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Ferricenium Salts as True Substrates of Glucose Oxidase

A Steady-State Kinetic Study

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ABSTRACT

The steady-state kinetics of D-glucose oxidation by ferricenium dyes $RFc^+PF_6^-$ (R = H, Me, Et, *n*-Bu, $MeCH_2CMe_2$, and Cl) and 1,1'-$Et_2Fc^+PF_6^-$ catalyzed by glucose oxidase from *Aspergillus niger* was investigated as a function of RFc^+ and D-glucose concentrations at pH 6.7, 25°C in the presence of 2% (v/v) Triton X-100. The enzymatic bleaching is characterized by large steady-state portions on the kinetic curves for all ferricenium ions studied. The reaction follows the Michaelis-Menten kinetics demonstrating a high affinity of RFc^+ toward the active site of reduced glucose oxidase (GO). The reaction rate is weakly sensitive to the nature of RFc^+, and the apparent $V_{m(app)}$ values decrease only twofold on going from the most to the least reactive salt in the series (1,1'-Et_2Fc^+ and $ClFc^+$, respectively), although their observed redox potentials differ by 160 mV. Remarkably, the reactivity of RFc^+ does not increase with increasing their oxidative power. The apparent Michaelis constants $K_{m(app)}$ are also weakly sensitive to the nature of RFc^+. The profiles for the steady-state rate vs [HFc^+] and [D-glucose] were rationalized in terms of the "ping-pong" mechanism typical of the catalysis by GO. Ferrocenecarboxylic acid (FcCOOH) appeared to be a competitive inhibitor of GO with the inhibition constant of $(3 \pm 1) \times 10^{-3} M$. The pH profile for the ferricenium fading is bell-shaped with the optimum around 7.5. A simple routine for a rapid *in situ*

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preparation of the ferricenium dye, which is ready for spectrophotometric assaying of the GO activity, is presented. The apparent $V_{m(app)}$ and $K_{m(app)}$ values for this substrate are similar to those for $HFc^+PF_6^-$.

Index Entries: Ferricenium ions; D-glucose; glucose oxidase; steady-state kinetics; mechanism; inhibition.

INTRODUCTION

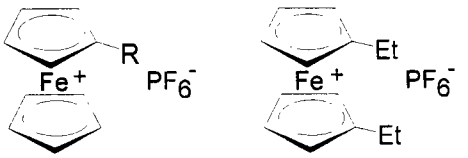
Glucose oxidase (GO) is a redox enzyme that catalyzes the oxidation of β-D-glucopyranose into γ-D-gluconolactone by dioxygen (*1*). Ferrocene (HFc) or bis(η^5-cyclopentadienyl)iron(II) is a typical organometallic compound that may undergo one-electron reversible oxidation to afford the so-called ferricenium ion or bis(η^5-cyclopentadienyl)iron(III) (*2*). Until 1984, it was difficult to imagine that these two species could have anything in common. That they would show a prominent cooperation resulted later in the buildup of a variety of practical devices. However, the pioneering work of Cass et al. has demonstrated that GO couples electrochemically with ferrocenes in the presence of D-glucose to produce large catalytic currents on cyclic voltammograms (*3*). This report initiated a "branched chain reaction," and the principle described by Cass and coworkers was widely realized in numerous amperometric glucose biosensors (*4*). Other oxidases couple with ferrocenes as well (*5*). The stoichiometric mechanism of this coupling is given by Eqs. (1)–(3).

$$E_{ox} + S \rightarrow E_{red} + P \quad (1)$$

$$E_{red} + 2\,RFc^+ \rightarrow E_{ox} + 2\,RFc \quad (2)$$

$$2\,RFc - 2\,e^- \rightarrow 2\,RFc^+ \quad (3)$$

where E_{ox} and E_{red} are oxidized and reduced enzymes, respectively, S is a substrate, and P is a product of an enzymatic reaction. Progress in this area has led to remarkable findings, among which are a covalent "wiring" of ferrocenes to the surface of GO (*6–8*), elaboration of the procedure for evaluation of the rate constants for step 2 from cyclic voltammetry data (*9*), coupling of GO with poorly water-soluble ferrocenes incorporated into micelles of various surfactants (*10*), and recognition by the enzyme of the micelle charge with solibilized *n*-alkylferrocene (*11*). Evidently, step 2, which involves the interaction of reduced enzyme with a ferricenium cation, is crucial for the catalysis of this type. Since ferricenium salts are deep-blue compounds that fade on reduction into ferrocenes (*2*) and can easily be prepared by different means (*2*), two research groups tested their behavior in the presence of GO and D-glucose to follow step 2 directly by the spectrophotometric technique (*12,13*). In fact, the procedure appeared to be very convenient for both monitoring the activity of GO and direct titration of D-glucose in solution (*14*), since minor fading was observed without either the enzyme or D-glucose. Successful utiliza-



