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# Influence of biologically relevant ligands on oxidation of reduced glucose oxidase by electrochemically generated $[Ru^{III}(bpy)_2XY]$ complexes

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**Abstract**

Complex formation between *cis*-$[Ru(bpy)_2(H_2O)_2]^{2+}$ (bpy = 2,2′-bipyridyl) and some biologically relevant ligands (pyridines, imidazole, histidines) increases the rate constant for the oxidation of reduced glucose oxidase (generated in situ by D-glucose from the native enzyme) by the corresponding $Ru^{III}$ derivatives generated electrochemically. Almost a 20-fold rate acceleration is demonstrated by the cyclic voltammetry for histidine as ligand, the rate constant being ca. $10^6\ M^{-1}\ s^{-1}$ at pH 7 (0.1 M phosphate). © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Glucose oxidase; Ruthenium complexes; Electron transfer; Kinetics

## 1. Introduction

Bioinorganic chemistry is often viewed as the chemistry of metal species in biological objects. This generally accepted approach masks a challenging field focused on the reactivity of transition metal complexes and organometallics themselves in, for example, enzyme-catalyzed reactions [1]. Electron-transfer processes are a particular area, the importance of which is dictated by the rapid development of amperometric biosensors based on the mediated electron transfer [2,3]. A fast electron exchange between enzyme active site and mediator, which is often a transition metal species [4–7], is a crucial aspect of the entire bioanalytical setup. In our previous works, we have investigated in detail the reactivity of ruthenium(III) and osmium(III) complexes toward reduced GO [8] and kinetics of horseradish peroxidase-catalyzed oxidation of ruthenium(II) derivatives by hydrogen peroxide [9]. Here, we report on the electrochemical study of the coupling between reduced by D-glucose GO enzyme and $Ru^{III}$ complexes obtained in situ from *cis*-$[Ru(bpy)_2(OH_2)_2]^{2+}$ (**1**) and biologically relevant ligands listed

*Abbreviations:* GO, glucose oxidase; FAD, flavin adenine dinucleotide; CVA, cyclic voltammetry; DPV, differential pulse voltammetry; im, imidazole; his, histidine

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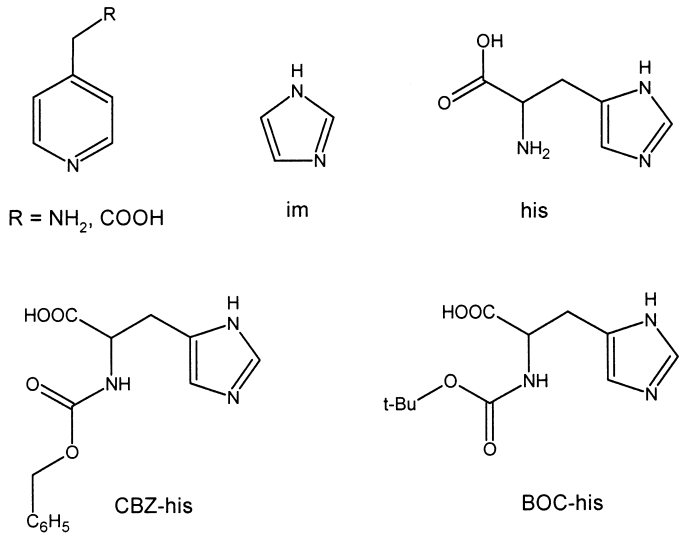


Scheme 1.

in Scheme 1 with a major goal to understand how the complex formation affects the ability of generated electrochemically $Ru^{III}$ species to oxidize GO(red) and to introduce simple ways for increasing the rate constants for the electron transfer.

## 2. Experimental

### 2.1. Materials

Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4., 228 and 282 u/mg) was purchased from Serva and used as received. *Cis*-$[Ru(bpy)_2Cl_2]$ was a Strem Chemicals reagent. Pyridine, 4-aminomethylpyridine, 4-pyridineaceticacid, im, his, BOC-his and CBZ-his were commercial products obtained from Aldrich. All pyridines except 4-pyridineaceticacid were distilled before use. Inorganic salts $KH_2PO_4$, NaCl and $NaClO_4$ used for the preparation of buffer solutions were Merck products and used as received.

