Enantioselectivity in Enzyme-Catalyzed Electron Transfer to and from Planar Chiral Organometallic Compounds

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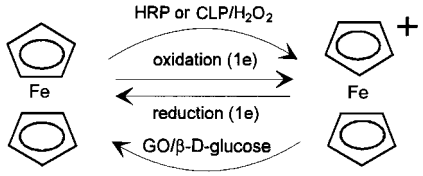
Abstract: Asymmetric cyclopalladation of dimethylaminomethylferrocene in the presence of *N*-acetyl-(*R*)- or (*S*)-leucine afforded enantiomerically enriched palladacycles (*S*)- and (*R*)-$[Pd\{C_5H_3(CH_2NMe_2)FeC_5H_5\}(\mu\text{-}Cl)]_2$, respectively. Carbonylation of each enantiomer followed by iodomethylation and reduction by sodium amalgam gave (*S*)- and (*R*)-2-methylferrocene carboxylic acid (**1**) with an optical purity of 80 and 93%, respectively. (*S*)- and (*R*)-**1** readily undergo one-electron (1e) oxidation to form the corresponding ferricenium cations by hydrogen peroxide, catalyzed by horseradish peroxidase (HRP) and chloroperoxidase (CLP) from *Caldariomyces fumago* (25 °C, pH 5–8 and 2.75, respectively). In the case of HRP, the reaction is strictly first-order with respect to (*S*)- and (*R*)-**1** (rate $= k$[HRP][**1**]), whereas Michaelis–Menten kinetics are observed for CLP. The strongly pH-dependent kinetic enantioselectivity is, however, only observed in the case of HRP. HRP-generated cations (*S*)-$\mathbf{1}^+$ and (*R*)-$\mathbf{1}^+$ have been used to demonstrate that their enzymatic reduction by reduced glucose oxidase (GO) is also enantioselective; the (*S*)-$\mathbf{1}^+$ enantiomer is more reactive than (*R*)-$\mathbf{1}^+$ by a factor of 1.54. The existence of the planar chiral enantioselectivity in the GO catalysis was also confirmed by the cyclic voltammetry study of (*S*)-**1** and (*R*)-**1** in the presence of GO and β-D-glucose with glassy carbon and pyrolytic graphite electrodes. The corresponding enantioselectivity factors $k(S)$-$\mathbf{1}^+/k(R)$-$\mathbf{1}^+$ are 1.7 and 1.6, respectively. Based on the known X-ray structural data for the active site of GO, it has been tentatively suggested that the enantioselectivity originates from the hydrophobic contact between the enzyme tyr-68 residue and the η^5-C_5H_5 ring of $\mathbf{1}^+$, and a hydrogen bond network formed by his-516 and/or his-559 residues and the carboxylic group of the ferrocene derivative. The findings reported confirm the existence of enantioselective electron transfer between oxidoreductases and organometallic compounds with a planar chirality. The lack of kinetic enantioselectivity may be a result of i) the incorrect rate-limiting step, ii) unfavorable pH region, and iii) the deficit of charged groups attached to ferrocenes.

Keywords: chiral recognition · electron transfer · ferrocenes · oxidoreductases · planar chirality

Introduction

Our recent investigations in the field of organometallic biochemistry[1, 2] have demonstrated that the ferrocene/ferricenium couple is unique for redox enzymes that are capable of efficient electron transfer to and from the ferricenium cation (Fc^+) and ferrocene (Fc) (Scheme 1). In particular, ferrocenes are oxidized by hydrogen peroxide in the presence of horseradish peroxidase (HRP) and chloroperoxidase (CLP). In the former case, the enzymatic reaction is characterized by unexpected first-order kinetics with respect to the ferrocene derivative, however the reactivity under the steady-state[3] and stopped-flow[4] conditions is comparable with that for typical organic substrates associated with HRP. Since 1984 ferricenium ions have been known to be excellent oxidants of reduced glucose oxidase {GO(red)}, which is produced during



Scheme 1. Electron transfer to and from ferrocene and the ferricenium cation.

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the oxidation of β-D-glucose to D-gluconolactone.[5] This was confirmed in a variety of electrochemical studies by many research groups[6–9] including ourselves.[10, 11] Recently, a detailed analysis of the steady-state kinetic data, which was obtained spectrophotometrically by monitoring the presence of ferricenium dyes $RFc^+PF_6^-$ (R = alkyl group), was carried out with respect to the interaction between GO(red) and RFc^+.[12] It was found that the reduction of RFc^+ follows Michaelis–Menten kinetics, and the intrinsic kinetic parameters for HFc^+ fall in the same range that is typical for β-D-glucose. Thus, there is evidence that ferrocene derivatives effectively mediate biocatalyzed reactions. This presents the possibility that the enzymatic transformations of ferrocenes might also proceed stereoselectively.