R = H, Me, Et, n-Bu, $MeCH_2CMe_2$, Cl

Table 1
Spectral Characteristics of Substituted Ferricenium Salts
(25°C, pH 6.7, Phosphate 0.1*M*, Triton X-100 2% v/v)

Ferricenium salt	λ_{max}, nm	ϵ, M^{-1}/cm
1, 1′-$Et_2Fc^+PF_6^-$	617	260
$EtFc^+PF_6^-$	623	296
$MeFc^+PF_6^-$	622	302
$HFc^+PF_6^-$	625	210
n-$BuFc^+PF_6^-$	626	200
$MeCH_2CMe_2Fc^+PF_6^-$	625	265
$ClFc^+PF_6^-$	621	120

tion of ferricenium ions in the systems and a high reactivity of these artificial substrates toward reduced GO strongly stimulated a search for the mechanism of step 2. Being interested in mechanisms of interaction of organometallic compounds with enzymes (*15*), we have undertaken a detailed investigation of the steady-state kinetics of fading of various ferricenium salts shown in Chart 1 in the presence of GO and D-glucose, as well as the influence of pH and the effect of ferrocenecarboxylic acid as an inhibitor of the enzymatic activity. The results of this study are reported in this article.

MATERIALS AND METHODS

Reagents

Ferrocene was a Feakhim reagent. Ethylferrocene was purchased from Strem Chemicals. 1,1′-Diethyl-, *n*-butyl-, and *tert*-pentylferrocene were obtained from Aldrich. Methyl- and chloroferrocene were kindly provided by M. D. Reshetova. Ferrocenecarboxylic acid was a Fluka reagent. Ferricenium salts $RFc^+PF_6^-$ were obtained by oxidation of the corresponding ferrocenes in the concentrated sulfuric acid in the air followed by dilution the reaction mixture with water and its treatment with the concentrated aqueous solution of KPF_6 (*16*). The spectral characteristics of the compounds are summarized in Table 1.

GO was purchased from Serva and standardized with respect to the active FAD as described by Weibel and Bright (*17*). Triton X-100 was obtained from Sigma. D-Glucose, buffer components, and other chemicals used were all Reakhim reagents. Stock solutions of $RFc^+PF_6^-$ (ca. $1 \times 10^{-2}M$) for assaying D-glucose were prepared daily by dissolving the salt in 5 m*M* HCl containing Triton X-100 (2% v/v). Insoluble admixtures of ferrocene were filtered off.

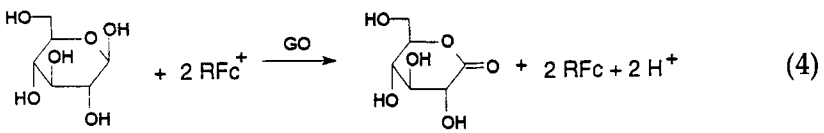
Procedures

Spectrophotometric measurements were made on a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A cell positioner/temperature controller and a Hitachi 150-20 spectrophotometer. The reduction of the ferricenium ions into RFc was followed at 25°C by monitoring a decrease in absorbance at the wavelength of maximal absorption indicated in Table 1 in solution of Triton X-100 (2% v/v) in 0.1*M* phosphate buffer, pH 6.7. The typical reaction mixture for spectrophotometric measurements contained 2 mL of the buffer, 50–600 μL of the $RFc^+PF_6^-$ solution (0.012*M*), and 180 μL of the solution of D-glucose (0.7*M*). The reaction was initiated by adding 20 μL of the solution of GO ($2.0 \times 10^{-5}M$). The changes in absorbance caused by the enzymatic reaction were normally registered every 10 s in a matter of 1 min. In some cases, background fading in the absence of GO was taken into account. All calculations were carried out on PC computers using SigmaPlot 5.0 and 1.01 packages.

