GDH

Enzyme active sites are usually located inside the protein globule, this hampering direct electron transfer from the active site on a biosensor detector. Thus, redox mediators are employed to achieve efficient electron transport paths from the enzyme active site onto the electrode. The ideal mediator of electron transport is usually a low-molecular-weight compound with a redox potential close to that of the enzyme active site [1, 2]. This avoids side oxidation/reduction processes on the electrode.

Transition metal compounds have already found a wide application as mediators of electron transport [3, 4]. Recent studies performed in our laboratory revealed that ruthenium(II/III) and osmium(II/III) complexes are efficient in the coupled oxidation of D-glucose in the presence of FAD-dependent glucose oxidase (GO) [5, 6]. The coordinative insertion of a ruthenium label into the GO protein globule significantly increased the catalytic currents in a glucose/modified GO/electrode system [6, 7].

The ruthenium mediators presented in this work can be divided into two groups. The first group consists of complexes with the common formula $[Ru(LL)_2X_2]$, where LL are ligands of 2,2′-bipyridine type or 1,10-phenanthroline and X are acido-ligands (Scheme 1a). The second group of substrates is presented by a new class of ruthenium-organic compounds, the cyclometallated derivatives of 2-phenylpyridine [8], containing a metal–carbon covalent bond (Scheme 1b). The principal difference of a second group is lowered redox-potential as well as high rates of self-exchange rate constants [8]. The latter provides high reactive ability of ruthenacycles in the enzymatic processes.

Investigation of ruthenium(III) interaction kinetics with D-glucose oxidizing enzymes is important both for practical tasks (development and improvement of mediator biosensors) as well as for fundamental issues (similarity and distinction of kinetic behavior and mechanisms of these biocatalysts differing in active site structure).

First mentioned in the literature in 1960, the story of quinoprotein research begins with the characterization of a new prosthetic group discovered by Hauge [9] in methanol dehydrogenase, containing a quinoid structure and two nitrogen atoms [10, 11]. The term "quinoproteins" itself [12] includes all proteins containing pyrroloquinoline-quinone prosthetic group [13]. The pyrroloquinoline-quinone-dependent (PQQ-dependent) glucose dehydro-

*Abbreviations*: GO) glucose oxidase; Gl) D-glucose; GDH) glucose dehydrogenase; PQQ) pyrroloquinoline quinone; phen) 1,10-phenanthroline; phpy) 2-phenylpyridine.

* To whom correspondence should be addressed.

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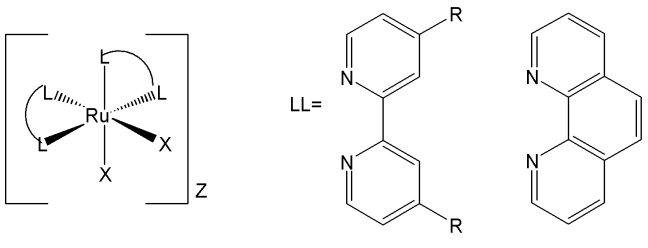
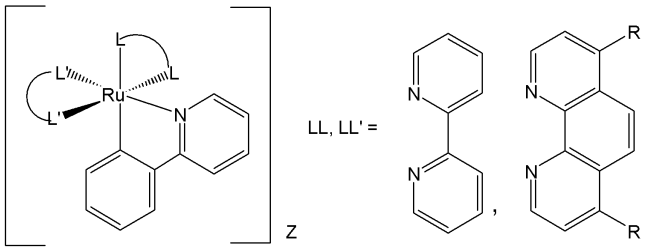
a




| | LL | R | X | Z |
|---|---|---|---|---|
| **1a** | bpy | H | $Cl^-$ | |
| **2a** | bpy | H | $1/2CO_3^{2-}$ | |
| **3a** | bpy | H | $SCN^-$ | |
| **4a** | bpy | H | $H_2O$ | $PF_6^-$ |
| **5a** | bpy | Me | $Cl^-$ | |
| **6a** | bpy | Me | $Cl^-$ | $PF_6^-$ |
| **7a** | phen | H | $Cl^-$ | |
| **8a** | phen | H | $1/2CO_3^{2-}$ | |

b




| | LL | LL' | R | Z |
|---|---|---|---|---|
| **1b** | bpy | bpy | H | $PF_6^-$ |
| **2b** | bpy | phen | H | $PF_6^-$ |
| **3b** | phen | phen | H | $PF_6^-$ |
| **4b** | phen | phen | Me | $PF_6^-$ |

**Scheme 1**

genase (GDH, EC 1.1.99.17) discovered 10 years ago [13] is a typical representative of the new class of quinoenzymes [14, 15]. GDH transfers electrons from glucose onto the non-oxygen type of oxidizing substrates [13]. Natural substrates of this enzyme are ubiquinones formed in the cell membrane [16]. PQQ-dependent GDH is characterized by high thermostability [17] along with the high number of redox cycles performed [18]. The aim of the present study was the construction of an artificial reversible redox system based on various ruthenium compounds for the development and optimization of oxygen insensitive biosensors.

## MATERIALS AND METHODS

Solutions of Ru(III) compounds for investigating the interactions between the oxidized form of ruthenium and glucose were prepared immediately before the measurements. $H_2O_2$ at the concentration of $2 \cdot 10^{-4}$ M was added to the solution of a corresponding $[Ru^{II}(LL)_2X_2]$ complex, to give the working $H_2O_2$ concentration of $1 \cdot 10^{-4}$ M. Oxidation of ruthenium compounds by hydrogen peroxide was initiated by adding a solution of horseradish peroxidase (Sigma, USA) to enzyme concentration of $1 \cdot 10^{-8}$ M [19]. The peroxidase was characterized by R/Z ratio of 1.5. The oxidation reaction catalyzed by peroxidase was monitored spectrophotometrically at a wavelength corresponding to maximum absorption of the ruthenium(II) compounds (Table 1).