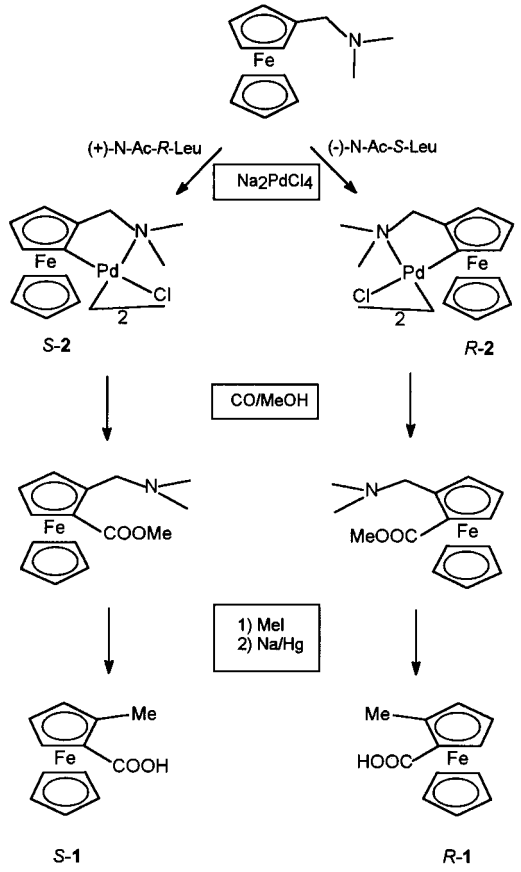
Ferrocene derivatives and related organometallics are nowadays recognized substrates of proteases and oxidoreductases in synthetically relevant reactions that are aimed at modifying side-chain functional groups.[13–16] Enantiomers of organometallics with planar chirality can display different reactivity or be accumulated with distinct rates that allows for kinetic resolution.[1, 2] These examples and our findings described above raise the question of whether it is possible to observe enantioselectivity in an electron-transfer process involving a redox enzyme and a planar chiral organometallic molecule, that is, unnatural substrate with unnatural chirality type? Whilst this manuscript was in preparation, a preliminary communication was published in which planar-chiral enantioselectivity was observed in oxidation catalyzed by wild-type and mutant forms of cytochrome *c* peroxidase.[17] However, the question is still intriguing when we take into consideration the controversial publications by Willner et al.[18] and Savéant et al.[19] The former has claimed that ferrocene derivatives with a central carbon chirality, namely the *R* and *S* enantiomers of Me_2NC^*MeHFc, show different reactivity in the oxidized state towards GO and glutathione reductase. The latter group has been unable to reproduce these results. In this work, we demonstrate that it is possible to achieve stereoselectivity for the planar chiral ferrocene molecules[20–23] in the presence of several oxidoreductases under properly selected conditions. In particular, we describe i) the optimized synthetic approach to (*S*)-2-methylferrocene carboxylic acid, (*S*)-**1**, and (*R*)-2-methylferrocene carboxylic acid, (*R*)-**1**, the key step of which is asymmetric cyclopalladation of dimethylaminomethyferrocene in the presence of either *N*-acetyl-(*R*)- or (*S*)-leucine; ii) the spectrophotometrically measured kinetic data of the HRP- and CLP-catalyzed oxidation of (*S*)-**1** and (*R*)-**1** by H_2O_2 revealing the pH-dependent enantioselectivity for the former enzyme and its absence for the latter; iii) the enantioselective reduction of ferricenium cations (*S*)-$\mathbf{1}^+$ and (*R*)-$\mathbf{1}^+$ by GO(red) in the presence of β-D-glucose evaluated by both conventional spectrophotometry and cyclic voltammetry with two different carbon electrodes.

Abstract in Russian:

Асимметрическое циклопалладирование диметиламинометилферроцена в присутствии N-ацетил-*R*- или *S*-лейцина приводит к энантиомерно обогащенным палладациклам *S*- и *R*-$[Pd\{C_5H_3(CH_2NMe_2)FeC_5H_5\}(\mu\text{-}Cl)]_2$ соответственно. Карбонилирование каждого энантиомера с последующим иодметилированием и восстановлением амальгамой натрия дает *S*- и *R*-2-метилферроценкарбоновые кислоты (**1**) с оптической чистотой 80 и 93% соответственно. Под действием H_2O_2 в присутствии пероксидазы из корней хрена (ПХ) или хлорпероксидазы из *Caldariomyces fumago* (ХЛП) *S* и *R* энантиомеры **1** легко превращаются в соответствующие феррициниевые катионы. При катализе ПХ реакция имеет первый порядок по отношению к *S*- и *R*-**1** (скорость=k[ПХ][**1**]), в то время как для реакции, катализируемой ХЛП, реализуется кинетика Михаэлиса-Ментен. Наблюдаемая только в случае ПХ энантиоселективность зависит от pH, причем наибольший эффект проявлялся при pH 7. Константы скорости окисления *R*- и *S*-**1** энантиомеров в этом случае соответственно равны 4.6×10^4 и 2.5×10^4 M^{-1} c^{-1}. Катионы *S*-$\mathbf{1}^+$ и *R*-$\mathbf{1}^+$, получаемые при окислении **1** пероксидом водорода в присутствии ПХ, использовались в качестве субстратов в катализируемой глюкозооксидазой (ГО) реакции с β-D-глюкозой. Ферментативное восстановление *S*-$\mathbf{1}^+$ и *R*-$\mathbf{1}^+$ также энантиоселективно. В катализируемой ГО реакции *S*-$\mathbf{1}^+$ энантиомер реакционноспособнее *R*-$\mathbf{1}^+$ в 1.54 раза. Энантиоселективность катализа ГО по отношению к молекулам с элементами планарной хиральности была подтверждена методом циклической вольтамперометрии на примере *S*-$\mathbf{1}^+$ и *R*-$\mathbf{1}^+$ с использованием стеклоуглеродного и пирографитового электродов. Соответствующие факторы энантиоселективности k(*S*-$\mathbf{1}^+$)/k(*R*-$\mathbf{1}^+$) равны 1.7 и 1.6. Основываясь на данных рентгеноструктурного анализа, полученных для активного центра ГО, предполагается, что энантиоселективность возможна благодаря гидрофобным взаимодействиям между тирозиновым остатком белковой глобулы фермента tyr68 и η^5-C_5H_5 кольцом $\mathbf{1}^+$, а также благодаря водородным связям, образующимся между остатками his516 и/или his559 и карбоксильной группой $\mathbf{1}^+$. Полученные результаты показывают, что перенос электрона между оксидоредуктазами и металлоорганическими веществами, обладающими элементами планарной хиральности, может происходить стереоселективно. Отсутствие энантиоселективности может быть обусловлено следующими причинами *(i)* "неправильной" скоростьлимитирующей стадией, *(ii)* неправильно выбранной областью pH, *(iii)* отсутствием заряженных групп в молекуле ферроцена.