RESULTS AND DISCUSSION

General Kinetic Scheme

The fading of the ferricenium dye in the presence of GO and D-glucose is shown in Fig. 1. The spectra were recorded with a 1-min interval to demonstrate a steady-state nature of the enzymatic reaction that follows the stoichiometric Eq. (4) (*14*).



(4)

Since it was demonstrated that the rate of ferricenium fading is practically unaffected by removing dioxygen (*12*), all kinetic measurements were carried out in the air. It was also confirmed that Triton X-100 taken at different concentrations had no effect on the steady-state rate. The steady-state kinetics of reaction (4) investigated at different concentrations of HFc^+ and D-glucose and these data are summarized in Fig. 2 and Table 2. The former shows that the Michaelis-Menten kinetics holds with respect to the both reagents, whereas the latter points to an approximate constancy

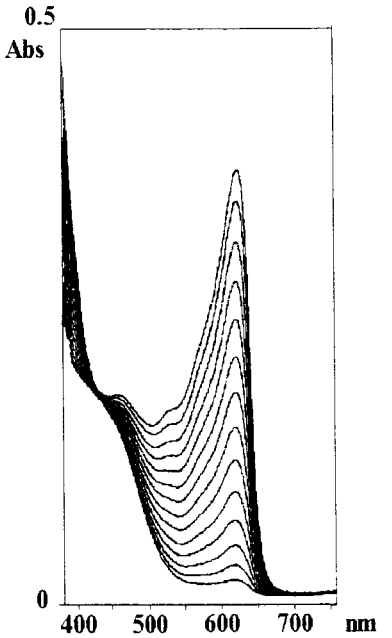


Fig. 1. Fading of $HFc^+PF_6^-$ in the presence of GO ($2.7 \times 10^{-7}M$) and D-glucose (0.1*M*) at 25°C, pH 6.7 (0.1*M* phosphate). Spectra were recorded with 1-min interval.

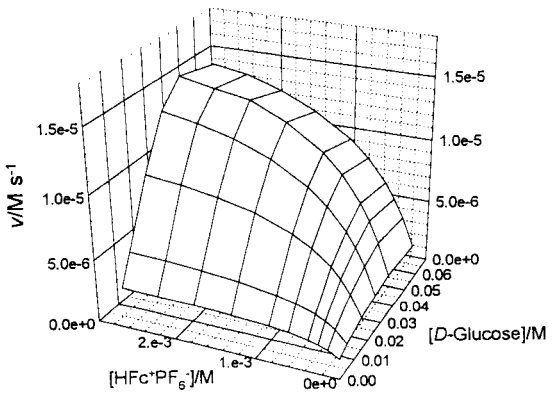


Fig. 2. Steady-state rate of GO-catalyzed reduction of $HFc^+PF_6^-$ as a function of $HFc^+PF_6^-$ and D-glucose concentrations. The conditions are as in Table 2.

of the ratio $V_{m(app)}/K_{m(app)}$ in the D-glucose concentration range (0.525–5.6) $\times$ $10^{-2}M$ indicating that the so-called ping-pong reaction mechanism (*18,19*), which is generally accepted in the glucose oxidase catalysis (*1*), might be operative. In the framework of the present investigation, it can be represented as follows

$$\text{GO(ox)} + \text{D-glucose} \leftrightharpoons \{\text{GO(ox)}, \text{D-glucose}\} \qquad (k_1, k_{-1}) \quad (5)$$

$$\{\text{GO(ox)}, \text{D-glucose}\} \rightarrow \text{GO(red)} + \delta\text{-D-gluconolactone} \qquad (k_2) \quad (6)$$

Table 2
Apparent Parameters of the Michaelis-Menten Equation of Fading of $HFc^+PF_6^-$, Obtained at Different Concentrations of D-Glucose (25°C, pH 6.7, Phosphate 0.1*M*, Triton X-100 2%, [GO] = $2 \times 10^{-7}M$)

