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Study of kinetics of *Aspergillus niger* glucose oxidase reaction with pentacyanoferrates containing nucleophilic ligands

L. Tetianec*, J. Kulys

Institute of Biochemistry, Mokslininkø 12, LT-08662 Vilnius, Lithuania

Complexes of pentacyanoferrate(III) and nucleophilic ligands were synthesized and reactivity with glucose oxidase (GO) from *Aspergillus niger* was determined at pH 7.2. As nucleophilic ligands ammonia, imidazole or pyrazole were used. For comparison the reactivity of ferricyanide with GO was measured at the same conditions. Calculated apparent bimolecular constants of reduced enzyme interaction with the complexes varied from $2.4 \cdot 10^2$ to $3.0 \cdot 10^3$ $M^{-1} \cdot s^{-1}$.

The distance of electron transfer between the enzyme active center and the complexes was calculated using outer sphere electron transfer theory (Marcus theory). It varied between 8.1–10.2 Å.

Key words: glucose oxidase, pentacyanoferrate, hexacyanoferrate(III), electron transfer

INTRODUCTION

Structure / function relation is a fundamental question of biological electron transfer processes and has been of considerable interest in the last 30 years [1–7]. Beginning from the pioneering works of Wherland and Gray [1] a great deal of data has been collected on the dependence of electron transfer rate on the process free energy, electron transfer distance, and structure of active centre [2–8].

The glucose oxidase (GO) is notable for several reasons. One reason for the extensive exploration of glucose oxidase catalysis is the large practical significance of this reaction. Glucose biosensors are examples of successful utilization of glucose oxidase catalysis [9–13]. Other reason is a fundamental question of flavin oxidoreductases action. The gene of *Aspergillus niger* glucose oxidase has been isolated and cloned [14, 15]. The three-dimensional structure of the protein has been established and substrate docking in the active center has been calculated [15]. The structure activity relationship of this enzyme was explored using organic and inorganic electron acceptors [4–8]. As inorganic acceptors Fe(III), Ru(III) and Os(III) complexes were used [6, 7]. Many of the investigated acceptors were successfully used in the preparation of glucose biosensors.

Despite the numerous investigations of GO catalysis, the factors determining the reactivity of substrates are still not completely clear. In attempt to investigate a role of ligands to the reactivity of inorganic complexes in this work we used pentacyanoferrate(III) with nucleophilic compound as the sixth ligand. The changing structure of the ligand permits to change redox potential and other molecular properties of the complex and to expose the characteristics of substrates which determinate the reactivity. As ligands ammonia, imidazole, pyrazole were used for complexes preparation and GO reactivity probing.

*Corresponding author. E-mail: lidija@bchi.lt. Fax: + (370-5) 2729 196.

MATERIALS AND METHODS

Enzymes and substrates

Glucose oxidase from *Aspergillus niger* (type VII) was a product of Sigma. The concentration of enzyme was determined by using differential molar extinction coefficient of flavin cofactor as $1.31 \cdot 10^4$ M^{-1} cm^{-1} at 450 nm [16]. Catalase from *Aspergillus niger* was purchase from Novozymes A/S (Denmark).

A solution of glucose for kinetic measurements was prepared from glucose powder (Cerestar, Germany) and allowed to mutarotate for 24 h before use. Imidazole (IM) was received from Reanal (Budapest, Hungary), potassium ferricyanide was from Sigma, pyrazole (PYR) was from Aldrich. Sodium nitroprusside was obtained from Polskie Odczynniki Chemiczne (Gliwice, Poland), sodium nitrite, acetic acid and other analytical grade reagents were obtained from Reachim (Moscow, Russia). 1-(N,N-dimethylamine)-4-(4-morpholine)benzene (AMB) was synt-

hesized as described in [5]. All buffers and solutions were prepared using double distilled water. $Na_3[Fe(CN)_5NH_3] \cdot 3H_2O$ was synthesized from sodium nitroprusside and then oxidized to $Na_2[Fe(CN)_5NH_3] \cdot 2H_2O$ following the procedure described in [17].

Synthesis of the complexes

The complex formation between amino pentacyanoferrate(III) and ligands was carried out *in situ* in 0.1 M phosphate buffer, containing 0.1 M KCl, pH 7.2. The incubation time was 48–72 h at room temperature. The formation of complexes was monitored spectrophotometrically using Beckman DU-8V spectrophotometer (Beckman Instruments, Inc., California, USA). The constants of ligand exchange rate (k_{ex}) have been calculated from the data of absorbance change at fixed wavelength:

$$A = A_\infty \cdot (1- \exp(-k_{ex} \cdot t)), \quad (1)$$

where t is time, A – absorbance, A_∞ – final absorbance of the complex at the same wavelength.