Complexes (**1**-**7**)**a** were synthesized as described in literature [20-22]. The synthesis of ruthenium cyclometalated complexes (**1**-**4**)**b** was described earlier [8].

GO from *Aspergillus niger* (EC 1.1.3.4) had an activity of 250 units/mg (Serva, Germany) and was used without additional purification. Stock solutions of GO were stored in a freezer. PQQ-dependent GDH from *Erwinia* sp. was extracted according to [17]. The enzyme preparation was stored in 0.02 M phosphate buffer (pH 7.0) containing 10% glycerol; PQQ-dependent GDH had specific activity 12 units/mg.

**Table 1.** Spectral characteristics of the investigated complexes. Spectra were recorded at 30°C, pH 7.3, in 0.01 M potassium phosphate buffer

| Complex | $\lambda_{max}$, nm | $\varepsilon \times 10^{-3}$, $M^{-1} \cdot cm^{-1}$ |
|---|---|---|
| **1a** | 490 | 7.41 ± 0.03 |
| **2a** | 484 | 6.83 ± 0.01 |
| **3a** | 485 | 7.69 ± 0.05 |
| **4a** | 484 | 5.05 ± 0.06 |
| **5a** | 484 | 2.76 ± 0.03 |
| **6a** | 480 | 3.52 ± 0.01 |
| **7a** | 470 | 7.98 ± 0.03 |
| **1b** | 494 | 8.71 ± 0.07 |
| **2b** | 484 | 9.50 ± 0.04 |
| **3b** | 480 | 12.52 ± 0.08 |
| **4b** | 485 | 12.98 ± 0.09 |

Solutions of D-glucose (ICN, USA) were prepared the day before the experiments to reach the equilibrium between α- and β-anomers.

Spectrophotometric measurements were performed using a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A thermostat. All calculations were performed using software packages SigmaPlot 4.0 and 7.0.

**Kinetic experiments.** The reaction rates were monitored spectrophotometrically within the wavelength range of 470-490 nm, which corresponds to the maximum absorbance of Ru(II) [19]. The tangent slope of the linear range of a kinetic curve was taken as the initial reaction rate, which was calculated according the equation:

$$V_0 = \Delta A/\varepsilon l t, \quad (1)$$

where $\Delta A$ is an increment of optical density during the time $t$, ε molar absorption coefficient for ruthenium(II) compounds, and $l$ optical pathlength (1 cm).

To determine the rate of D-glucose enzymatic oxidation by ruthenium(III) compounds, 60 μl of 1 M glucose solution were added to a cuvette containing a solution of **1a** at the concentration of $2 \cdot 10^{-4}$ M. The final concentration of glucose in the cuvette was 0.03 M. The electronic spectrum (range of 300-800 nm) of **1a** in the absence of enzyme remained unchanged for 2 h. Dramatic increase in optical density at the corresponding wavelength was observed after the addition of GO or GDH.

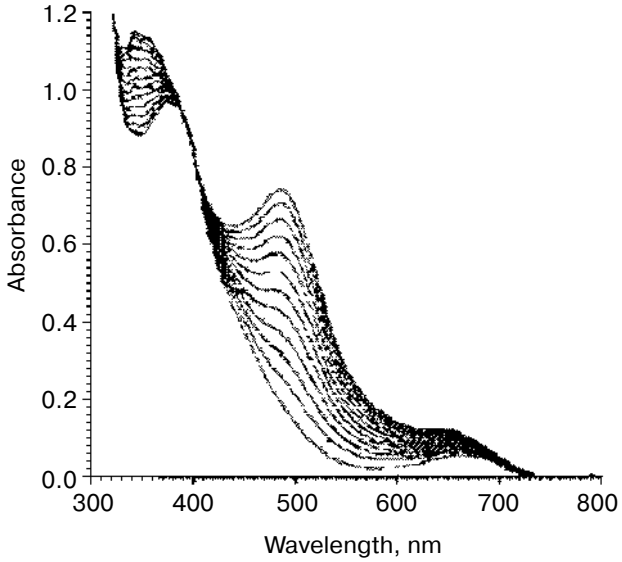



**Fig. 1.** Increase in solution optical density caused by **1a** reduction by D-glucose in the presence of GDH. Spectra were recorded every 5 min; [**1a**] = $2 \cdot 10^{-4}$ M, [D-glucose] = 0.02 M, [GDH] = $1.13 \cdot 10^{-8}$ M, 0.01 M phosphate buffer, pH 7.0, 30°C.

## RESULTS AND DISCUSSION

**Stoichiometry of D-glucose oxidation by ruthenium(III) compounds in the presence of GO or GDH.** Enzymatic oxidation of D-glucose by ruthenium(III) compounds is accompanied by changes in the visible spectral range due to the reduction of ruthenium(III) complexes to ruthenium(II) (Fig. 1). The increase in optical density at 490 nm corresponds to the accumulation of Ru(II). The existence of an isobestic point at 412 nm (**1a**) indicates that the solution contains only oxidized and reduced forms of ruthenium and no other side products.

To determine the stoichiometry of D-glucose oxidation by Ru(III) in the presence of GO and GDH, aliquots of D-glucose were added to a solution, containing enzyme and **1a**, to ensure glucose deficiency compared to **1a**. Figure 2 illustrates the dependency of optical density of oxidized **2a** complex on time during consecutive additions of glucose. Each stair step on Fig. 2 is initiated by addition of an additional portion of D-glucose.