[Gl], *M*	$K_{m(app)}$, *M*	$V_{m(app)}$, *M*/s
5.25×10^{-3}	$(2.4 \pm 0.2) \times 10^{-4}$	$(2.03 \pm 0.05) \times 10^{-6}$
1.05×10^{-2}	$(3.9 \pm 0.2) \times 10^{-4}$	$(5.17 \pm 0.08) \times 10^{-6}$
2.1×10^{-2}	$(7.3 \pm 0.5) \times 10^{-4}$	$(1.20 \pm 0.05) \times 10^{-5}$
3.15×10^{-2}	$(7.3 \pm 0.5) \times 10^{-4}$	$(1.68 \pm 0.04) \times 10^{-5}$
4.2×10^{-2}	$(7.3 \pm 0.5) \times 10^{-4}$	$(1.96 \pm 0.06) \times 10^{-5}$
5.6×10^{-2}	$(7.9 \pm 0.9) \times 10^{-4}$	$(1.82 \pm 0.09) \times 10^{-5}$

$$GO(red) + RFc^+ \rightleftharpoons \{GO(red), RFc^+\} \qquad (k_3, k_{-3}) \quad (7)$$

$$\{GO(red), RFc^+\} \rightarrow GO(ox) + RFc \qquad (k_4) \quad (8)$$

Here GO(ox) and GO(red) are the oxidized (native) and reduced forms of the enzyme, respectively. The key simplifications employed in Scheme 1 concern (1) the neglecting of the effect of dioxygen and (2) the assumption that the transfer of the first electron from the reduced flavin adenine dinucleotide by ferricenium ion is the rate-limiting step, whereas transfer of the second electron occurs much faster and does not affect the overall steady-state kinetics. Applying the steady-state approximation with respect to all intermediates involved in Scheme 1 and taking into account the mass balance equation with respect to all forms of GO, one arrives at Eq. (9) for the steady-state rate of ferricenium fading.

$$v = (k_2k_4[E]_0[Gl]_0[HFc^+]_0) / \{k_4K_m^{Gl}[HFc^+]_0 + k_2K_m^{Fc}[Gl]_0 + (k_2 + k_4)[Gl]_0[HFc^+]_0\} \quad (9)$$

Here $[E]_0$, $[Gl]_0$, and $[HFc^+]_0$ are the total concentrations of glucose oxidase, D-glucose, and ferricenium ion, respectively; $K_m^{Fc} = (k_4 + k_{-3})/k_3$ and $K_m^{Gl} = (k_2 + k_{-1})/k_1$. At a fixed concentration of D-glucose Eq. (9) rearranges into Eq. (10).

$$v = a_1[HFc^+]_0 / (a_2[HFc^+]_0 + a_3) \quad (10)$$

where $a_1 = k_2k_4[E]_0[Gl]_0$, $a_2 = (k_2 + k_4)[Gl]_0 + k_4K_m^{Gl}$, and $a_3 = k_2K_m^{Fc}[Gl]_0$. We attempted to derive the parameters a_1, a_2, and a_3 of Eq. (10) at different concentrations of D-glucose by the nonlinear least-squares method using data, such as shown in Fig. 2. The experimental data were fitted to Eq. (10) varying initial setting values of the parameters and the constraints. The parameters calculated with the best precision in terms of the standard errors and the dependencies were considered valid. All three must be a linear function of glucose concentration, i.e., $a_1 = \alpha_1[Gl]_0 + \beta_i$, without and with the intercept for a_1 or a_3 ($\beta = 0$) and a_2, respectively. Thus, the parameters a_1, a_2, and a_3 obtained at different concentrations of D-glucose

Table 3
Values of α_i and β_i Derived from the Best-Fit Parameters of Eq. (10) Obtained at Different [D-glucose][a]

	Coefficients of the equation $a_i = \alpha_i[Gl] + \beta_i$	
Parameter of Eq. (10)	α	β
a_1	1.87 ± 0.19	0
a_2	(1.7 ± 2.5) × 10^4	(4.3 ± 0.97) × 10^3
a_3	95 ± 12	0

[a]The conditions are as in Table 2.

