Molecular Recognition of Artificial Single-Electron Acceptor Cosubstrates by Glucose Oxidase?

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Previous investigation of several artificial single-electron acceptors (mostly ferroceniums) as cosubstrates for the oxidation of glucose by glucose oxidase has revealed that their reactivity does not obey the kinetic laws expected for a simple outer-sphere electron transfer process.[2] Indeed, predicted variations of the kinetics with the standard free energies corresponding to each cosubstrate in the series, based on the standard potentials and pK_a values characterizing the $FADH_2/FAD + 2e^- + 2H^+$ couple,[3] were not observed experimentally. Specific interactions between these artificial single electron cosubstrates and glucose oxidase may be responsible for this kinetic behavior.[2]

Specificity of these interactions seemed to receive a spectacular confirmation when chiroselective electron transfer from glucose oxidase to a ferrocenium was reported,[4] implying a very precise recognition of the ferroceniums ions by glucose oxidase.

Intrigued by inconsistencies in the kinetic treatment[4] of the cyclic voltammetric data (supporting information), we set out to repeat the experiments. Out of a large number of tries, Figure 1 shows a typical example of the catalytic cyclic voltammetric responses obtained with the (*S*) and the (*R*) enantiomers and with glucose oxidase from Boeringer Mannheim.[5a] We found that glassy carbon electrodes give better behaved responses than do gold electrodes. In particular they allow an easy and precise calibration of the concentrations of the two enantiomeric ferrocenes. Cyclic voltammetric titration of the ferrocenes in the absence of glucose oxidase was thus used for preparing solutions with the same concentration of each enantiomer. Figure 1 shows that, within experimental uncertainty, there is no detectable difference between the catalytic responses of the two enantiomers in the presence of the same amounts of glucose and glucose oxidase. These experiments were repeated at several other scan rates (0.02, 0.03, 0.04, and 0.06 V/s) and repeated several times at each scan rate. In all cases, the responses of the (*S*) and the (*R*) enantiomers were found to be the same. The same experiments were also performed at a gold disk electrode, as in ref 4, still using a glassy carbon electrode for initial concentration calibration. Again, the responses were the same for the two enantiomers (supporting information). The same experiments were repeated with other batches of the (*S*) and the (*R*) enantiomers, the purity of which was checked by polarimetry and with glucose oxidase from Sigma (as in ref 4).[5b] The catalytic responses were again the same for the two enantiomers (supporting information). We thus conclude that the electron transfer reaction is not chiroselective.

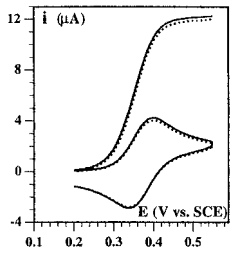

Figure 1. Cyclic voltammetry of (*S*)- (0.24 mM, solid line) and (*R*)-(0.24 mM, dotted line) *N*,*N*-dimethyl-1-ferrocenylethylamine in a 0.025 M NaH_2PO_4/0.025 M Na_2HPO_4 buffer (pH = 6.9; ionic strength, 0.1 M) at a 3 mm diameter glassy carbon electrode in the presence of 0.444 M glucose.[5] Scan rate: 0.08 V/s. Lower reversible curves: no glucose oxidase present. Upper plateau-shaped curves: in the presence of 1.3×10^{-6} M glucose oxidase.[5]

Scheme 1

$$\text{FAD} + \text{G} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{FADG}$$

$$\text{FADG} \xrightarrow{k_2} \text{FADH}_2 + \text{GL}$$

$$\text{FADH}_2 + 2\text{Q} \xrightarrow{k} \text{FAD} + 2\text{P}$$

(P, Q are the reduced and oxidized forms of the single electron cosubstrate respectively).

Table 1. Thermodynamics and Kinetics of the Rate-Determining Steps

	single-electron cosubstrates					
	ferrocene methanol[a]		ferrocene carboxylate[b]		(dimethylamino)-methylferrocene[c]	
acid medium	E1	E3	E1	E3	E1	E3
SFE[d]	−0.26	−0.28	−0.36	−0.38	−0.44	−0.46
K_AK_E/K_B	2.2×10^4	4.6×10^4	1.0×10^6	2.2×10^6	2.2×10^7	4.6×10^7
log k_{ac}[e]	5.1		5.0		5.7	
basic medium	E2	E4	E2	E4	E2	E4
SFE[d]	−0.52	−0.71	−0.62	−0.81	−0.70	−0.289
K_AK_E/K_B	4.6×10^8	6.8×10^{11}	2.2×10^{10}	3.2×10^{13}	4.6×10^{11}	6.8×10^{14}
log k_{bas}[e]	7.4		5.6		7.7	

[a] Standard potential (V vs SCE) 0.19. [b] Standard potential (V vs SCE) 0.29. [c] Standard potential (V vs SCE) 0.37. Protonated form. [d] Standard free energy, eV. [e] M^{-1} s^{-1}.