Electrochemical measurements

Cyclic voltammetry was performed using an electroanalytical system (Cypress Systems, USA) equipped with a glassy carbon electrode (model CS-1087, Cypress Systems, USA). A saturated calomel electrode (SCE saturated with KCl, model K-401, Radiometer, Denmark) was used as a reference electrode, and as an auxiliary electrode a Pt wire (diameter 0.2 mm, length 4 cm) was mounted on the end of the reference electrode. Measurements were performed in 0.1 M phosphate buffer solution containing 0.1 M KCl, pH 7.2, at room temperature. The glassy carbon electrode was freshly polished with aluminum oxide before the measurements and treated ultrasonically in water for 10 min. The formal redox potential was calculated as the midpoint potential of the reduction and oxidation peak potentials.

Kinetic measurements and calculations

The kinetics of enzymatic complexes reduction was measured in aerobic conditions. The catalase was added to the reaction mixture to reduce small amounts of hydrogen peroxide impurities which can form during reaction with oxygen. The reduction of the complexes was monitored spectrophotometrically using a computer-assisted Gilford Instrument 2600 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH, USA). The kinetics was recorded at a maximum absorbance of oxidized complexes. The kinetic curves were fitted by the first order reaction integral equation, and the rate constant and initial concentration were used for initial reaction rate calculation. The analysis of the dependence of the initial rate on the concentration of the complex was performed by applying a ping-pong scheme of the enzyme action [16]:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} E_{ox}\text{-}S \xrightarrow{k_2} E_{red} + P, \quad (2)$$

$$E_{red} + M_{ox} \xrightarrow{k_3} E_{ox} + M_{red}, \quad (3)$$

where E_{ox}, E_{red}, E_{ox}-S, S, P, M_{ox} and M_{red} are abbreviations of the oxidized, reduced forms of enzyme, enzyme-substrate complex, substrate, product, oxidized and reduced forms of the mediator. Following this scheme, the dependence of the initial reaction rate on the complex concentration is expressed:

$$[E]_t / V = (1 / k_{cat}) + 1 / (k_{red} \cdot [S]_0) + 1 / (k_{ox} \cdot [complex]_0), \quad (4)$$

where $[E]_t$ is a total enzyme concentration, V – initial rate, $k_{cat} = k_2$, $k_{red} = k_1 \cdot k_2 / (k_{-1} + k_2)$ and $k_{ox} = k_3$ are catalytic, reductive and oxidative constants, $[complex]_0$ and $[S]_0$ are the initial concentration of the complex and glucose, respectively. k_{ox} describes the apparent bimolecular constant of the interaction of an oxidized complex with reduced GO.

At a large excess of glucose, the dependence of the rate on complex concentration can be simplified:

$$V = k_{cat} \cdot k_{ox} \cdot [E]_t \cdot [complex]_0 / (k_{ox} \cdot [complex]_0 + k_{cat}). \quad (5)$$

At a low complex concentration when $k_{cat} > k_{ox} \cdot [complex]_0$, equation (5) transforms into equation (6), and a linear dependence of the initial reaction rate on $[complex]_0$ has been indicated:

$$V = k_{ox} \cdot [E]_t \cdot [complex]_0. \quad (6)$$

To calculate self-exchange constants (k_{11}), the reaction of $[Fe(CN)_5L]^{n-}$ complexes and hexacyanoferrate(III) with AMB was investigated with a stopped-flow Otsuka RA-401 spectrophotometer (Japan) interfaced with a computer system. The kinetics of absorbance of the oxidized AMB increase was registered.

The measurements were made in 0.1 M phosphate buffer solution containing 0.1 M KCl, pH 7.2, at 25 °C. The initial concentrations of AMB and $[Fe(CN)_5L]^{n-}$ complexes or hexacyanoferrate(III) were 10 μM and 100 μM, respectively.