The dependency of the amount of ruthenium(II) formed on total amount of added D-glucose is presented on Fig. 2, inset. It is a straight line with a slope close to 2: 2.3 ± 0.2 in the case of GDH, or 1.8 ± 0.1 if GO was employed as the catalyst. Hence, it can be assumed that two mediator molecules are involved in the oxidation of one D-glucose molecule according to Eq. (2). The lower value in the case of GO is probably explained by concurrent participation of di-oxygen in the oxidation of a reduced GO form.

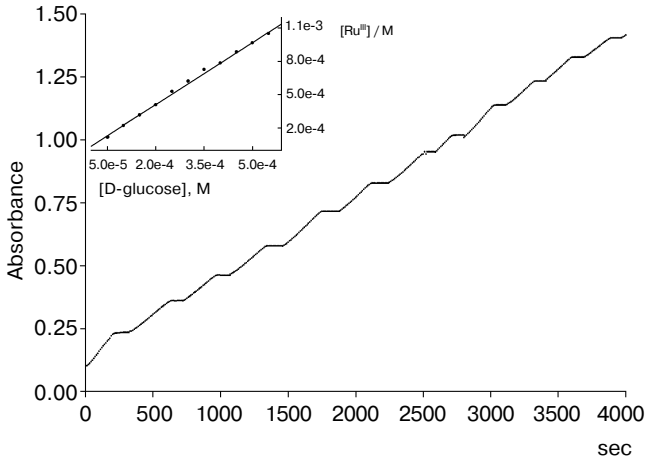



**Fig. 2.** Increase in the optical density caused by the addition of 50 μM D-glucose aliquots into the cuvette. [**3a**] = $2 \cdot 10^{-4}$ M, [GO] = $1.13 \cdot 10^{-6}$ M, pH 7.0, 0.01 M phosphate buffer, 30°C. Inset: the dependence of Ru(III) concentration on D-glucose concentration: [GO] = $1.13 \cdot 10^{-6}$ M, pH 7.0, 0.01 M phosphate buffer, 30°C.

$$2[Ru^{III}(LL)(LL')XY] +$$

$$+ \text{D-glucose} \xrightarrow{\text{GO or GDH}} 2\,[Ru^{II}(LL)(LL')XY] +$$

$$+ \delta\text{-D-gluconolactone} + 2H^+. \qquad (2)$$

**Kinetics of GO catalyzed D-glucose oxidation by ruthenium(III) complexes.** Ru(III) is not reduced by D-glucose in the absence of GO. The influence of GO on initial reduction rate of ruthenium(III) complexes by D-glucose was investigated in the range of $1\cdot10^{-6}$-$1\cdot10^{-9}$ M of **1a** concentrations. The dependence was linear.

To investigate the influence of D-glucose and Ru(III) on reaction (2) kinetics and elucidate the mechanism of GO catalyzed oxidation of D-glucose by ruthenium(III) compounds, the dependence of initial reaction rate on ruthenium(III) concentration at various glucose concentration was studied. The obtained three-dimensional graph is presented in Fig. 3. Qualitative analysis reveals that in the range of D-glucose concentrations between 0.05 and 3 mM the dependence of initial reaction rate on Ru(III) concentration always follows Michaelis kinetics. The value of the $V_{max}/K_m^{Ru}$ ratio is not dependent on D-glucose concentration within the investigated range. The dependence of reaction (2) rate on D-glucose concentration at Ru(III) concentrations of 20 to 100 μM is also of Michaelis type. Similar data were earlier observed by us while using ferricinium $[(\eta^5\text{-}C_5H_5)_2Fe^+]$ cations as D-glucose oxidant [23] in the presence of GO. Since the value of the $V_{max}/K_m^{Ru}$ ratio is not dependent on D-glucose concentration [6], the quantitative interpolation of the data is given within the framework of the "ping-pong" mechanism illustrated in Scheme 2.

$$\text{E(ox.)} + \text{D-glucose} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \{\text{E(ox.), D-glucose}\},$$

$$\{\text{E(ox.), D-glucose}\} \xrightarrow{k_2} \{\text{E(red.)} + \delta\text{-D-glucose}\},$$

$$\text{E(red.)} + Ru^{III} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \{\text{E(red.)}, Ru^{III}\},$$

$$\{\text{E(red.)}, Ru^{III}\} \xrightarrow{k_4} \text{E(semiox.)} + Ru^{III},$$

$$\text{E(semiox.)} + Ru^{III} \xrightarrow{\text{fast}} \text{E(ox.)} + Ru^{II}.$$

**Scheme 2**

E(ox.) and E(red.) are oxidized and reduced forms of GO containing FAD and $FADH_2$, accordingly; E(semiox.) is semioxidized form of GO containing semireduced FADH cofactor

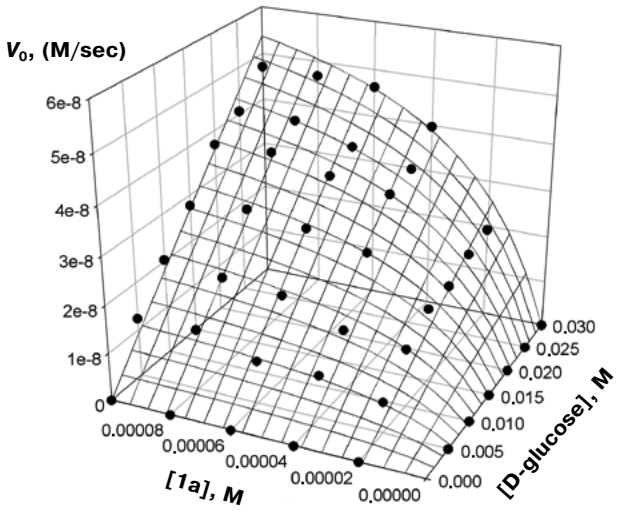



**Fig. 3.** Dependence of GO catalyzed D-glucose oxidation rate on the concentrations of **1a** and D-glucose; [GO] = $1.13\cdot10^{-9}$ M, pH 7.0, 0.01 M phosphate buffer, 30°C.