Analysis of the responses obtained at a glassy carbon electrode (Figure 1 and supporting information) allowed the kinetic characterization of Scheme 1 along the same procedures as previously described.[2]

Starting from $k_2 = 700$ s^{-1} and $k_{red} = k_1k_2/(k_{-1} + k_2) = 1.1 \times 10^4$ M^{-1} s^{-1} as previously determined, [2] we found that log k (M^{-1} s^{-1}) = 6.8 ± 0.1, a value which is, as expected, close to the value found for (dimethylamino)methylferrocene (7.1) at the same pH (6.9).[2] Other experiments were also carried out with different concentrations of the enantiomers (supporting information). The same preceding value of k was found in each case.

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(4) (a) As shown in Figure 1 of ref 4b, the catalytic cyclic voltammetric response obtained with (*S*)-*N*,*N*-dimethyl-1-ferrocenylethylamine was found to be about twice the response obtained with the (*R*) enantiomer. (b) Marx-Tibbon, S.; Katz, E.; Willner, I. *J. Am. Chem. Soc.* **1995**, *117*, 9925.

(5) (a) The electrochemical instrumentation and procedures as well as the procedures for preparing the glucose and glucose oxidase stock solutions were the same as previously described. [2] Overnight mutarotation of the glucose solutions was particularly important for obtaining reproducible results. The ferrocenium concentrations were calibrated by comparison with solutions of ferrocene methanol of known concentrations. (*S*)- and (*R*)- *N*,*N*-dimethyl-1-ferrocenylethylamine were purchased from Aldrich and used as received. Glucose was purchased from Prolabo and *A. niger* glucose oxidase from Boehringer Mannheim (grade I). (b) Polarimetry gave the following results. *R*-(+)-*N*,*N*-dimethyl-1-ferrocenylethylamine, $[\alpha]_D = +13°$; *S*-(−)-*N*,*N*-dimethyl-1-ferrocenylethylamine, $[\alpha]_D = -13°$ (10 mg/mL; C_2H_5OH; optical path, 1 dm; Perkin Elmer 241 polarimeter). *A. niger* glucose oxidase from SIGMA (type X-S) was used in this case.

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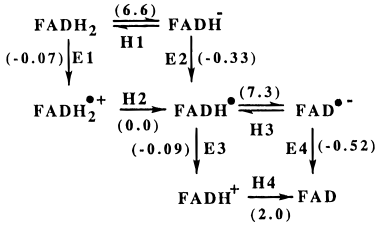
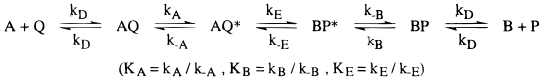
Scheme 2



Scheme 3



Since the publication of our kinetic results,[2] the X-ray crystal structure of glucose oxidase from *Aspergillus niger*, refined at a 2−3 Å resolution, has been reported,[6] allowing the re-examination of these data in the light of the structural characteristics. The main feature of interest for this discussion is that the flavin prosthetic group sits at the bottom of a funnel-shaped pocket with an opening of ca. 10 Å × 10 Å at the enzyme surface.[6] The ferrocenium cosubstrates may thus experience some difficulty in reaching a positioning suitable for efficient electron transfer.

$$\frac{1}{k} = \frac{1 + (K_{a,H1}/[H]^+)}{k_{E1} + (K_{a,H1}/[H]^+)k_{E2}} + \frac{1 + (K_{a,H3}/[H]^+)}{k_{E3} + (K_{a,H3}/[H]^+)k_{E4}} \quad (1)$$

Previous pH-dependent studies revealed that the oxidation of the reduced flavin by the single-electron cosubstrates follows the mechanism depicted in Scheme 2.[2] The vertical reactions are oxidations by Q regenerating P. From the values (V vs SCE) of the standard potentials of the four flavin redox couples[3] involved that are indicated in Scheme 2 and those of the mediators (Table 1), all four oxidation steps may be regarded as irreversible. The horizontal reactions are deprotonations by the bases present in the buffer. From the pK_a values of the various flavin acid−base couples indicated in Scheme 2,[3] reactions H2 and H4 may be regarded as irreversible and reactions H1 and H3 as reversible in the pH range of interest (4−8.5). Experiments where the concentration of the buffer was varied showed that the kinetics of the acid base steps do not interfere. It follows that the rate constant k_1 may be related to the various steps of Scheme 2 according to eq 1. The variation of k with pH thus plateaus off below pH 4 and above pH 8 (see Figure 8 in ref 2), reaching values, k_{ac} and k_{bas}, that are related to the rate constants of the electron transfer steps according to eqs 2 and 3, respectively.