Table 4
Intrinsic Kinetic Characteristics of GO Toward D-Glucose and the Ferricenium Ion in Terms of Eqs. (5)-(8) Calculated from the Data Shown in Table 3[a]

Parameter	Estimate
k_2, s^{-1}	570
$K_m^{Gl} = (k_2 + k_{-1})/k_1$, M	0.27
k_4, s^{-1}	1.6 × 10^4
$K_m^{Fc} = (k_4 + k_{-3})/k_3$, M	0.17

[a]The conditions are as in Table 2.

were used for evaluation of three pairs of α_i and β_i (Table 3). The latter provide a system of four equations with four unknowns k_2, K_m^{Gl}, k_4, and K_m^{Fc}, which are believed to be intrinsic characteristics of the enzymatic system given by Eq. (4). The calculated parameters are summarized in Table 4. It should be noted that the solution of the quadratic equation gives two sets of data. The one presented in Table 4 was chosen on the basis of our current knowledge on the reactivity of GO toward D-glucose and ferricenium ions. The second set, although also possible, does not seem that probable because the numerical values of the parameters found are in worse agreement with the data known from the literature. The set placed in Table 4 deserves a comment.

The value of k_2 is in a perfect agreement with that of 800 s^{-1} reported by Weibel and Bright (*17*), which was obtained using a basically different approach. Second, the ratio k_4/K_m^{Fc}, which refers to the bimolecular interaction of reduced GO with the ferricenium ion, equals ca. 1 × $10^5 M^{-1}$/s, and this must be compared with that of 0.8 × $10^5 M^{-1}$/s found directly from the electrochemical measurements under practically identical conditions (*10*). The second-order rate constant for the oxidation of reduced GO by dioxygen equals 2 × $10^6 M^{-1}$/s (*17*), by an order of magnitude higher

Table 5
Apparent Parameters of the Michaelis-Menten Equation for Substituted Ferricenium Salts (25°C, pH 6.7, Phosphate 0.1*M*, Triton X-100 2%, [D-glucose] = 5×10^{-2}*M*, [GO] = 2×10^{-7}*M*)

Ferricenium salt	$K_{m(app)}$, *M*	$V_{m(app)}$, *M*/s	$E_{1/2}$, pH 7.0, phosphate, Triton, X-100, 0.05*M*
1,1′-$Et_2Fc^+PF_6^-$	$(7.4 \pm 1.3) \times 10^{-4}$	$(1.8 \pm 0.1) \times 10^{-5}$	180
$EtFc^+PF_6^-$	$(8.1 \pm 1.3) \times 10^{-4}$	$(1.5 \pm 0.1) \times 10^{-5}$	190
$MeFc^+PF_6^-$	$(8.6 \pm 0.9) \times 10^{-4}$	$(1.4 \pm 0.05) \times 10^{-5}$	190
$HFc^+PF_6^-$	$(8.9 \pm 0.7) \times 10^{-4}$	$(1.37 \pm 0.03) \times 10^{-5}$	195
n-$BuFc^+PF_6^-$	$(1.2 \pm 0.2) \times 10^{-3}$	$(1.3 \pm 0.1) \times 10^{-5}$	240
$MeCH_2CMe_2Fc^+PF_6^-$	$(1.5 \pm 0.2) \times 10^{-3}$	$(1.2 \pm 0.1) \times 10^{-5}$	245
$ClFc^+PF_6^-$	$(1.5 \pm 0.7) \times 10^{-3}$	$(0.9 \pm 0.2) \times 10^{-5}$	340
$HFc^+PF_6^-$ with 5% EtOH	$(3.7 \pm 0.3) \times 10^{-4}$	$(1.7 \pm 0.04) \times 10^{-5}$	
$HFc^+PF_6^-$	$(6.8 \pm 2.3) \times 10^{-4}$	$(1.47 \pm 0.2) \times 10^{-5}$	

than that by HFc^+, but since the concentration of HFc^+ can be made much higher than that of O_2 in salty aqueous solutions, it becomes clear why the GO–ferricenium couple is practically oxygen-independent.