The concentration of oxidized AMB was calculated from the absorbance change at 604 nm using the experimentally determined extinction coefficient of 13.1 $mM^{-1} cm^{-1}$. The bimolecular rate constant of AMB reaction with oxidized complex (k_{12}) was calculated applying a bimolecular reaction scheme with

equilibrium establishment. Whereas AMB charge was assumed equal to 0, k_{12} was not corrected for non-specific electrostatic effects.

k_{11} was calculated from the Marcus relationship [18]:

$$k_{12}^{\infty} = (k_{11} \cdot k_{22} \cdot K_{12} \cdot f)^{1/2}, \quad (7)$$

where

$$\log(f) = [\log(K_{12})]^2 / [4 \cdot \log(k_{11} \cdot k_{22}/Z^2)]. \quad (8)$$

In equations (7), (8), Z is the collision frequency (taken to be 10^{11} $M^{-1}s^{-1}$ at 25 °C), K_{12} is the equilibrium constant calculated at 25 °C from the redox potential difference (ΔE) of the reactants:

$$\log(K_{12}) = 16.9 \cdot \Delta E \text{ (V)}, \quad (9)$$

where k_{22} is the self-exchange constant of AMB. The AMB self-exchange constant was calculated in the same manner from the measurements of AMB oxidation with hexacyanoferrate(III). For calculations, the hexacyanoferrate(III) self-exchange constant of $2.4 \cdot 10^2$ $M^{-1}s^{-1}$ [19] and potentials of AMB and ferricyanide equal to 146 mV [5] and 190 mV (experimentally determined), respectively, were used.

RESULTS AND DISCUSSION

Synthesis and characterization of the complexes

The reaction of ammonia replacement with ligands proceeds according to equation (10):

$$[Fe(CN)_5NH_3]^{2-} + L \rightarrow [Fe(CN)_5L]^{2-} + NH_3. \quad (10)$$

Reaction (10) is associated with a strong absorbance change (Fig. 1). During complexe formation a new band of absorbance appears at the visible area of the spectrum. The spectra of the synthesized complexes are presented in Fig. 1. Measurement of the kinetics of ammonia replacement gave $k_{ex} = 9.3 \cdot 10^{-5}$ s^{-1} for the complex with PYR and $7.0 \cdot 10^{-5}$ s^{-1} for the complex with IM.

The replacement of ammonia with a heterocyclic compound changed the formal redox potential of the complexes. Data depicted in Fig. 2 represent cyclic voltammograms (CV) of $[Fe(CN)_5NH_3]^{2-}$, $[Fe(CN)_5PYR]^{2-}$ and $[Fe(CN)_5IM]^{2-}$. The calculated redox potentials of the complexes are presented in Table.

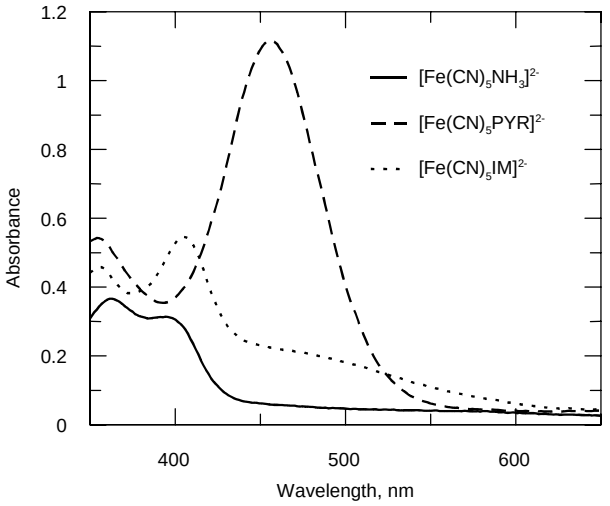

Fig. 1. Absorbance spectra of $[Fe(CN)_5NH_3]^{2-}$, $[Fe(CN)_5PYR]^{2-}$ and $[Fe(CN)_5IM]^{2-}$. 0.1 M phosphate buffer solution containing 0.1 M KCl, pH 7.2, concentrations of complexes 0.5 mM. $[Fe(CN)_5NH_3]^{2-}$ was incubated with imidazole and pyrazole for 2 days

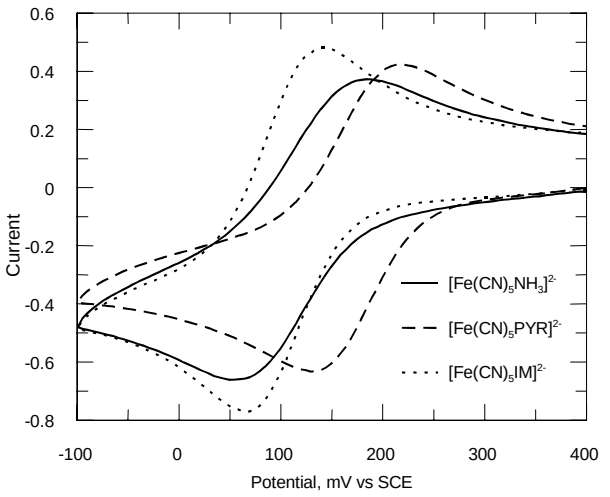

Fig. 2. Cyclic voltammograms of $[Fe(CN)_5NH_3]^{2-}$, $[Fe(CN)_5PYR]^{2-}$ and $[Fe(CN)_5IM]^{2-}$. 0.1 M phosphate buffer solution containing 0.1 M KCl, pH 7.2, scan rate 100 mV/s. Concentrations of complexes 0.5 mM. $[Fe(CN)_5NH_3]^{2-}$ was incubated with imidazole and pyrazole for 2 days