Results

Synthesis of planar chiral ferrocene by cyclopalladation: A synthetic approach to a pair of planar chiral enantiomers is shown in Scheme 2. The key step is asymmetric cyclopalladation of dimethylaminomethylferrocene by Na_2PdCl_4 in the presence of sodium salts of *N*-Ac-(*R*)-Leu or *N*-Ac-(*S*)-Leu. The ability of enantiomerically pure *N*-acetyl amino acids to



Scheme 2. Reaction scheme for the preparation of (*S*)-**1** and (*R*)-**1**.

induce the preferential formation of planar chiral enantiomers has already been established.[24] However, the whole reaction sequence was previously performed for only one enantiomeric series.[20, 22] Here we report on the substantially improved preparative synthesis of both series in order to obtain a pair of enantiomerically pure target molecules. The absolute configuration and the enantiomeric purity of the key organopalladium compounds **2** and their derivatives were determined as previously described.[21]

The procedure in Scheme 2 is easier and more efficient than previously reported methods of asymmetric synthesis of planar chiral ferrocene derivatives,[25, 26] in which interest has markedly increased in recent years.[27] Its most advantageous feature is an easy access to both planar chiral enantiomers. Moreover, an alternative chemical approach to such molecules[26] is more laborious and hardly applicable to the preparation of 2-methylferrocene carboxylic acid. The successful realization of the transformations in Scheme 2 demonstrates once again the high potential of palladacycles in a variety of organic syntheses.[28]

HPR-catalyzed oxidation of (*R*)- and (*S*)-1 by H_2O_2: Compound **1** is soluble in water at pH > 6, that is, when the acid is deprotonated.[29] This means that the HRP-catalyzed oxidation of ferrocenes by H_2O_2, which follows 2:1 stoichiometry, can be carried out in the absence of surfactants that were previously necessary in order to increase the solubility of alkylferrocenes.[3] The steady-state rate of oxidation of (*R*)- and (*S*)-**1** by H_2O_2 at pH 7 in the presence of HRP as a function of [(*R*)-**1**] and [(*S*)-**1**] is shown in Figure 1. As in

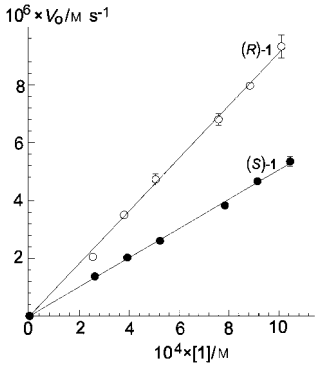


Figure 1. Steady-state rate of HRP-catalyzed oxidation of (*R*)-**1** and (*S*)-**1** by H_2O_2 (2×10^{-4} M) as a function of concentration of **1**: pH 7, 25 °C, [HRP] = 10^{-7} M.

previous work,[3] the first-order kinetics associated with **1** emphasize the different reactivities of the *R* and *S* enantiomers. Since the reaction is first-order in HRP, the corresponding observed second-order rate constants ($k = v_o/[\mathbf{1}][\mathrm{HRP}]$) are $(4.6 \pm 0.4) \times 10^4$ and $(2.5 \pm 0.3) \times 10^4$ $M^{-1}s^{-1}$ for (*R*)-**1** and (*S*)-**1**, respectively, ([H_2O_2] = 2×10^{-4} M, pH 7, and 25 °C). As expected, the rate constant for the racemate (*R*,*S*)-**1**, $(3.8 \pm 0.6) \times 10^4$ $M^{-1}s^{-1}$, lies between the two values. The effect appears to be strongly pH dependent and the resulting curve is bell-shaped (Figure 2b). The enantioselectivity almost

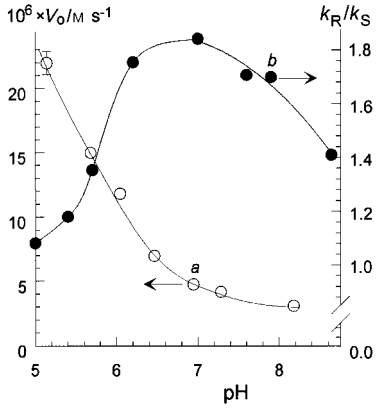


Figure 2. a) Steady-state rate of HRP-catalyzed oxidation of (*R*,*S*)-**1** as a function of pH: [**1**] = 8×10^{-4} M, [H_2O_2] = 2×10^{-4} M, [HRP] = 10^{-7} M, 25 °C. b) Effects of pH on the HRP enantioselectivity in terms of k_R/k_S ratio for HRP-catalyzed oxidation of **1** versus solution pH: [H_2O_2] = 2×10^{-4} M, [HRP] = 10^{-7} M, 25 °C.

vanishes at pH 5. Curiously, the activity of HRP is higher at lower pH, (Figure 2a). Therefore, the highest enzymatic activity is not required for achieving the highest enantioselectivity; this is observed for the electron-transfer enzymatic process that occurs without kinetically meaningful enzyme–substrate binding.

CLP-catalyzed oxidation of (*R*)- and (*S*)-1 by H_2O_2: Our recent study of the mechanism of CLP-catalyzed halogenation led to the conclusion that stereoselectivity cannot be achieved in this process.[30] However, selectivity is observed when CLP displays peroxidase activity, that is, when the CLP-catalyzed oxidation by H_2O_2 proceeds in the absence of halide ions.[31–33] The pH optimum of CLP is around 3 and the enzyme is inactive toward ferrocenes at pH 5. Therefore, the oxidation kinetics of **1** were measured at pH 2.75 and 25 °C in the presence of Triton X-100, since the protonated acid is not soluble enough in acidic aqueous solution. The steady-state rate of generation of the ferricenium dye as a function of concentration of racemic and enantiomerically pure forms of **1** is shown in Figure 3. The figure shows a Michaelis–Menten-type dependence that is not, however, accompanied by enantioselective discrimination. The maximal rates $V_{m,obs}$ and the Michaelis constants $K_{m,obs}$ with different concentrations of Triton X-100 are summarized in Table 1.

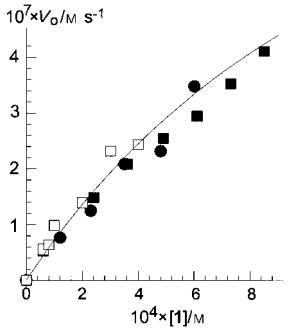


Figure 3. Steady-state rate of CLP-catalyzed oxidation of (*R*)-**1** (■), (*S*)-**1** (□), and (*R*,*S*)-**1** (●) by H_2O_2 (1.4×10^{-4} M) as a function of concentration of **1**: pH 2.75, 25 °C, [CLP] = 10^{-7} M.

The different behavior of HRP and CLP with respect to (*R*)- and (*S*)-**1** may be accounted for in terms of the pH effect as suggested by the pH-dependent enantioselectivity in the case of HRP for which the k_R/k_S ratio is almost unity at pH 5.