Neglecting the influence of oxygen on the reaction kinetics and assuming stationary approach to intermediate products {E(ox.), D-glucose}, {E(red.), Ru(III)} analogously to the work investigating ferricinium cation behavior in GO catalysis [23] results in Eq. (3).

$$V_0 = \frac{k_2 k_4 [E][Ru][Gl]}{k_4 K_m^{Gl}[Ru] + k_2 K_m^{Ru}[Gl] + (k_4 + k_2)[Ru][Gl]}, \qquad (3)$$

where [E], [Gl], [Ru] are total concentrations of enzyme, D-glucose, and ruthenium(III), respectively; $K_m^{Gl} = (k_{-1} + k_2)/k_1$ and $K_m^{Ru} = (k_4 + k_{-3})/k_3$.

Based on the suggested reaction scheme and using $k_2 = 520$ $sec^{-1}$ and $K_m^{Gl} = 0.27$ M values obtained earlier [23], the quantitative estimation of true kinetic parameters of GO catalyzed reaction of D-glucose oxidation by ruthenium(III) compounds was given. The effective parameters presented in Table 2 and obtained by varying ruthenium complexes concentration have the following connection with true kinetic parameters of the reaction. When [GO] $\to \infty$, Eq. (3) transforms into (4):

$$\underset{Gl\to\infty}{V_0} = \frac{k_4[E][Ru^{III}]}{K_m^{Ru} + \frac{(k_2 + k_4)}{k_2}[Ru^{III}]}, \qquad (4)$$

assuming that

$$\frac{(k_2 + k_4)}{k_2} \approx 1,$$

$$V_0 = \frac{k_4[\mathrm{E}][\mathrm{Ru}]}{(K_m^{Ru} + [\mathrm{Ru}])}. \quad (5)$$

Kinetic parameters of GO catalyzed reaction of D-glucose oxidation by different complexes were calculated from Eq. (5) (Table 2). Comparative analysis of these data demonstrated that the investigated complexes can be assigned to two groups both by structure and reactive ability. The first group (Scheme 1a) consists of complexes with a common formula $[Ru(LL)_2X_2]$, where LL are ligands of 2,2′-bipyridine type or 1,10-phenanthroline, X are acido-ligands. The more reactive second group of substrates (Scheme 1b) is represented by a new class of ruthenium-organic molecules containing a metal–carbon covalent bond. The presence of metal–carbon bonds makes the mediator molecule more rigid [8], provides high self-exchange rate constants [24] and high oxidation rates of the reduced enzyme [24].

**Influence of PQQ-dependent GDH on Ru(III) reduction rate affected by D-glucose.** Glucose oxidation was initiated by adding PQQ-dependent GDH. The dependence of initial reduction rate of Ru(III) compounds on enzyme concentration was investigated with **1a** complex as an example. The dependence is linear, pointing to the fact that one molecule of enzyme is involved in the transition state.

**Mutual interaction of GDH substrates on glucose oxidation rate by ruthenium(III) compounds.** In terms of determining the key substrate features in oxidation of D-glucose by ruthenium(III) compounds as well as the reaction mechanism in the presence of GDH, the study of reaction rate dependence on glucose concentration at different concentrations of **1a** ruthenium substrate was performed. The obtained three-dimensional dependence is presented in Fig. 4. The detailed kinetic investigation of complex formation stage {GDH, D-glucose} performed by Duine's group [14] revealed that complex formation

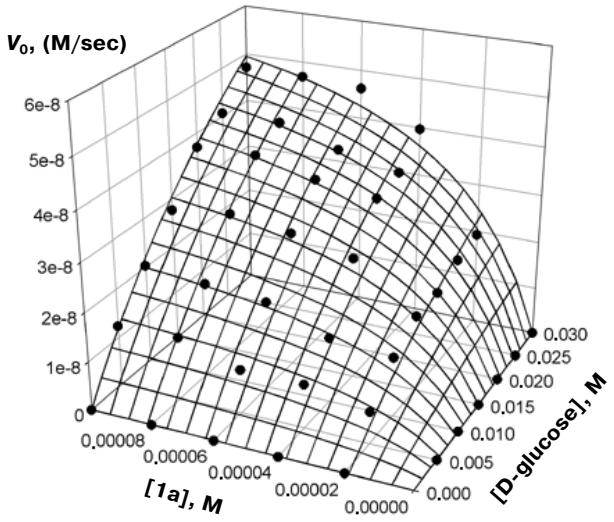


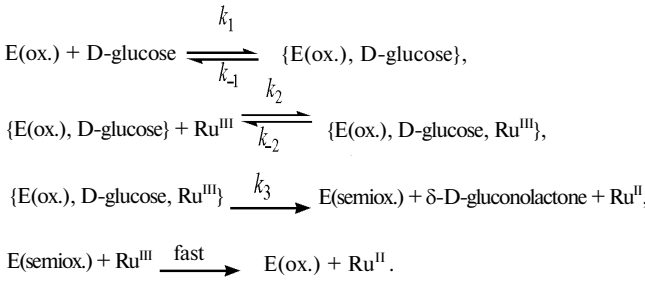

**Fig. 4.** Dependence of PQQ-dependent GDH catalyzed D-glucose oxidation rate on the concentrations of **1a** and D-glucose: [GDH] = $1.13 \cdot 10^{-8}$ M, pH 7.0, 0.01 M phosphate buffer, 30°C.