Substituted Ferricenium Salts

A rich chemistry of ferrocenes (*2*) gives an attractive chance to vary systematically the electronic and steric properties of ferricenium substrates. Their bulkiness was changed with care, since exhaustive methylation of ferrocene makes the corresponding oxidized decamethylferrocene unreactive toward GO (*10*). All salts shown in Chart 1 were tested in reaction (4) to reveal again the Michaelis dependence of the steady-state rate on $RFc^+PF_6^-$ concentration and the data, i.e., the apparent values $V_{m(app)}$ and $K_{m(app)}$ at 0.05*M* D-glucose, are summarized in Table 5, together with the observed redox potentials $E_{1/2}$, which cover the range of 180–340 mV vs SCE. Rather unexpectedly, both parameters are poorly sensitive to the nature of RFc^+. In particular, the $K_{m(app)}$ increases, but $V_{m(app)}$ decreases by a factor of two on going from 1,1′-Et_2Fc^+ to $ClFc^+$, although their $E_{1/2}$ differs by 160 mV. Thus, reaction (4) is insensitive to the nature of ferricenium salt. It seems natural that the values of $K_{m(app)}$ decrease slightly as substituents become bulkier. More surprising is the highest $V_{m(app)}$ value for the ferricenium salt with the lowest observed redox potential, viz. for 1,1′-Et_2Fc^+. It appears that the weakest oxidant is the most efficient in oxidation of reduced flavin adenine dinucleotide in GO(red). Only nonsubstituted ferricenium ion is competitive with 1,1′-Et_2Fc^+. This fact is difficult to interpret bearing in mind the existence of a good linear dependence of $\ln V_{m(app)}$ against $E_{1/2}$ (not shown).

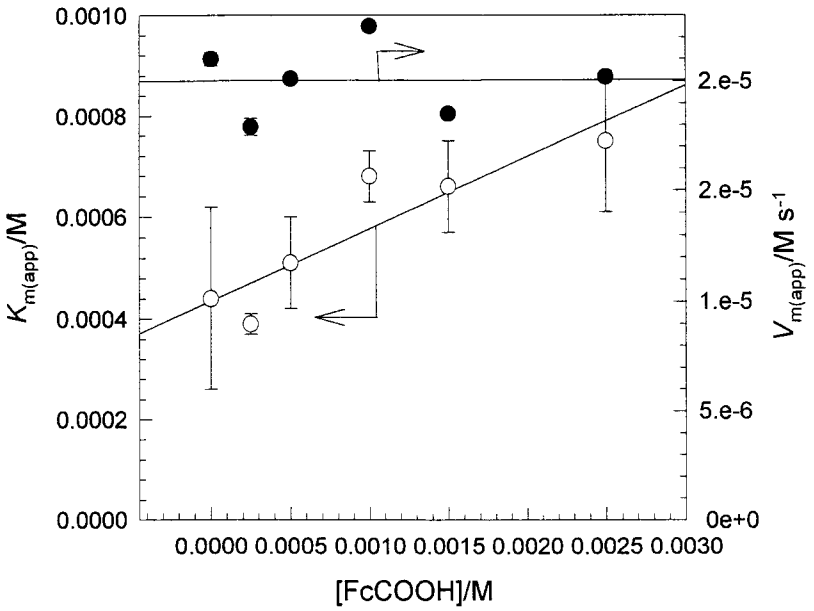


Fig. 3. Dependence of $K_{m(app)}$ (opened circles) and $V_{m(app)}$ (closed circles) on concentration of FcCOOH at [D-glucose] = 0.05*M*, [GO] = 2×10^{-7}*M*, 25°C, pH 6.7 (0.1*M* phosphate), [Triton X-100] = 2% (v/v).

Ferrocenecarboxylic Acid as Competitive Inhibitor

An evident binding of ferricenium salts with reduced GO and a close geometrical similarity between cations RFc^+ and reduced ferrocenes RFc (*2*) suggests that the latter might be inhibitors of GO. In order to investigate such a possibility, the apparent values of $V_{m(app)}$ and $K_{m(app)}$ in the case of HFc^+ were evaluated at different concentrations of ferrocenecarboxylic acid. The choice of FcCOOH was dictated by a higher redox potential of the acid ($\Delta E_{1/2}$ = 0.110 V) precluding the rapid electron exchange between HFc^+ and FcCOOH. The data are given in Fig. 3. It is seen that $V_{m(app)}$ is fairly constant, whereas $K_{m(app)}$ increases with increasing [FcCOOH], indicative of a competitive inhibition. A slope of the linear plot of $K_{m(app)}$ vs [FcCOOH] (Fig. 3) provides an estimate for the inhibition constant $K_i = (3 \pm 1) \times 10^{-3}M$, since $K_{m(app)} = K_s (1 + [FcCOOH]/K_i)$ (*20*). The inhibition constant is by an order of magnitude higher than the apparent Michaelis constants, suggesting that the inhibiting ability of ferrocenes is only marginal.

pH Profile for Ferricenium Fading

Catalysis by GO is known for its different sensitivity to pH depending on the nature of the substrate, and this makes the basis for classification of substrates of GO (*1*). Therefore, the pH dependence of reaction (4) was