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Table 1. The values of $V_{m,obs}$ and $K_{m,obs}$ for the CLP-catalyzed oxidation of **1** and RFc at different concentrations of Triton X-100. Conditions: pH 2.75, 25 °C, [CLP] = 10^{-7} M.

	[Triton X-100] [mM]	$10^6 \times V_{m,obs}$ [M s^{-1}]	$10^6 \times V_m$[a] [M s^{-1}]	$10^3 \times K_{m,obs}$ [M]
1[b]	50	1.2 ± 0.2	1.3 ± 0.2	8.7 ± 1.18
	67.5	1.6 ± 0.4		11 ± 4
	85.3	1.2 ± 0.2		15.7 ± 3.0
	100	1.04 ± 0.07		16.4 ± 1.6
HFc	50	0.4 ± 0.05	0.49 ± 0.07	7.2 ± 1.3
	67.5	0.5 ± 0.05		13.3 ± 1.5
	100	0.54 ± 0.28		17 ± 11
	116	0.54 ± 0.31		18 ± 11
EtFc	3.25	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.3
	8.14	1.4 ± 0.1		2.3 ± 0.4
	16.4	0.93 ± 0.06		3.3 ± 0.6
	33.1	1.0 ± 0.1		5.2 ± 1.4
	85.3	0.9 ± 0.1		12 ± 3
BuFc	16.4	0.44 ± 0.04	0.53 ± 0.08	3.4 ± 0.9
	33.1	0.48 ± 0.07		5.4 ± 2.1
	50.1	0.60 ± 0.09		7.6 ± 2.4
	67.5	0.63 ± 0.17		14.6 ± 8.2
	85.3	0.49 ± 0.14		16.5 ± 8.4

[a] Mean value. [b] Since no enantioselectivity was found for CLP, each value was calculated from the data for (*R*)-, (*S*)-, and (*R*,*S*)-**1**.

Also, a neutral molecule of **1** is oxidized in the case of CLP. One may speculate that a charged substrate is also a key feature necessary for the enzymatic chiral recognition. It is also possible that the lack of enantioselectivity in the CLP catalysis results from the fact that the electron transfer from ferrocene is not the rate-limiting step under the steady-state conditions. To provide evidence for the latter, we have tested the reactivity of a series of monoalkyl-substituted ferrocenes.

CLP-catalyzed oxidation of alkylferrocenes by H_2O_2: We investigated the CLP-catalyzed oxidation of HFc, EtFc, and BuFc. The data for EtFc in Figure 4 show that the reaction

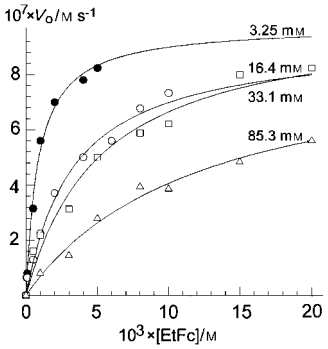

Figure 4. Steady-state rate of CLP-catalyzed oxidation of EtFc by H_2O_2 (1.4×10^{-4} M) as a function of concentration of EtFc at different [Triton X-100]: pH 2.75, 25 °C, [CLP] = 10^{-7} M.

rate levels off on increasing [EtFc] as opposed to the HRP catalysis where a clean first-order behavior with respect to RFc was observed.[3] The Michaelis–Menten equation holds, and the values of $V_{m,obs}$ and $K_{m,obs}$ with different [Triton X-100] are given in Table 1. The same rate law is valid for ferrocene and *n*-butylferrocene and the corresponding parameters are also in Table 1.

As for **1**, the values $V_{m,obs}$ for RFc are almost independent of both the nature of ferrocene and Triton X-100 concentration, Table 1. The former contrasts with the HRP case for which the rate decreases strongly with increaseing length of alkyl radical; the rate decreases by a factor of 32 on going from HFc to BuFc.[3] The independence of $V_{m,obs}$ on the nature of RFc supports the fact that for CLP the electron transfer from ferrocene is not the rate-limiting step, but rather the interaction of CLP with H_2O_2 to form the compound CLP–**I**, Equation (1). Precedents for such a mechanism exist in the literature,[34] and an estimate for k_1 is available.[35] Assuming that k_1 is rate-limiting, it follows that $V_m = k_1[\text{CLP}][H_2O_2]$, and with concentrations of CLP and H_2O_2 used in this work one arrives at $k_1 \approx 10^5$ M^{-1} s^{-1}, which is comparable with the rate constant reported previously of 1.1×10^5 M^{-1} s^{-1} (pH 3.4).[35] This coincidence also suggests that k_1 refers to rate-limiting steps and the simplest scheme to account for the kinetics observed is given by Equations (1) and (2).

$$\text{CLP} + H_2O_2 \xrightarrow{k_1} \text{CLP}-\mathbf{I} \quad (1)$$

$$\text{CLP}-\mathbf{I}(\mathbf{II}) + \text{RFc} \xrightarrow{k_2} \text{CLP} + \text{RFc}^+ \quad (2)$$

We do not specify here which oxidized form of CLP, namely CLP–**I** or CLP–**II** (two and one oxidation equivalents above the native state, respectively), contributes to the overall kinetics. What is important is that CLP is not the best enzyme for demonstrating kinetic enantioselectivity under the steady-state conditions. In fact, the resulting rate equation [Eq. (3)] shows that the key step driven by k_2 does not play a role when $k_1[H_2O_2] \ll k_2[\text{RFc}]$.

$$\frac{d[\text{RFc}^+]}{dt} = \frac{k_1 k_2 [\text{E}]_0 [H_2O_2]_0 [\text{RFc}]}{k_1 [H_2O_2]_0 + k_2 [\text{RFc}]} \quad (3)$$