**Table 2.** Kinetic parameters of GO reduction of ruthenium(III) compounds by D-glucose (0.02 M), at 30°C, pH 7.3 (0.01 M potassium phosphate buffer), [GO] = $1.36 \cdot 10^{-8}$ M

| Complex $Ru^{III}$ | $k_4$, $sec^{-1}$ | $K_m^{Ru}$, M | $V_{max}$, $M \cdot sec^{-1}$ | $k_4/K_m$, $M^{-1} \cdot sec^{-1}$ |
|---|---|---|---|---|
| **1a** | 35.4 ± 0.3 | $(1.9 \pm 0.05) \cdot 10^{-4}$ | $(1.59 \pm 0.1) \cdot 10^{-7}$ | $(1.8 \pm 0.4) \cdot 10^5$ |
| **1a*** | | $(6.6 \pm 0.7) \cdot 10^{-3}$ | $(4.59 \pm 0.06) \cdot 10^{-6}$ | |
| **2a** | 38.6 ± 0.2 | $(9.22 \pm 0.4) \cdot 10^{-5}$ | $(1.96 \pm 0.1) \cdot 10^{-7}$ | $(4.1 \pm 0.4) \cdot 10^5$ |
| **3a** | 31.4 ± 0.8 | $(5.13 \pm 0.9) \cdot 10^{-5}$ | $(3.51 \pm 0.3) \cdot 10^{-7}$ | $(6.1 \pm 0.3) \cdot 10^5$ |
| **4a** | 8.3 ± 0.2 | $(1.53 \pm 0.04) \cdot 10^{-4}$ | $(8.73 \pm 0.3) \cdot 10^{-7}$ | $(5.4 \pm 0.3) \cdot 10^4$ |
| **5a** | 10.5 ± 0.3 | $(5.2 \pm 0.05) \cdot 10^{-4}$ | $(2.36 \pm 0.1) \cdot 10^{-7}$ | $(2.0 \pm 0.2) \cdot 10^4$ |
| **6a** | 13.8 ± 0.4 | $(3.13 \pm 0.05) \cdot 10^{-5}$ | $(2.03 \pm 0.1) \cdot 10^{-7}$ | $(4.4 \pm 0.2) \cdot 10^5$ |
| **7a** | 10.47 ± 0.3 | $(6.94 \pm 0.05) \cdot 10^{-4}$ | $(2.00 \pm 0.1) \cdot 10^{-7}$ | $(1.5 \pm 0.2) \cdot 10^4$ |
| **7a*** | | $(6.6 \pm 0.4) \cdot 10^{-3}$ | $(3.85 \pm 0.06) \cdot 10^{-7}$ | |
| **1b**** | 158 ± 2 | $(5.7 \pm 0.4) \cdot 10^{-5}$ | $(5.0 \pm 0.6) \cdot 10^{-7}$ | $(2.77 \pm 0.09) \cdot 10^6$ |
| **4b**** | 50.3 ± 4 | $(1.9 \pm 0.8) \cdot 10^{-5}$ | $(2.4 \pm 0.2) \cdot 10^{-8}$ | $(2.6 \pm 0.5) \cdot 10^6$ |

* Dependence on glucose concentration. [GO] = $1.5 \cdot 10^{-7}$ M, **1a** or **7a** = $2 \cdot 10^{-4}$ M, 30°C, pH 7.3 (0.01 M phosphate buffer).
** [GO] = $3.08 \cdot 10^{-9}$ M, D-glucose (0.02 M), 30°C, pH 7.3 (0.01 M phosphate buffer).

rate is not dependent on β-D-gluconolactone concentration, which implies the enzyme mechanism illustrated in Scheme 3 [14].




**Scheme 3**

E is oxidized enzyme form; {E, D-glucose} is complex of GDH and D-glucose; {E, D-glucose, $Ru^{III}$} is triple complex of GDH, D-glucose, and Ru(III)

Glucose dehydrogenase interacts with D-glucose resulting in formation of enzyme–substrate complex, which reacts with oxidizing substrate yielding a triple complex {GDH, D-glucose, Ru(III)}. The latter decomposes and forms semioxidized form of GDH, β-D-gluconolactone, and Ru(II). The semioxidized GDH is further oxidized by the second mediator molecule. Assuming stationary approach to intermediate products by complex {GDH, glucose}, by reduced form of GDH and by complex {GDH-reduced, ruthenium}, Eq. (6) can be written:

$$V_0 = \frac{k_3[E][Gl][Ru]}{(K_m^{Gl} + [Gl])(K_m^{Ru} + [Ru])}, \qquad (6)$$

where [E] is GDH concentration, [Gl] concentration of D-glucose, [Ru] concentration of ruthenium(III), and $K_m^{Gl} = (k_{-1} + k_2)/k_1$ and $K_m^{Ru} = (k_{-2} + k_3)/k_2$.

The dependence of initial reaction rate on concentrations of **1a** and D-glucose was calculated from Eq. (6).