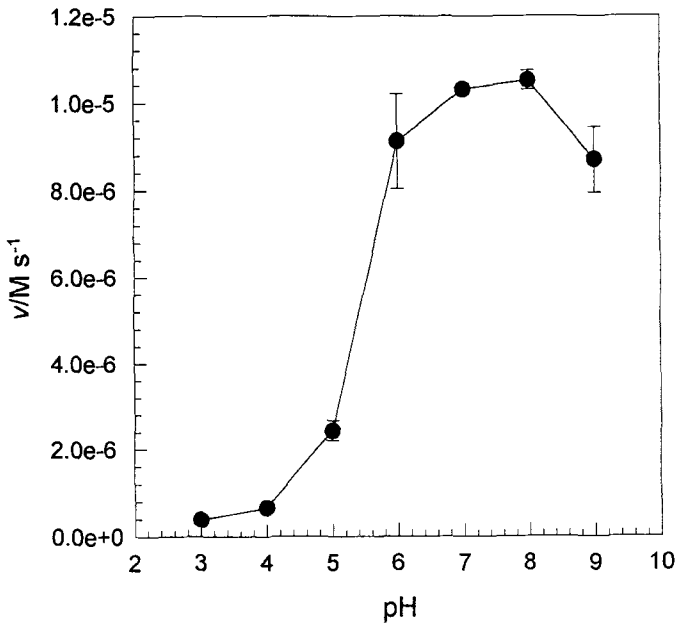


Fig. 4. pH profile for the steady-state rate of GO-catalyzed reduction of $HFc^+PF_6^-$ at [$HFc^+PF_6^-$] = 0.002*M*, [D-Glucose] = 0.05*M*, [GO] = $2 \times 10^{-7}M$, 25°C, phosphate buffer, [Triton X-100] × 2% (v/v).

investigated, and the results are in Fig. 4. The maximal rate is observed around pH 7.5 in the phosphate buffer, and this gives additional evidence that ferricenium salts refer to the group II together with methylene blue and toluidine blue, as well as tetrathiafulvalene, tetracyanoquinodimethane, and benzylviologen (*1*).

Effect of Ethanol

In our previous electrochemical study (*10*), we found that GO seemed to be more active in the presence of 5% EtOH (v/v). To prove that the effect is not owing to an electrochemical artifact, we have measured the rate of HFc^+ fading in the presence of low content of the organic cosolvent. It is seen from Fig. 5 that there is a rate increase, then the rate reaches its maximal value, and finally decreases as [EtOH] is ca. 10–12%. The apparent values of $V_{m(app)}$ and $K_{m(app)}$ determined in the presence of 5% EtOH (Table 3) show that both parameters are affected such that the observed reactivity does increase. The ratio $V_{m(app)}/K_{m(app)}$ is by a factor of 3 higher in 5% EtOH compared to the ethanol-free system. Thus, an easy tool increase the activity of GO is to add the small amount of the alcohol.

Express Preparation of Ferricenium Dyes

A practical conclusion of this study is that ferricenium dyes are convenient substrates of GO, which are rather inexpensive and now commercially available from Aldrich as the $HFc^+PF_6^-$ and $HFc^+BF_4^-$ salts.