Here $[\text{E}]_0$ and $[H_2O_2]_0$ are the total concentrations of CLP and hydrogen peroxide; [RFc] is the concentration of a ferrocene in the aqueous pseudophase. To relate this with the total concentration, one should take into account the fact that ferrocenes bind with micelles of Triton X-100, Equation (4). If we assume that $[\text{M}]_0 \gg [\text{RFc}]_0$ (the condition holds in a limited concentration range, $[\text{M}]_0$ is the total micelle concentration), we obtain $[\text{RFc}] = [\text{RFc}]_0/(K_4[\text{M}]_0 + 1)$. Substitution into Equation (3) gives Equation (5), which shows that $K_{m,obs}$ should be a linear function of micelle concentration as seen from the data in Table 1 for all ferrocenes tested.

$$\text{M} + \text{RFc} \overset{K_4}{\rightleftharpoons} \{\text{M,RFc}\} \quad (4)$$

$$\frac{d[\text{RFc}^+]}{dt} = \frac{k_1 k_2 [\text{E}]_0 [H_2O_2]_0 [\text{RFc}]_0}{[H_2O_2]_0 \frac{k_1}{k_2}(K_4[\text{M}]_0 + 1) + [\text{RFc}]_0} \quad (5)$$

GO-catalyzed reduction of (*R*)- and (*S*)-$\mathbf{1}^+$ by β-D-glucose: a spectral study. We have found that the HRP-catalyzed oxidation of **1** into $\mathbf{1}^+$ affords a catalytically active material that can then be reduced by GO(red). Thus, a direct spectrophotometric study of the kinetics of this process given by stoichiometric Equation (6) is possible.

$$\text{GO(red)} + 2\,\text{RFc}^+ \rightarrow \text{GO(ox)} + 2\,\text{RFc} + 2\,\text{H}^+ \quad (6)$$

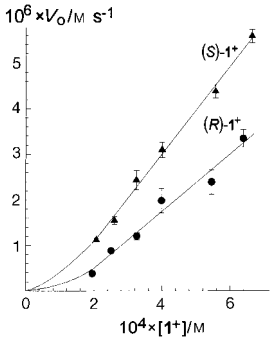

Figure 5. Steady-state rate of GO-catalyzed reduction of (*R*)-**1**$^+$ and (*S*)-**1**$^+$ by β-D-glucose (0.1M) as a function of concentration of **1**: pH 7, 25 °C, [GO] = 10^{-7}M.

The kinetic data is presented in Figure 5. Evidently, the (*S*)-**1**$^+$ enantiomer is more reactive than (*R*)-**1**$^+$. The reactivity order in the reduction reaction is thus reversed compared with the oxidation reaction, that is, the following relative reactivity (*R*)-**1** > (*S*)-**1** and (*S*)-**1**$^+$ > (*R*)-**1**$^+$ is observed in the HRP- and GO-catalyzed electron-transfer reactions, respectively. Another feature worth comment in Figure 5 is that the straight lines do not go through origin. We ascribe this effect to the necessity of performing kinetic measurements in the presence of appreciable concentrations of (*S*)-**1** and (*R*)-**1** (ca. 3.6×10^{-4}M), that is, 40% of the highest concentration of **1**$^+$ in both series. We have previously shown that ferrocene carboxylic acid is a competitive inhibitor of GO(red) in reactions with Fc$^+$.[12] Assuming that **1** can also suppress the enzymatic activity, the lower reaction rate under the condition [**1**] ≫ [**1**$^+$] does not seem very surprising. When the excess of **1** is not very large, the reaction follows first-order kinetics with respect to **1**$^+$. The slopes of the linear portions of the curves are $(9.6 \pm 0.3) \times 10^{-3}$ and $(6.25 \pm 0.52) \times 10^{-3}$ s^{-1} for (*S*)-**1**$^+$ and (*R*)-**1**$^+$ ferricenium cations, respectively (total [GO] = 2×10^{-7}M, [D-glucose] = 0.05M, 25 °C, and pH 7). The ratio of 1.54 is evidently the enantioselectivity factor in the GO-catalyzed reaction. It should, however, be pointed out that the ratio must be treated as a rough estimate, since the rate constants were obtained in the presence of (*S*)-**1** and (*R*)-**1** in both experimental series, and the two enantiomers may have different ability to suppress the GO activity. In order to minimize the latter effect and to gain additional evidence for the existence of planar chiral enantioselectivity in the GO catalysis, we have estimated the reactivity of (*R*)- and (*S*)-**1**$^+$ towards GO(red) by means of cyclic voltammetry (CV).

GO-catalyzed reduction of (*R*)- and (*S*)-1$^+$ by β-D-glucose: a CV study. This electrochemical method is very useful to gain information about the coupling between GO(red) and electrochemically generated ferricenium ions.[5] CV data reported in this work was obtained on glassy carbon and pyrolytic graphite electrodes. Cyclic voltammograms for both enantiomers of **1** in the absence and in the presence of GO and β-D-glucose at a glassy carbon electrode are shown in Figure 6. The voltammograms of the both enantiomers in the absence of the enzyme are very similar, whereas in the presence of GO the (*S*)-**1** enantiomer gives rise to a larger peak current provided all other conditions except the enantiomeric nature of **1** are kept constant. The quantitative information on the efficacy of the electron transfer from GO(red) to **1**$^+$ was obtained by the procedure developed by Bourdillon et al.[8] The typical plot for evaluation of the rate constant k_3 (in terms of the formalism adopted in ref. [8]),

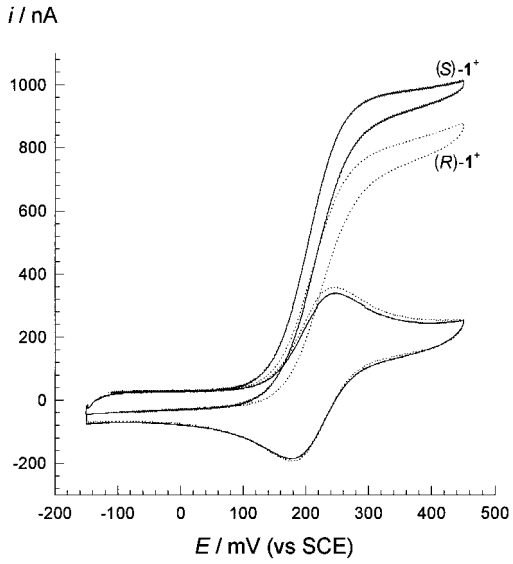

Figure 6. Cyclic voltammograms of (*S*)-**1** (solid line) and (*R*)-**1** (dotted line) in aqueous solution of pH 7.0 (0.1M phosphate) in the absence (below) and in the presence (above) of GO (1.1×10^{-6}M) and β-D-glucose (0.1M). Glassy carbon electrode, scan rate 2 mV s^{-1}, 25 °C.

which most probably refers to the rate-limiting transfer of the first electron from GO(red) at **1**$^+$, is shown in Figure 7.