The resulting values of Michaelis constants for GDH and different substrates were 4.0 ± 0.5 mM for D-glucose and 0.020 ± 0.005 mM for **1a**. Equation (6) allows a number of approaches coupling the effective kinetic parameters of GDH catalyzed D-glucose oxidation by ruthenium(III) compounds with its true kinetic parameters.

Investigation of the dependence of initial reaction rate of GDH catalyzed D-glucose oxidation on ruthenium(III) concentration were performed in the presence of excess D-glucose. Under these conditions:

$$V_0 = \frac{k_3[E][Ru]}{(K_m^{Ru} + [Ru])}. \qquad (7)$$

True kinetic parameters of D-glucose oxidation by ruthenium(III) compounds calculated based on the

**Table 3.** Kinetic parameters of reduction of ruthenium(III) compounds by D-glucose in the presence of PQQ-dependent GDH. D-Glucose (0.02 M) at 30°C, pH 7.3 (0.01 M potassium phosphate buffer); [GDH] = $1.36 \cdot 10^{-8}$ M

| Complex $Ru^{III}$ | $k_3$, $sec^{-1}$ | $K_m^{Ru}$, M | $V_{max}$, $M \cdot sec^{-1}$ | $k_3/K_m^{Ru}$, $M^{-1} \cdot sec^{-1}$ |
|---|---|---|---|---|
| **1a** | 1.90 ± 0.03 | $(1.33 \pm 0.05) \cdot 10^{-5}$ | $(2.6 \pm 0.1) \cdot 10^{-8}$ | $(1.42 \pm 0.4) \cdot 10^5$ |
| **2a** | 11.0 ± 0.3 | $(1.02 \pm 0.08) \cdot 10^{-4}$ | $(1.5 \pm 0.1) \cdot 10^{-7}$ | $(1.08 \pm 0.4) \cdot 10^5$ |
| **2a*** | 7.3 ± 0.04 | $(3.7 \pm 0.4) \cdot 10^{-3}$ | $(1.0 \pm 0.1) \cdot 10^{-7}$ | |
| **3a** | 3.97 ± 0.02 | $(5.4 \pm 0.9) \cdot 10^{-5}$ | $(5.4 \pm 0.3) \cdot 10^{-8}$ | $(7.3 \pm 0.3) \cdot 10^4$ |
| **3a*** | 2.7 ± 0.02 | $(4.2 \pm 0.4) \cdot 10^{-3}$ | $(3.4 \pm 0.3) \cdot 10^{-8}$ | |
| **4a** | 12.29 ± 0.5 | $(3.1 \pm 0.4) \cdot 10^{-5}$ | $(1.66 \pm 0.3) \cdot 10^{-7}$ | $(3.95 \pm 0.3) \cdot 10^5$ |
| **5a** | 0.92 ± 0.03 | $(3.5 \pm 0.3) \cdot 10^{-5}$ | $(1.26 \pm 0.2) \cdot 10^{-8}$ | $(2.6 \pm 0.4) \cdot 10^4$ |
| **6a** | 0.26 ± 0.05 | $(1.33 \pm 0.05) \cdot 10^{-5}$ | $(3.5 \pm 0.1) \cdot 10^{-9}$ | $(1.96 \pm 0.2) \cdot 10^4$ |
| **7a** | 1.27 ± 0.05 | $(4.2 \pm 0.9) \cdot 10^{-5}$ | $(1.7 \pm 0.5) \cdot 10^{-8}$ | $(3.0 \pm 0.2) \cdot 10^4$ |
| **8a** | 3.11 ± 0.04 | $(8.6 \pm 0.8) \cdot 10^{-6}$ | $(4.2 \pm 0.3) \cdot 10^{-8}$ | $(3.6 \pm 0.2) \cdot 10^5$ |
| **8a*** | 0.54 ± 0.04 | $(5.01 \pm 0.6) \cdot 10^{-3}$ | $(7.4 \pm 0.3) \cdot 10^{-9}$ | 107 ± 5 |
| **1b**** | 45.4 ± 1 | $(1.7 \pm 0.1) \cdot 10^{-5}$ | $(1.4 \pm 0.4) \cdot 10^{-7}$ | $(2.62 \pm 0.09) \cdot 10^6$ |
| **2b**** | 9.4 ± 0.2 | $(2.3 \pm 0.1) \cdot 10^{-5}$ | $(2.9 \pm 0.2) \cdot 10^{-8}$ | $(4.1 \pm 0.2) \cdot 10^5$ |
| **3b**** | 20.4 ± 0.1 | $(7.9 \pm 0.1) \cdot 10^{-6}$ | $(6.3 \pm 0.4) \cdot 10^{-8}$ | $(2.58 \pm 0.09) \cdot 10^6$ |
| **4b**** | 11.3 ± 0.04 | $(2.7 \pm 0.3) \cdot 10^{-5}$ | $(3.5 \pm 0.2) \cdot 10^{-8}$ | $(4.2 \pm 0.4) \cdot 10^5$ |

* Dependence on D-glucose concentration. [$Ru^{III}$] = $2 \cdot 10^{-4}$ M; pH 8.0 (0.01 M phosphate buffer).
** [PQQ-dependent GDH] = $3.08 \cdot 10^{-9}$ M, D-glucose (0.02 M), 30°C, pH 7.3 (0.01 M phosphate buffer).