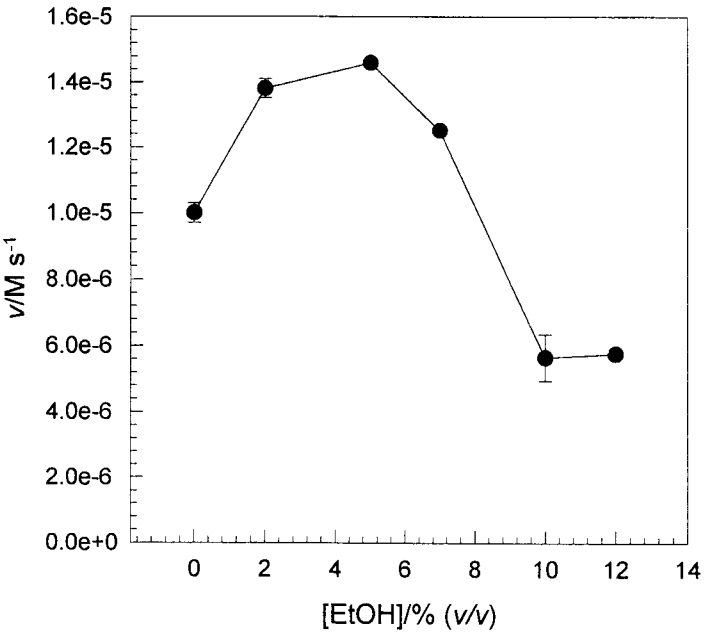


Fig. 5. Effect of EtOH on the steady-state rate of GO-catalyzed reduction of $HFc^+PF_6^-$ at [$HFc^+PF_6^-$] = 0.002*M*, [D-Glucose] = 0.05*M*, [GO] = 2×10^{-7}*M*, 25°C, pH 6.7 (0.1*M* phosphate), [Triton X-100] = 2% (v/v).

However, it is possible to prepare *in situ* a ferricenium substrate from ferrocene just before use. The procedure is very trivial and involves a treatment of HFc with a small amount of concentrated H_2SO_4 followed by dilution with the phosphate buffer, pH 6.7. The solution can be standardized using the extinction coefficient for HFc^+ from Table 1. The values of $V_{m(app)}$ and $K_{m(app)}$ obtained for these prepared substrates are practically the same as those obtained in the case of $HFc^+PF_6^-$, Table 5.

Mechanistic Considerations: a "Ball-Funnel" Model

The crystal structure of GO from *Aspergillus niger* was recently reported (*21*), and the structural data give a clue for understanding why ferricenium ions behave like true substrates of the enzyme. It has been found that the access to flavin comprises a large deep pocket, which is a funnel-shaped with the cross-section at the top approximately 10 × 10 Å. Let us imaginarily transform the ferricenium cation into a ball, the diameter of which is around 5.6 Å,** that fits perfectly the above-mentioned funnel. Probably, there is no better trap for a ball than a funnel provided the strict geometric correspondence of the two objects. Therefore, it seems very reasonable that ferricenium ions enter the funnel-shaped binding site where they are kept bound via the hydrophobic interactions involving Trp426, which is known to form a small hydrophobic area opposite to the flavin system

**The estimate comes from the mean Fe-H value of 2.81 Å observed in ferrocene, *see* p. 10 of ref (*2*).

(*21*). Other candidates for stabilizing the hydrophobic contacts are Tyr68 and especially Tyr515 (*21*). It seems plausible that additionally affinity of RFc^+ toward the active site arises from the presence of an excessive negative charge at the reduced flavin system. A proper settlement of RFc^+ at the vicinity of the reduced flavin may facilitate the following electron transfer. The "funnel-ball" model described accounts for both the high affinity of RFc^+ toward GO manifested in $K_{m(app)}$ and high rates of electron transfer manifested in k_4. It helps to understand why a reasonably bulky, but a single group R does not dramatically decrease both $K_{m(app)}$ and $V_{m(app)}$, whereas exhaustive methylation makes the corresponding ferricenium cation completely unreactive. In the former case, a single R group does not preclude the entering of the tailored ball into the funnel provided the tail is always directed at its 10 × 10 Å top, since the diameter of the tailored ball is basically unchanged. The diameter of permethylated ferricenium must be much bigger, approx 9 Å. The size would either completely preclude the entrance into the funnel, or such a substrate will finally be fixed too far away from the reduced flavin system and the electron transfer will be hampered. A too long alkyl chain is also very unfavorable. In particular, the cation derived from *n*-dodecylferrocene does not couple with GO (*10*). This may be because of the ability of the molecule to increase its diameter at the expense of covering the bis(η^5-cyclopentadienyl)iron(III) core by the dodecyl radical.

CONCLUSION

The analysis of the steady-state kinetics of the GO-catalyzed reduction of ferricenium ions by D-glucose in terms of the "ping-pong" mechanism shown in Scheme 1 gives an entry to the intrinsic rate constants for the oxidation of D-glucose by glucose oxidase and oxidation of reduced GO by the ferricenium ion. The values obtained show that the efficacy of the organometallic substrate is quite competitive with that of D-glucose. This high reactivity was accounted for in terms of the "ball-funnel" model according to which the ferricenium ion is treated as a ball that fits the funnel-shaped access to the flavin moiety of glucose oxidase.

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