From the slopes of the linear plots passing through the origin, defined by $3.17 \times (k_3RT/F)^{1/2}$, values of k_3 of $(7.4 \pm 0.4) \times 10^4$ and $(4.4 \pm 0.2) \times 10^4$ M^{-1}s^{-1} were calculated for (*S*)-**1**$^+$ and (*R*)-**1**$^+$ ferricenium cations, respectively ([D-glucose] = 0.1M, pH 7, and 25 °C). The enantioselectivity factor k(*S*)-**1**$^+$/k(*R*)-**1**$^+$ is 1.7. Experiments carried out with a pyrolytic

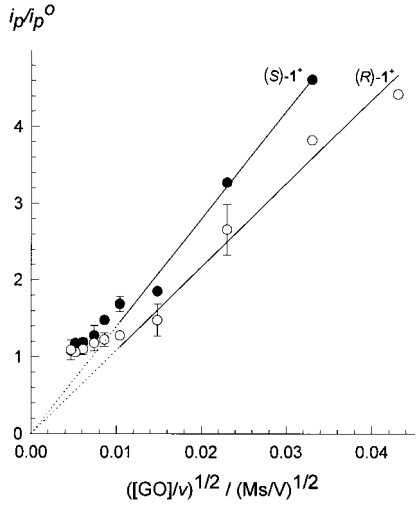

Figure 7. Plot for evaluation of the rate constants k_3 as described by Bourdillon et al.,[8] by the example of (*S*)-**1** (●) and (*R*)-**1** (○). For conditions, see legend to Figure 6.

graphite working electrode led to the same conclusions and the values of k_3 calculated were $(9.3 \pm 0.3) \times 10^4$ and $(5.7 \pm 0.4) \times 10^4$ M^{-1}s^{-1} for (*S*)-**1**$^+$ and (*R*)-**1**$^+$, respectively, with an enantioselectivity factor of 1.6. The increase of the enantioselectivity factors obtained by CV is insignificant compared with that obtained by the spectrophotometric technique.

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However, the CV experiment appears to be more accurate, since on the one hand the reaction mixture does not contain HRP, and on the other the rate constants are evaluated at a low total concentration of **1**, which is better for minimizing the inhibition. It should also be pointed out that in the present case, when the reaction is first-order with respect to $\mathbf{1}^+$ (see Figure 5), the rate constants evaluated spectrophotometrically and electrochemically show reasonable agreement. The slopes in Figure 5 were obtained at $[GO] = 10^{-7}$ M and the corresponding second-order rate constants (9.6×10^4 and 6.2×10^4 $M^{-1}s^{-1}$) are close to those obtained by CV at both electrodes.

Discussion

Three oxidoreductases, horseradish peroxidase (HRP) and chloroperoxidase (CLP) from *C. fumago*, and glucose oxidase (GO) from *A. niger*, were screened for the planar chiral enantioselectivity in a single electron transfer processes to and from a 1,2-disubstituted ferrocene derivative. Glucose oxidase was investigated by visible spectrophotometry and cyclic voltammetry. The principal results obtained are summarized in Table 2. The enantioselective electron transfer in the case planar chiral ferrocene derivatives was established for HRP and GO; no kinetic preference was observed for CLP.

Table 2. Enantioselectivity factors observed for the three oxidoreductases.

Enzyme	Enantioselectivity factor
HRP	(R)-**1**/(S)-**1** = 1.84
GO (spectral control)	(R)-$\mathbf{1}^+$/(S)-$\mathbf{1}^+$ = 0.65
GO (CV, pyrolytic graphite)	(R)-$\mathbf{1}^+$/(S)-$\mathbf{1}^+$ = 0.625
GO (CV, glassy carbon)	(R)-$\mathbf{1}^+$/(S)-$\mathbf{1}^+$ = 0.58
CLP	(R)-**1**/(S)-**1** = 1

It is interesting to note that the compounds that followed first-order kinetics or, in other words, that did not display kinetically meaningful enzyme–substrate binding, showed different reactivity for *R* and *S* enantiomers. We have recently put forward the argument that the electron-transfer between HRP and alkylferrocenes has features typical of an outer-sphere electron-transfer process.[3] Nevertheless, enantioselectivity was observed. It is also interesting to note that the highest enantioselectivity factor of 1.84 observed in the case of HRP is very similar to that reported (1.81) for wild-type cytochrome *c* peroxidase-catalyzed oxidation of *R*- and *S*-enantiomers of 1-hydroxymethyl-2-dimethylaminomethylferrocene.[17] Remarkably, the *R* enantiomers reacted faster in both the cases.

The expectation to observe enantioselectivity in the CLP catalysis failed. However, this example might be representative for discussing the origin of enantioselectivity or the lack of it. The fact that enantioselectivity is not observed in this case may be a result of i) the incorrect rate-limiting step, ii) unfavorable pH region, and iii) the lack of charged groups attached to ferrocenes. The second and third issues are most probably interrelated. The charged ferrocene must be properly oriented in the vicinity of the enzyme active center as a result of weak interactions made, for example, by the enzyme–substrate hydrogen-bond network and/or hydrophobic contacts. The hydrogen bonding within the enzyme active center should be regulated by pH and, therefore, the enantioselectivity appears to be strongly pH sensitive. The network seems to be more efficient in the case of charged ferrocene substrates. A possible detailed mechanistic picture can be provided by the example of GO for which the composition of the active site is known from the X-ray structural data.[36] Figure 8 shows the amino acid residues