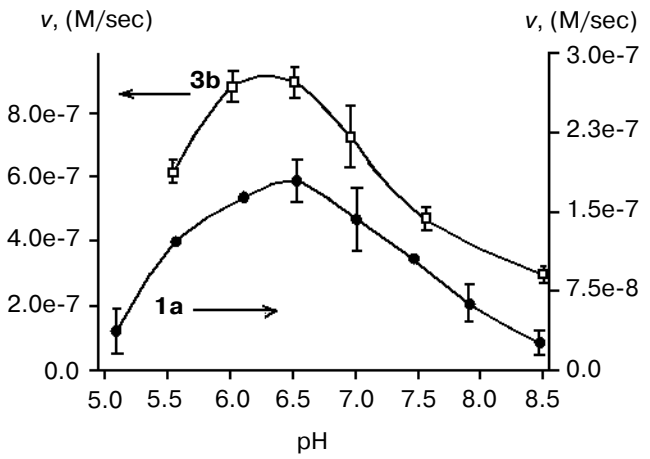



**Fig. 5.** pH profile of GO catalyzed reduction of ruthenium(III) compounds by D-glucose; 0.01 M phosphate buffer, 30°C; 1a) [GO] = $1.13 \cdot 10^{-9}$ M, [**1a**] = $1 \cdot 10^{-4}$ M, [D-glucose] = 0.03 M; 3b) [GO] = $1.13 \cdot 10^{-7}$ M, [**3b**] = $1 \cdot 10^{-4}$ M, [D-glucose] = 0.03 M.

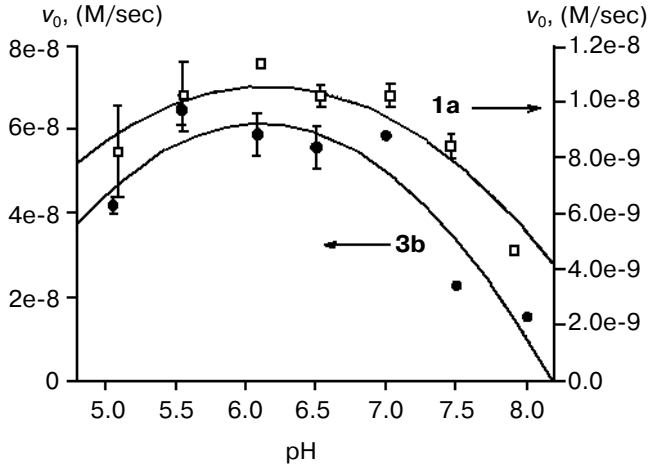



**Fig. 6.** pH profile of GDH catalyzed reduction of ruthenium(III) compounds by D-glucose; 0.01 M phosphate buffer, 30°C; 1a) [GDH] = $1.13 \cdot 10^{-9}$ M, [**1a**] = $1 \cdot 10^{-4}$ M, [D-glucose] = 0.03 M; 3b) [GDH] = $1.13 \cdot 10^{-7}$ M, [**3b**] = $1 \cdot 10^{-4}$ M, [D-glucose] = 0.03 M.

dependence of initial reaction rate of GDH catalyzed D-glucose oxidation on ruthenium(III) concentration are presented in Table 3.

The expression describing the dependence of initial reaction rate of GDH catalyzed D-glucose oxidation on D-glucose concentration obtained at excessive ruthenium(III) concentration takes the form:

$$\underset{\mathrm{Ru}\to\infty}{V_0} = \frac{k_3[\mathrm{E}][\mathrm{Gl}]}{(K_\mathrm{m}^{\mathrm{Gl}} + [\mathrm{Gl}])(1 + \frac{K_\mathrm{m}^{\mathrm{Ru}}}{[\mathrm{Ru}]})}. \qquad (8)$$

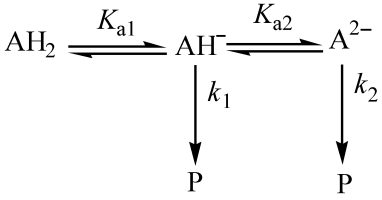
True kinetic parameters for the reaction of ruthenium complexes were calculated using Eq. (8) and are presented in Table 3.

The values of Michaelis constants for D-glucose and PQQ-dependant GDH are equal for **3a** and **1a** complexes with a value of 0.002 M. Michaelis constant calculated for phenazine methosulfate using D-glucose and GDH from *Erwinia* sp. 34-1 was 3 mM. Michaelis constants for 2,6-dichloroindophenol or phenazine methosulfate as the oxidizing substrate using D-glucose and GDH from *Escherichia coli* [25] was 2 mM. For ruthenium(III) compounds used in this study Michaelis constant for GDH using glucose was close to the literature data.

**pH profile for GO or GDH catalyzed reaction of D-glucose oxidation by ruthenium(III) compounds.** The dependences of initial GO and GDH reaction rates on pH are presented in Figs. 5 and 6, accordingly. The pH profile of **1a** reduction by D-glucose in the presence of GO is a bell-shaped curve with pH optimum of 6.5. The electron transfer in the case of ruthenium(III) compounds is characterized by the same pH optimum as for other electron acceptors that were active within the investigated pH range [26]. The mechanism of electron transfer includes two independent single-electron processes without adduct formation between the mediator molecule and FAD. The semiquinoid FAD form is an intermediate product in this reaction [27].

The fact that GDH has no intrinsic pH optimum that is independent of the nature of electron acceptor is widely known [28-30]. Previously it was established that the value of the pH optimum is 6.0 when using 2,6-dichloroindophenol as an electron acceptor [28]. The use of 2,6-dichloroindophenol and phenazine methosulfate mixture [29] shifts the pH optimum toward 7.0, while the application of Wurster's Blue [30] results in the pH optimum of 9.0. Such diversity is explained by different nature of amino acid residues capable of binding to oxidizing substrate and involved in the reaction between the enzyme reduced form and electron acceptor, since there is no particular amino acid residue in GDH responsible for binding to the oxidizing substrate [14].