### 2.2. Instrumentation

Cyclic and differential pulse voltammetry measurements were carried out using a potentiostat Model 273A (EG&G Princeton Applied Research). A three-electrode electrochemical cell with glassy carbon disk working electrode, saturated Ag/AgCl reference electrode and Pt auxiliary electrode was thermostated by circulating water. The working electrode was carefully polished with a 0.1 μm diamond paste before each measurement. A Cary 300 Bio UV–VIS spectrophotometer was used for assaying GO concentration using the extinction coefficient of $1.31 \times 10^3\ M^{-1}\ cm^{-1}$ at 450 nm [10].

### 2.3. Procedures

Stock solutions of complex **1** ($4.5 \times 10^{-4}$ M) in a phosphate buffer (0.01 or 0.1 M, pH 7.0) were prepared by stirring a suspension of *cis*-$[Ru(bpy)_2Cl_2]$ overnight. Buffered solution of D-glucose (1 M) was kept overnight for equilibration between the anomers. For the measurement of the peak current of $Ru^{II}$ species unaffected by the enzymatic reaction ($i_p°$), the protocol was standardized as follows. Buffered solutions of $Ru^{II}$ (2.9 ml) and D-glucose (0.1 ml) were introduced into the electrochemical cell. Cyclic voltammograms were recorded at seven different scan rates ($\upsilon$) in the range 2–50 $mV\ s^{-1}$. For the measurement of the peak current of $Ru^{II}$ in the presence of GO and D-glucose ($i_p$), a solution of GO (20 μl, final concentration in the cell $3 \times 10^{-6}$ M)

was added to the electrochemical cell and cyclic voltammograms were recorded as previously described [11]. The ratio $i_p/i_p^\circ$ was plotted against $([GO]/\upsilon)^{1/2}$ and the rate constants $k$ were evaluated from the slope of the linear plot using the procedure of Bourdillon et al. assuming that $k$ is independent of GO concentration [12].

Complex formation between $Ru^{II}$ and different ligands was carried out in situ in 0.1 M phosphate buffer, pH 7.0, at the metal–ligand ratios of 1:1, 1:2, 1:3 and 1:10. The reaction time was 24 or 2 h depending on the temperature used (ambient or 50°C, respectively). The redox potentials of the complexes obtained were registered using CVA and differential DPV techniques. The GO activity toward the complexes was measured using the same electrochemical procedure as described above for complex **1**.

## 3. Results and discussion

### 3.1. Electrochemical and spectral studies of $Ru^{II}$ speciation

The cyclic voltammogram recorded for complex **1** in 0.01 M phosphate buffer (pH 7.0) (Fig. 1) indicates the presence of two complexes in solution with reduction potentials around 100 and 300 mV. The peak at 100 mV disappears after several days and is not observed in 0.1 M phosphate. A peak–peak potential separation at 300 mV is ca. 60–75 mV in accord with a quasi-reversible behavior at higher scan rates. These observations confirm that *cis*-$[Ru(bpy)_2Cl_2]$ undergoes hydrolysis in the buffered phosphate solutions with dissociation of both chlorides according to Eqs. (1) and (2) [13–15].

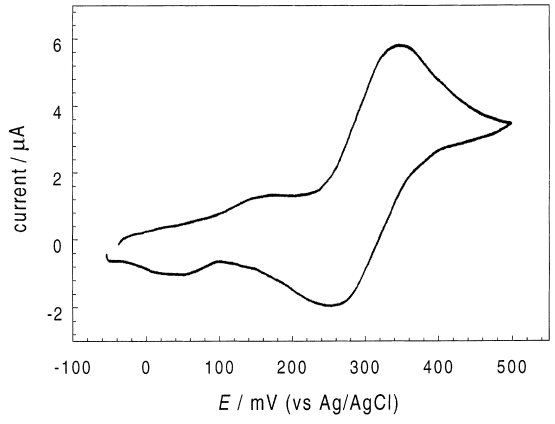


Fig. 1. Cyclic voltammogram of complex **1** ($5.56\times10^{-4}$ M) at pH 7.0: 0.01 M phosphate; scan rate 20 mV $s^{-1}$.