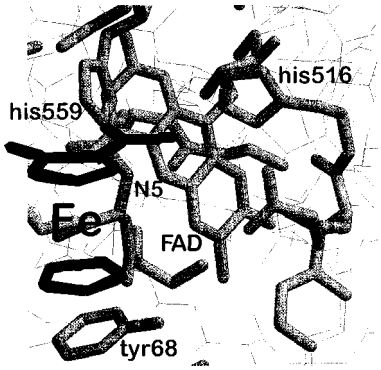

Figure 8. Modeling of the active site architecture of GO to show the possible binding of (S)-$\mathbf{1}^+$ in the vicinity of FAD. For details, see text.

separated from the N5 atom of FAD 600 by about 8 Å and the (S)-$\mathbf{1}^+$ molecule incorporated into the architecture of the active site of GO. Tyr-68 has been suggested as a residue involved in the substrate binding.[36] Its aromatic ring can be viewed as a platform to facilitate a hydrophobic or stacking contact with the cyclopentadienyl ring of ferrocene. Located in the active center, histidines 516 and 559 might be involved in the interactions with the carboxylic group of the substrate. Involvement of these amino acid residues is strongly suggested by the shape of the rate versus pH profile for the reduction of ferricenium ion by reduced GO with the maximum of activity in the pH range 7.5–8.[12] Thus, the most reactive enantiomer may be fixed in the active site by at least two weak interactions that involve Tyr-68, His-559 and/or His-516. In the case of (R)-$\mathbf{1}^+$ enantiomer, similar interactions do not appear to be very advantageous because the methyl group would be a natural barrier between the ferricenium ion and reduced FAD. It should also be mentioned that the results of the modeling shown in Figure 8 should be considered as a first level of approximation. The enantioselectivity factors reported are not that high and, alternatively, their origins could stem from as yet unspecified weak interactions of a substrate with surface chiral amino acid residues that are not necessarily very close to the enzyme active site.

Another interesting feature, which naturally may just be a coincidence, is the reactivity order in the oxidation and reduction reactions (Table 2). In particular, (R)-**1** is more reactive than (S)-**1** in the HRP-catalyzed oxidation, whereas (S)-$\mathbf{1}^+$ is reduced faster than (R)-$\mathbf{1}^+$ in the presence of GO. However, this does not seem very surprising in light of the fact that the enantioselectivity factor inverts on going from a wild-type to a mutant form of cyctochrome *c* peroxidase.[17]

Conclusions

In addition to the related previous recent[17, 18] and relevant older work[37] we have presented here several new examples of the enantioselective electron transfer to or from ferrocenes with the elements of planar chirality. HRP and GO proved to be the enzymes with different reactivity toward both enantiomers of 2-methylferrocene carboxylic acid and the corresponding ferricenium cations. CLP did not show kinetic preference for any enantiomer, most likely because of the incorrect rate-limiting step. Taking into account the results reported here and elsewhere it seems likely that the question mark at the end of the title of the paper, "Molecular Recognition of Artificial Single-Electron Acceptor Cosubstrates by Glucose Oxidase?" by Savéant et al.[19] can now be omitted. Perspectives of this study are clear. First, the results help us to understand the structural factors that bring about the highest stereoselectivity in the enzymatic electron transfer involving planar chiral organometallics and the elucidation of intimate mechanisms of the enantioselectivity. Second, the observation found suggests an approach to the kinetic resolution of planar chiral molecules based on electron transfer to or from oxidoreductases. Although the best enantioselectivity factor equals 1.84, high rates of enzymatic reactions and an easy recycling of partly resolved material makes this approach very challenging.

Experimental Section

General: Enzymes HRP ($R/Z = 3$), CLP ($R/Z = 1$), and GO from *Aspergillus niger* (250 U per mg) were purchased from Dia-M, Sigma, and Serva, respectively, and all used as received. Spectrophotometric measurements were carried on a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A cell positioner/temperature controller. CV measurements were performed on a PC-interfaced potentiostat-galvanostat IPC-3 (Institute of Physical Chemistry, RAS). A three-electrode scheme was used with working glassy carbon (Moscow State University (Russia), diameter 1.8 mm) and pyrolytic graphite electrodes (Tokaii (Japan), diameter 1.8 mm), saturated calomel reference electrode, and ancillary Pt electrode. An electrochemical cell was thermostated at 25 °C by circulating water. The opticatl rotation measurements were carried out at 22 °C.

Preparation of (*R*)-1.

*Synthesis of (+)-**2***: A solution of (−)-*N*-acetyl-(*S*)-leucine (Reakhim, 3.41 g, 0.02 mol) and NaOH (0.8 g) in water (75 mL) was added to a solution of Na_2PdCl_4 (5.85 g, 0.02 mol) in MeOH (225 mL), and the pH of the resulting solution was adjusted to 7.85 with 50% NaOH. A solution of dimethylaminomethylferrocene (4.9 g, 0.02 mol) in MeOH (75 mL) was then added to the stirred solution and a precipitate began to appear after 10 min. The mixture was allowed to stand overnight, the precipitate was then filtered off, washed with water, and dried over P_2O_5. The solid was dissolved in benzene, and the insoluble admixtures were removed by filtration. *n*-Heptane was added to the filtrate until a slight clouding was evident, the mixture was concentrated in vacuo, *n*-heptane was again added, and the solution filtered. The filtrate was evaporated almost to dryness, the precipitate was separated and dried to obtain 3.34 g of (+)-(*R*)-**2** $[\alpha]_D = +537.6°$, ($c = 1$, CH_2Cl_2), 80.7% optical purity. The same procedure was applied to the methanolic mother liquor to obtain 1.86 g of (+)-(*R*)-**2** $[\alpha]_D = +463°$, ($c = 1$, CH_2Cl_2), 69.5% optical purity. Total yield 67%.

1-Methoxycarbonyl-2-dimethylaminomethylferrocene: Carbon monoxide was bubbled for 1 h through a suspension of (+)-(*R*)-**2** (2.0 g, 0.005 mol, $[\alpha]_D = +537.6°$) in methanol (50 mL). Palladium metal was filtered off, the filtrate treated with sodium bicarbonate, extracted with diethyl ether, dried over $MgSO_4$, and the solvent was finally removed in vacuo. 1.25 g (79%) of the ester was obtained. The product was however contaminated with traces of Me_2NCH_2Fc.