The pH profile of D-glucose oxidation by ruthenium(III) compounds is a bell-shaped curve within the investigated range. The reaction rate increases significantly while pH is decreased from 8 to 7. The reaction rate is not changed so dramatically when pH is varied between 7 and 5. Figure 6 illustrates that semiprotonated enzyme form, $AH^-$, formed within the pH range of 5.0-7.5 is catalytically more active (Scheme 4).




**Scheme 4**

The p$K_a$ values of amino acid residues responsible for binding of ruthenium(III) compounds were calculated according to the equation:

$$k_{obs} = \frac{k_1 K_{a1} K_{a2} + k_2 K_{a1}[H]}{[H]^2 + K_{a1}[H] + K_{a1}K_{a2}}, \quad (9)$$

where $k_{obs}$ is observed rate constant of GDH catalyzed reaction of D-glucose oxidation by ruthenium(III) compounds, $k_1$ observed rate constant of first order reaction catalyzed by $AH^-$ form of GDH, $k_2$ observed rate constant of first order reaction catalyzed by completely deprotonated enzyme form ($A^{2-}$).

The values of p$K_{a1}$ and p$K_{a2}$ obtained from the dependence of $k_{obs}$ on pH for **3b**, were 4.8 ± 0.9 and 7.7 ± 0.5, respectively. The numeric values indicate that it is a histidine residue [25] in the enzyme active site that is most likely involved in binding of the oxidizing substrate.

**Comparative analysis of GO and GDH catalytic behavior in the reduction of ruthenium(III) compounds by D-glucose.** The influence of substrate concentration on D-glucose oxidation by ruthenium(III) compounds displays a common tendency for the two enzymes. The dependence of initial reaction rate on substrate concentration has a shape of a saturation curve. The proposed scheme of GO catalysis matches the mechanism implicated in the case of ferricinium cations in GO catalysis [23]. The oxidized GO binds to D-glucose with formation of {GO(ox.), D-glucose} complex, which later decomposes into gluconolactone and reduced GO. The reduced GO reacts with ruthenium(III) compounds forming {GO(red.), ruthenium} complex, which decomposes into ruthenium(II) and oxidized GO.

The mechanism of GDH catalysis differs from that of GO. The substrates are bound in same order as in the case of GO [18]. The oxidized form of PQQ-dependent GDH binds to D-glucose resulting in {GDH, D-glucose} complex formation. In turn, {GDH, D-glucose} complex reacts with oxidizing substrate forming a triple {GDH, D-glucose, $Ru^{III}$} complex [14], which later breaks down into native GDH, β-D-gluconolactone, and Ru(II). Rate constant values of D-glucose oxidation by ruthenium(III) compounds in the case of GO and GDH are comparable. Due to the differences in enzyme kinetic behavior in the reaction of D-glucose oxidation by ruthenium(III) compounds, the comparison of kinetic parameter values does not allow correlating the efficiency of ruthenium mediators in enzymatic catalysis.

The specific activity of each enzyme toward the ruthenium substrate was considered as an efficiency criterion. The amount of ruthenium mediator micromoles that oxidizes one activity unit of enzyme in 1 min under the standard conditions [17] corresponds to a specific activity unit. The data are shown in Table 4. As seen from the data presented, the specific GDH activity toward the ruthenium substrates exceeds that of GO by one order of magnitude. This fact can be accounted for by the presence of bound D-glucose (potential of glucose/gluconolactone pair is 364 mV [31]) that lowers the potential of {GDH, D-glucose} complex and, therefore, increases the electromotive force of the reaction.

Interest in PQQ-dependent enzymes stems from the fact that their interaction is not associated with oxygen, and hence, the search for more efficient substrates for PQQ-dependent enzymes is an important task. A detailed kinetic study of the interaction of Ru(III) complexes (Scheme 2) with PQQ-dependent GDH and GO in the presence of D-glucose was carried out. All complexes efficiently reoxidize the reduced enzyme form, i.e., reaction (2) takes place.

Complexes of Ru(III) exhibited high catalytic activity in the reaction with GDH. The reaction has first kinetic order in the enzyme. Both GDH substrates, i.e., ruthenium(III) and D-glucose follow Michaelis kinetics. Reaction (2) has a broad pH optimum in the range of 6-7, whereas the GDH pH optimum is broader compared to GO. The kinetic parameters are summarized in Tables 2 and 3. Complex **8a** is characterized by higher rate constant values. At lower ruthenium concentrations, when $k_4/K_m$ ratio should be applied, the most preferred is **1a** complex. The study of D-glucose oxidation kinetics by ruthenium cyclic compounds in the presence of GO and GDH reveals that kinetic parameter values for ruthenacycles and their efficiency are by an order of magnitude higher than in the case of ruthenium acido-complexes, both for GO and GDH catalysis.

**Table 4.** Specific activities of GDH and GO enzymes toward the ruthenium substrates measured under standard conditions (30°C, pH 7.0, 0.01 M potassium phosphate buffer, 0.03 M D-glucose)

| Complex | GDH | GO |
|---|---|---|
| **1a** | 0.28 | 0.044 |
| **2a** | 1.63 | 0.055 |
| **3a** | 0.58 | 0.098 |
| **4a** | 1.81 | 0.245 |
| **5a** | 0.013 | 0.066 |
| **6a** | 0.038 | 0.057 |
| **7a** | 0.188 | 0.056 |
| **1b** | 6.8 | 0.14 |
| **2b** | 1.4 | |
| **3b** | 2.9 | |
| **4b** | 1.6 | 0.677 |

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