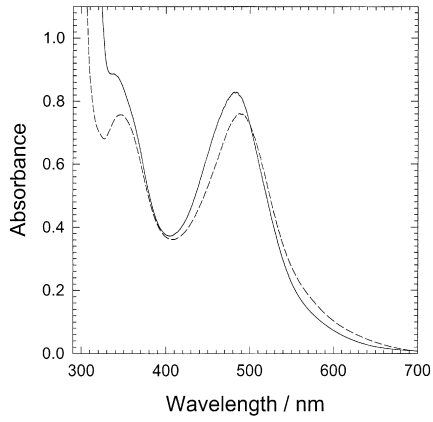


Fig. 2. UV–VIS spectra of **1** ($1.12\times10^{-4}$ M) in 0.01 M (solid) and 0.1 M (broken line) $KH_2PO_4$ buffer, pH 7.0.

$$cis\text{-}[Ru(bpy)_2Cl_2] + H_2O \rightleftharpoons cis\text{-}[Ru(bpy)_2Cl(H_2O)]^+ + Cl^- \quad (1)$$

$$cis\text{-}[Ru(bpy)_2Cl(H_2O)]^+ + H_2O \rightleftharpoons cis\text{-}[Ru(bpy)_2(H_2O)_2]^{2+} + Cl^- \quad (2)$$

**1**

Phosphate seems to speed up the process as a general base accounting for faster hydrolysis of chloride in the 0.1 M solution. CVA studies of solutions of *cis*-$[Ru(bpy)_2Cl_2]$ pretreated with 1 and 2 equivalents of silver(I) nitrate are in accord with Eqs. (2) and (3). Identical voltammograms were registered in the absence and in the presence of either 1 or 2 equivalents of $AgNO_3$. Although it was claimed by Allen et al. [14] that complex **1** exists as a diaqua species in aqueous buffered solutions, phosphate seems to interact with $[Ru(bpy)_2(H_2O)_2]^{2+}$, since the UV–VIS spectrum of the complex is sensitive to the phosphate concentration in the buffer (Fig. 2). It is difficult, however, to specify whether this interaction is a true anation by phosphate (P) (Eq. (3), charges not shown) or an outer sphere complex

formation to afford the associate {[Ru(bpy)$_2$-(H$_2$O)$_2$]$^{2+}$,P}. The reported p$K_a$ values for [Ru(bpy)$_2$(H$_2$O)$_2$]$^{2+}$ are 10.26 [16] and 8.9 and 9.3 for *cis* and *trans* isomers, respectively [14], hence, diaqua (**1**) rather than hydroxo(aqua) species dominates in the solution.

$$cis\text{-}\left[\mathrm{Ru(bpy)_2(H_2O)_2}\right]^{2+} + \mathrm{P} \rightleftharpoons cis\text{-}\left[\mathrm{Ru(bpy)_2(P)(H_2O)}\right] \quad (3)$$

It should be mentioned that the 487 nm maximum shifts in 0.1 M phosphate by 3 nm only (to higher wavelengths) referenced to that in 0.01 M phosphate, whereas the peak positions in CVA of **1** are identical in the two solutions. This is probably an indication that phosphate only slightly affects Ru$^{II}$ and that the outer-sphere complex formation is likely responsible for the spectral changes.

The complex formation was followed by UV–VIS spectroscopy, CVA and DPV. UV–VIS data presented in Fig. 3 indicate changes in maximum position and band intensity in accord with the complex formation. The changes, which were ascribed to equilibrium 4 on the basis of the UV–VIS and $^1$H NMR measurements, are similar to those observed in the py case in 0.01 M phosphate [9]. There is a small maximum shift to lower wavelengths in all cases accompanied by an increase in absorbance, the largest being observed for his.