(+)-2-Methylferrocene carboxylic acid, (*R*)-***1***: 1-Methoxycarbonyl-2-dimethylaminomethylferrocene was converted into the corresponding iodomethylate.[38] A suspension of the iodomethylate (1.7 g) in water (120 mL) was added to sodium amalgam prepared from Hg (7 mL) and Na (2.8 g). The reaction mixture was refluxed for 9 h. The solution was separated from the amalgam and dimethylaminomethylferrocene was extracted with *n*-hexane. The aqueous layer was acidified with 50% H_3PO_4 and the precipitate formed was filtered off. Additional amount of the acid was obtained after its extraction by ether from the acidic aqueous solution. Compound (*R*)-**1** was purified by column chromatography on SiO_2 eluting with hexane/ether (5:1). Yield 0.5 g (52%); $[\alpha]_D = +49.26°$ ($c = 2$, EtOH), 92.9% optical purity; ^1H NMR ($CDCl_3$): $\delta = 2.3$ (s, 3H; CH_3), 4.18(s, 5H; C_5H_5), 4.30, 4.37, 4.78 (m, 3H; C_5H_3); $C_{12}H_{12}FeO_2$ (244.073): calcd C 59.05, H 4.96; found C 59.08, H 5.07.

(−)-2-Methylferrocene carboxylic acid, (*S*)-***1***: Compound (*S*)-**1** was obtained in a similar way from (+)-*N*-acetyl-(*R*)-leucine (provided by Dr. Yu. A. Davidovich) in the asymmetric cyclopalladation: $[\alpha]_D = -42.27°$ ($c = 2.37$, EtOH), 79.7% optical purity.

Peroxidase reaction: Solutions of (*S*)-**1** and (*R*)-**1** (0.001 M) were prepared by dissolving 0.0099 g (4×10^{-5} mol) **1** in phosphate buffer (40 mL, 0.013 M, pH 7). Ferrocene derivative **1** has an absorption maximum at 442 nm ($\varepsilon = 182\ M^{-1}\ cm^{-1}$). The spectral characteristics of its 1e oxidation product were determined by titrating **1** with hydrogen peroxide in the presence of HRP to give $\lambda(max) = 647.5$ nm and $\varepsilon = 431\ M^{-1}\ cm^{-1}$ as described previously.[39] Almost all kinetic data were obtained at $[HRP] = 1 \times 10^{-7}$ M and $[H_2O_2] = 2 \times 10^{-4}$ M. The oxidation reaction was initiated by addition of HRP solution (26 μL, 7.7×10^{-6} M) to a 1 cm spectrophotometric cuvette containing a solution of **1** (1.94 mL) and H_2O_2 (38 μL, 0.01 M). An increase in absorbance was monitored at 647.5 nm and the data obtained were processed as described in the recent work.[3] The pH dependence of the HRP activity towards (*R*,*S*)-**1** was studied at a fixed $[H_2O_2]$ (2×10^{-4} M), [**1**] being in excess. This enabled us to estimate ε of the product in the same run. The extinction coefficient appeared to be pH independent in this pH region.

CLP oxidation: Owing to the insolubility of ferrocene, *n*-butylferrocene (Aldrich), ethylferrocene (Strem), and **1** in aqueous solution at pH 2.75, the compounds were dissolved in the phosphate buffer in the presence of Triton X-100 (3 – 116 mM). Kinetic data were obtained at $[CLP] = 1 \times 10^{-7}$ M and $[H_2O_2] = 1.4 \times 10^{-4}$ M. The kinetics of the CLP-catalyzed oxidation was measured and the data analyzed as described for alkylferrocenes[3] or above for **1**.

Reduction of (*S*)-1$^+$ and (*R*)-1$^+$ by reduced GO: spectral control. The ferricenium cations were generated in situ by HRP-catalyzed oxidation of **1** by H_2O_2. In a typical experiment, solutions of hydrogen peroxide (0.28 mL, 7.4×10^{-3} M) and HRP (0.129 mL, 8.1×10^{-6} M) were added to **1** (10 mL, 0.001 M) in phosphate buffer (0.01 M, pH 7). Such an optimal reagent ratio provided 60% yield of **1**$^+$. The oxidation was registered spectrophotometrically by following a decrease in absorbance at 647 nm. The GO-catalyzed reduction of (*S*)-**1**$^+$ and (*R*)-**1**$^+$ was monitored by following the protocol described below. The reaction mixture was composed by addition of solutions of D-glucose (0.18 mL, 0.7 M), GO (0.02 mL, 2×10^{-5} M), and **1**$^+$ (0.3 – 1.8 mL) to a 1 cm spectrophotometric cuvette with phosphate buffer (0.01 M, pH 7) to achieve the total volume of 2 mL. Since the solution of **1**$^+$ always contained 40% of **1**, which is known to be a competitive inhibitor of GO,[12] the required amount of **1** was added, when necessary, to the reaction solution to achieve constant $[\mathbf{1}] = 3.6 \times 10^{-3}$ M in the whole series. The rate of **1**$^+$ fading at 647 nm was registered every 10 s over a peroid of 1 min. It has been confirmed that HRP has no effect on the GO-catalyzed reaction under the conditions used.

Reduction of (*S*)-1$^+$ and (*R*)-1$^+$ by reduced GO: a CV study. Stock solutions of **1** (1.1×10^{-3} M) were prepared in phosphate buffer (0.1 M, pH 7). An aqueous solution of β-D-glucose (1 M) was prepared beforehand and kept overnight. For the measurement of i_p^o the protocol was standardized as follows: a buffered solution of **1** (2.7 mL) and glucose aqueous solution (0.3 mL, 0.1 M) were introduced into the thermostated electrochemical cell. The cyclic voltammograms were recorded with glassy carbon and pyrolytic graphite electrodes at seven to nine different scan rates from

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0.6 to 50 mV s^{-1}. For the measurement of i_p, GO solution (0.01 mL, its final concentration in the cell was 1.1×10^{-6} M) was added to the electrochemical cell and cyclic voltammograms were recorded as previously described.[10] Both carbon electrodes were polished by 0–1 μm diamond paste before every series of measurements consisting of 7–9 runs either with or without GO and D-glucose. It was confirmed that a higher frequency of polishing does not affect both the shape of voltammograms and peak currents. The rate constant k_3 was calculated by the described procedure.[8]

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