Table. **Spectral, electrochemical and kinetic properties of the complexes in 0.1 M phosphate buffer solution pH 7.2, containing 0.1 M KCl at 25 °C. The k_{11} value for ferricyanide was taken from [19]**

Complex	λ_{max}, nm	k_{ex}, s^{-1}	E, mV vs. SCE	k_{11}, $M^{-1} \cdot s^{-1}$	k_{ox}, $M^{-1} \cdot s^{-1}$	R_p, Å
$[Fe(CN)_6]^{3-}$	420	–	190	240 ± 10	240 ± 20	10.3
$[Fe(CN)_5NH_3]^{2-}$	360	–	120	470 ± 110	2000 ± 500	8.2
$[Fe(CN)_5PYR]^{2-}$	456	$9.3 \cdot 10^{-5}$	172	1300 ± 400	3000 ± 200	8.8
$[Fe(CN)_5IM]^{2-}$	475	$7.0 \cdot 10^{-5}$	101	790 ± 90	1250 ± 50	8.5

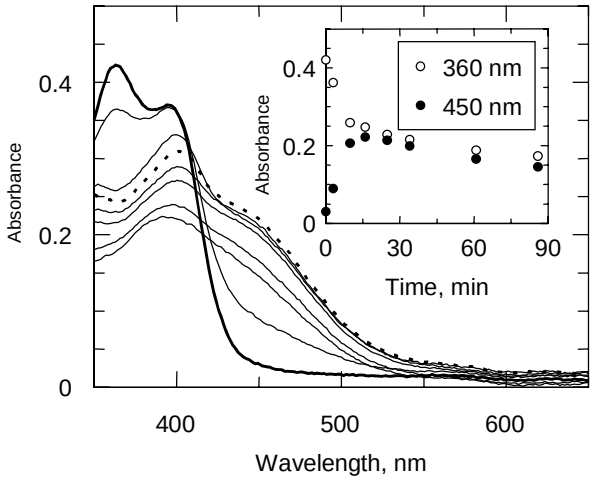

Fig. 3. $[Fe(CN)_5NH_3]^{2-}$ absorbance changes in the presence of 0.1 M of glucose, 0.1 μM of catalase and 2 μM of GO at pH 7.2. Aerobic conditions, 0.1 M phosphate buffer solution containing 0.1 M KCl, pH 7.2, 25 °C. The spectra were recorded at 0 (bold line), 3, 10, 16 (bold dotted line), 25, 34, 61 and 86 min. The insert shows the kinetics of absorbance change at 360 and 450 nm

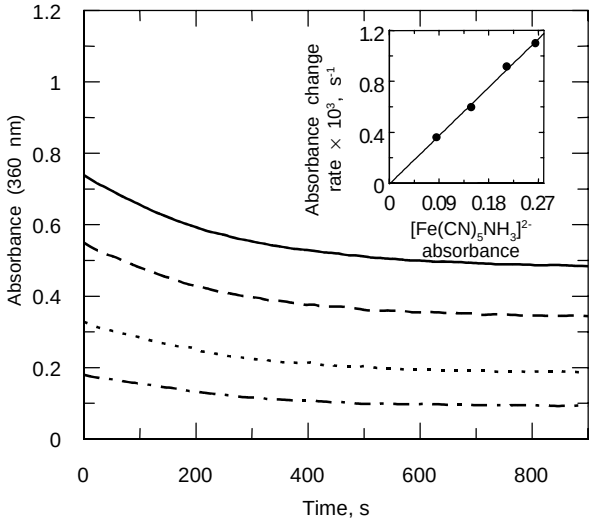

Fig. 4. Kinetics of $[Fe(CN)_5NH_3]^{2-}$ absorbance change in presence of 2 μM GO and 0.1 M glucose. Initial concentrations of $[Fe(CN)_5NH_3]^{2-}$ were 1 mM (continiuos line), 0.75 mM (dashed line), 0.5 mM (dotted line) and 0.25 mM (dashed and dotted line). Aerobic conditions, 0.1 M phosphate buffer solution containing 0.1 M KCl, pH 7.2, 25 °C. The inserted graph shows dependence of absorbance change rate on complex absorbance. The linear fit corresponds to a slope 4.2 s^{-1}

The synthesized complexes and ferricyanide reacted with AMB. The bimolecular rate constant (k_{12}) of AMB oxidation with the complex was calculated from the kinetic curve of oxidized AMB concentration increase. The complexs self-exchange constants were calculated using equations 7 and 8 (Table).