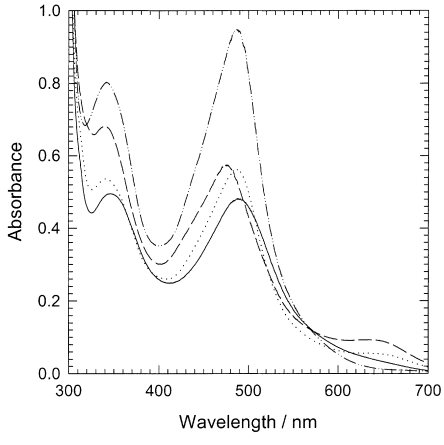


Fig. 3. UV–VIS spectra of **1** ($0.8\times10^{-4}$ M) in 0.1 M KH$_2$PO$_4$ buffer, pH 7.0 in the absence (solid line) and in the presence of two equivalents of py (broken), im (dotted), and his (broken dotted line).

Table 1
Rate constants $k$ for the oxidation of reduced GO by Ru$^{III}$ complexes [Ru(bpy)$_2$L(OH)]$^{n+}$ electrochemically generated in situ from [Ru(bpy)$_2$(H$_2$O)$_2$]$^{2+}$ and their formal redox potentials[a]

| Ligand | $E^{\circ\prime}$ (mV) (CVA) | $E^{\circ\prime}$ (mV) (DPV) | $10^{-5}\times$ $k$/M$^{-1}$ s$^{-1}$ |
|---|---|---|---|
| None | 300 | 290 | 0.54 ± 0.01 |
| None[b] | 300 | 290 | 0.18 ± 0.01[b] |
| py | 490 | 485 | 4.1 ± 0.1 |
| HOOCCH$_2$py | 485 | 470 | 3.6 ± 0.2 |
| H$_2$NCH$_2$py | 510 | 490 | 4.2 ± 0.2 |
| im | 415 | 410 | 4.4 ± 0.9 |
| his | 425 | 405 | 9.6 ± 0.4 |
| | 625 | 585 | 8.8 ± 0.4 |
| BOC-his | 415 | 415 | 0.48 ± 0.03 |
| | 620 | 625 | 0.77 ± 0.06 |
| CBZ-his | 415 | 400 | 0.73 ± 0.03 |
| | 625 | 600 | 0.31 ± 0.04 |

[a] Accuracy in determining potentials is ±5 mV. Conditions: 0.03 M D-glucose; $3\times10^{-6}$ M GO; 0.1 M KH$_2$PO$_4$; pH 7.0; 25°C; Ag/AgCl.
[b] In 0.01 M phoshate.

$$cis\text{-}\left[\mathrm{Ru(bpy)_2(H_2O)_2}\right]^{2+} + \text{R-py} \rightleftharpoons cis\text{-}\left[\mathrm{Ru(bpy)_2(R\text{-}py)(H_2O)}\right]^{2+} \quad (4)$$

Electrochemical methods are more sensitive to changes in the ligand environment at ruthenium. All electrochemical data obtained for Ru$^{II}$ complexes derived from **1** and monodentate ligands in Scheme 1 are in a qualitative agreement with the free-energy predictions of Lever [17]. For pyridine ligands an anodic shift of 185–210 mV was observed (Table 1), but the peaks around 300 mV were always seen on cyclic or differential pulse voltammograms indicative of the presence of the diaqua complex together with complexes of the type [Ru(bpy)$_2$(py)(H$_2$O)]$^{2+}$ even in 10-fold excess of the ligand. For im and his equilibria of type 4 are completely shifted to the right even in the case of two-fold excess of the ligand and traces of **1** were no longer observed on cyclic and differential pulse voltammograms. The

time required to complete the anation can be reduced by increasing the ligand concentration. It is interesting to compare the electrochemical data obtained for im, his, and BOC- and CBZ-protected histidines (Table 1). The im and his are characterized by close reduction potentials at 415 and 425 mV, respectively (CVA data). For his, additional anodic transition is observed at ca. 625 mV. Similar values of reduction potentials suggest identical coordination of im and his to $Ru^{II}$ and this should be the im nitrogen. The peak at 625 mV the intensity of which is always lower than that at 415 mV refers most likely to the $Ru^{III} \rightarrow Ru^{IV}$ transition [16]. It should be mentioned that similar transition is also observed as a shoulder in the im case at 605 mV. As seen in Table 1, the more anodic reduction potentials for all his derivatives are observed at 620–625 mV suggesting that similar species undergo redox transformations and these are $Ru^{III}$ complexes with monodentate his ligands coordinated via im nitrogen. Better defined $Ru^{III} \rightarrow Ru^{IV}$ transitions manifest for his derivatives and to a less extent for im. This is tentatively due to an anchoring effect of the amino acid backbone which facilitates the electron exchange with the electrode [18]. It should also be taken into account that the $pK_a$ value for the $Ru^{III}$ species $[Ru(bpy)_2(py)(H_2O)]^{3+}$ is as low as 0.85 [16]. This implies that complexes similar to $[Ru(bpy)_2(py)(OH)]^{2+}$ should be considered as the reacting species at pH 7. In contrast to water, hydroxide is substitutionally inert and this rules out the formation of chelated species at the $Ru^{III}$ center for his derivatives.

### 3.2. Electrochemical kinetics

In the presence of GO and D-glucose cyclic voltammograms of the Ru complexes undergo characteristic changes indicative of the interaction between electrochemically generated $Ru^{III}$ species and reduced GO (Eq. (6)) which is formed during the enzymatic oxidation of D-glucose into D-gluconolactone (Eq. (5)).

$$\text{D-glucose} + \text{GO(ox)} \rightarrow \text{D-gluconolactone} + \text{GO(red)} \quad (5)$$

$$2Ru^{III} + \text{GO(red)} \xrightarrow{k} 2Ru^{II} + \text{GO(ox)} \quad (6)$$

As seen in Fig. 4, each Ru species which coexists in solution demonstrate the coupling with GO(red) and the data obtained in one set of experiments (scanning at different scan rates) allow us to calculate the rate

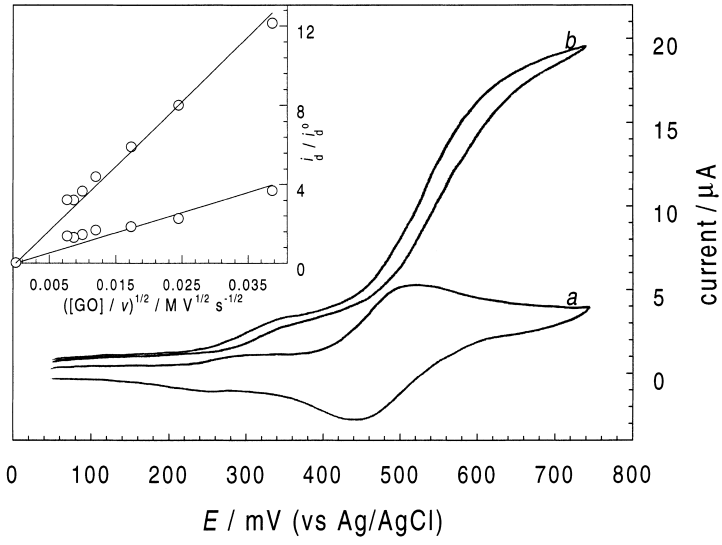


Fig. 4. Cyclic voltammograms of $[Ru(bpy)_2(py)(H_2O)]^{2+}$ ($4.5 \times 10^{-4}$ M) in 0.1 M phosphate buffer, pH 7.0; in the absence (a) and in the presence (b) of $3 \times 10^{-6}$ M GO and 0.03 M D-glucose; scan rate 10 mV $s^{-1}$, 25°C. Inset: plots for evaluating the rate constant $k$ for oxidation of reduced GO by $[Ru(bpy)_2(OH)(H_2O)]^{2+}$ (lower) and $[Ru(bpy)_2(py)(OH)]^{2+}$ (upper).

constant for the slowest step of two-electron process 6 using the approach introduced by Bourdillon, et al. [12]. The inset in Fig. 4 shows that two species present, viz. $[Ru(bpy)_2(H_2O)_2]^{2+}$ and $[Ru(bpy)_2(py)(H_2O)]^{2+}$, display different reactivity and the latter is more reactive in accord with its higher reduction potential.