Reaction of complexes with a reduced glucose oxidase

During the incubation of $[Fe(CN)_5NH_3]^{2-}$ with glucose oxidase and glucose, the absorbance spectra of the complex changed as shown in Fig. 3. No spectral changes were detected if pure glucose or the enzyme was added to the complex solution. Analysis of the spectra revealed that during the first 15 min of the reaction the changes are associated with complex reduction. The spectrum recorded at 16 min after reaction start match the spectra of $[Fe(CN)_5NH_3]^{3-}$ (not shown). The ammonia of aminopentacyanoferrate(II) are rapidly replaced with water in buffer solutions [17, 20], so changes of the spectrum after 16 min of reaction can be associated with the ligand exchange reaction. Investigations of $[Fe(CN)_5NH_3]^{2-}$ reduction kinetics at various glucose oxidase concentrations and at a wavelength of 360 nm showed only the initial reaction rate to depend on the concentration of GO. After the fast reduction reaction, a slow reaction of complex ligand exchange begins, its rate being independent of the enzyme concentration.

In the case of $[Fe(CN)_6]^{3-}$, $[Fe(CN)_5IM]^{2-}$ and $[Fe(CN)_5PYR]^{2-}$, changes of absorbance spectra in the presence of glucose and glucose oxidase are associated with reduction of Fe^{3+} by reduced glucose oxidase. The kinetics of enzymatic reduction of $[Fe(CN)_6]^{3-}$, $[Fe(CN)_5IM]^{2-}$ and $[Fe(CN)_5PYR]^{2-}$ was investigated at 420, 475 and 456 nm, respectively. The values were selected considering the largest difference of absorbance of the oxidized and reduced forms of the complexes at a particular wavelength.

The enzymatic reaction rate depended on the concentration of complexes (Fig. 4). The oxidation constants (k_{ox}) determined according to the ping-pong scheme of the enzyme action varied from $2.4 \cdot 10^2$ to $3.0 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (Table).

The application of outer sphere electron transfer theory [18] allows to calculate the distance of electron transfer between the active centre of GO and pentacyanoferrates. Assuming the redox potential of GO –0.048 V *vs.* SHE [21] and applying equations (7)–(9) give an apparent self-exchange constant k_{11}^* of GO for different pentacyanoferrates. According to Mauk and co-workers [2], the protein self-exchange constant is related to the electron transfer distance (R_p) by equation (11):

$$R_p(Å) = 6.2 - 0.35 \ln k_{11}^* (M^{-1}s^{-1}). \quad (11)$$

The calculations gave an electron transfer distance of 8.1–10.2 Å (Table). The electron transfer distance reduced by the van der Waals contact (1.85 Å) gives the shortest distance from the active site to the surface of the protein. This distance varied from 6.3 to 8.4 Å. The largest distance was indicated for ferricyanide which contains the largest charge and is

the most solvated among all the electron acceptors studied. It is possible to generalize that the rather low reactivity of pentacyanoferrates is a result of a highly negative charge, high solvation energy and low self-exchange constants of the compounds.

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L. Tetianec, J. Kulys

GLIUKOZËS OKSIDAZËS REAKCIJA SU PENTACIANOFERATO-NUKLEOFILINIØ LIGANDØ KOMPLEKSAIS

S a n t r a u k a

Buvo susintetinti pentacianoferato(III) ir ligandø – amoniako, pirazolo bei imidazolo – kompleksai, nustatytos jø spektrinës, elektrocheminës savybës, elektronø mainø konstantos bei reaktingumas su *Aspergillus niger* gliukozës oksidaze (GO) esant pH 7,2. Tomis paèiomis sàlygomis buvo nustatytos fericianido oksidacinës-redukcinës savybës ir jo reaktingumas su GO. Kompleksø reaktingumo su redukuotu fermentu apskaièiuotos konstantø (k_{ox}) reikðmës kinta nuo $2{,}4 \cdot 10^2$ iki $3{,}0 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. Pritaikius iðorinës sferos elektronø transporto teorijà (Markuso teorija) apskaièiuotas elektronø transporto atstumas, kuris keitësi tarp 8,1 ir 10,2 angstremø.