Similar data were obtained for other ligands listed in Scheme 1. The enzymatic reaction serves here as an amplifier that helps to visualize coexisting species in solution which are not in fast equilibrium. By the example of $[Ru(bpy)_2(H_2O)_2]^{2+}$ it was demonstrated that the rate constant $k$ is independent of glucose concentrations in the range 0.03–0.1 M. Since the rate constants are rather high, they are weakly sensitive to the bubbling of argon through the reaction solutions before the measurements. On the other hand, the reaction rate depends on the concentration of phosphate. The rate constant is three times higher in 0.1 M than in 0.01 M phosphate buffer at the same pH, but is insensitive to the ionic strength. Similar rate constants were obtained in pure 0.01 M phosphate and with 0.1 M NaCl or $NaClO_4$, viz. $(1.8 \pm 0.1) \times 10^4$ and $(1.58 \pm 0.07) \times 10^4\ M^{-1}\ s^{-1}$, respectively.

Inspection of the rate constants in Table 1 allows one to specify the most reactive complexes generated in situ. These are py, im, and his derivatives. High rate constant observed for py is not surprising since the reaction driving force increases substantially on going from the diaqua to (aqua)py complex. More surprising is that the im complex, although having an approximately 75 mV more cathodic redox potential, is even more reactive probably because of its lower size. The highest reactivity is observed for the his complex, the rate constant for which is as high as $10^6\ M^{-1}\ s^{-1}$. It is the same but easily achieved level of reactivity as that reported for the complex tris(4,4′-diamino-2,2′-dipyridyl)ruthenium(III), the synthesis of which is much more laborious [19]. Introducing the steric bulk on going from his to BOC- and CBZ-his results, as could be expected, in a noticeable rate decrease and the rate constants drop more than 10-fold despite identical reduction potentials. The easiest way to account for this observation is to assume that the size of complex is of primary importance and the bulkier BOC- and CBZ-his complexes of $Ru^{III}$ are less favorable for the closest approach of a transition metal oxidant to the GO active site, the FAD of which is deeply buried in the protein globule [20].

With the goal to estimate a plausible role of complexes of the type $[Ru(bpy)_2L_2]^{3+}$, the effect of excess of corresponding ligands on the rate constant $k$ was investigated. The most different results obtained are demonstrated in Fig. 5. There is practically no effect of py on $k$ and this is in accord with our previous conclusions that only one py molecule is coordinated with the diaqua species [9]. As opposed, even small excess of his decreases drastically the rate constant and this is due to the inactivation of GO by the amino acid. We have previously found by using UV–VIS technique that L-his and L-glutamic acid strongly diminish the enzymatic activity with respect to transition metal species which are resistant to ligand substitution and exchange. The electrochemical data reported here support previous conclusions about the poisoning effect of charged amino acids on activity of GO [8].

In conclusion, the complex formation between $[Ru(bpy)_2(H_2O)_2]^{2+}$ and some biologically important ligands such as im and his is favorable for increasing the rate constant for oxidation of reduced GO by electrochemically generated $Ru^{III}$ species. The highest rate constants are observed for the his complex but the disadvantage of the ligand is that it strongly inhibits the activity of GO.

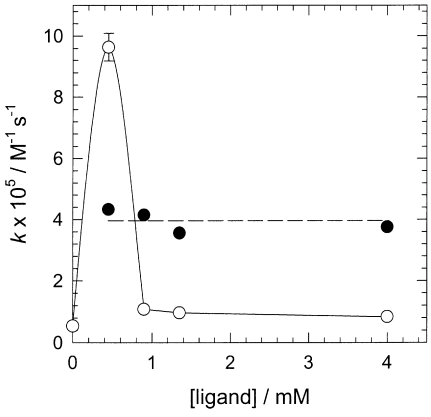


Fig. 5. Dependence of the rate constant $k$ for oxidation of GO(red) by $[Ru(bpy)_2(py)(OH)]^{2+}$ (●) and $[Ru(bpy)_2(his)(OH)]^{2+}$ (○) on the concentration of py and his, respectively: pH 7.0; 0.1 M phosphate; 25°C.

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