$$\frac{1}{k_{ac}} = \frac{1}{k_{E1}} + \frac{1}{k_{E3}} \quad (2)$$

$$\frac{1}{k_{bas}} = \frac{1}{k_{E2}} + \frac{1}{k_{E4}} \quad (3)$$

Table 1 provides a comparison between the variations of the rate constant and those of the standard free energy of the rate-determining step for both cases.

We may decompose each of the electron transfer reactions, in acid or basic medium, in three successive steps (Scheme 3), where A designates $FADH_2$ (or $FADH^{\bullet+}$) and B $FADH_2^{\bullet+}$ (or $FADH^+$), for the acid medium, while for the basic medium, A designates $FADH^-$ (or $FAD^{\bullet-}$) and B, $FADH^\bullet$ (or FAD).

Q first diffuses toward the opening of the pocket at the surface of the protein core of the enzyme, a location designated by AQ. It is then transferred close to the flavin in a position (AQ*) geometrically suited for electron transfer. Electron transfer then takes place, and a similar succession of steps eventually produces B while P diffuses out of the enzyme surface. In the framework of this mechanism, the rate constant of each electron transfer step, k_{et}, may be expressed by eq 4.

$$\frac{1}{k_{et}} = \frac{1}{k_D} + \frac{1}{k_A} + \frac{1}{K_A k_E} + \frac{K_B}{K_A K_E k_B} + \frac{K_B}{K_A K_E k_D} \quad (4)$$

$K_A K_E/K_B$ is at minimum equal to 2.2×10^4 (Table 1). There is thus little doubt that the last two terms in eq 4 can be neglected. From the diffusion coefficient of ferrocene ($D = 6.7 \times 10^{-6}$ $cm^2\ s^{-1}$ [7]) and an estimation of its size (equivalent diameter = 7.2 Å), $k_D \approx 5 \times 10^8\ M^{-1}\ s^{-1}$ in the whole series.[8] The rate constants in Table 1 are much smaller than this value, showing that diffusion to the surface opening of the pocket is not the rate-determining step of the reaction. The rate constants of the electron transfer steps are expected to follow a Marcus-type law.[9a] Since the positive and negative charges can be delocalized on the FAD structure, the intrinsic barrier is expected to be small, of the order of 0.15−0.20 eV.[9b] Furthermore, the thermodynamics of electron transfer is very favorable in all cases. We thus expect the electron transfer steps to be very fast. In this respect, a striking result is obtained with the uncharged ferrocenium deriving from ferrocene carboxylate; namely, the overall rate constant is only four times larger (36 meV difference in terms of activation free energy) in basic medium than in acidic medium in spite of a 260 meV advantage in reaction standard free energy. We are thus led to conclude that the electron transfer step in Scheme 3 is too fast to control the overall kinetics. It thus appears that the controlling rate constant in Scheme 3 is k_A. The results found with the positively charged ferrocenium may be explained along the same lines taking the electrostatic interactions into account. In acidic medium, the overall rate constant is about the same as with the ferrocenium carboxylate regardless of the large difference in reaction standard free energies. In basic medium, the rate constant is larger, by ca. 2 orders of magnitude but is still about independent from the value of the standard free energy for electron transfer. These observations indicate that the negative charge on the flavin group interacts with the positively charged ferrocenium to help position the cosubstrate in a spatial configuration appropriate for electron transfer to occur.

Two main conclusions emerge from the preceding discussion. Molecular recognition of ferrocenium cosubstrates by glucose oxidase is not precise enough to allow chiroselective electron transfer. There is, however, some recognition but in a negative manner: steric hindrance in the pocket connecting the prosthetic group to the enzyme surface slows down electron transfer, offering some resistance against a largely favorable thermodynamic driving force. Nevertheless, the resulting rate constants remain large, allowing ferrocenium ions to act as quite efficient cosubstrates.

Supporting Information Available: Discussion of inconsistencies in previous investigations; experimental results (4 pages). See any current masthead page for ordering and Internet access instructions.

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(8) This assumes that the diffusion coefficient of the enzyme is much smaller than that of the ferrocenium and that the diffusion of the latter species through the protein is much slower than in the surrounding solution and through the glycoside shell of the enzyme as suggested by the observation that the kinetics of the oxidation by the mediator is not affected by extensive glycosylation of the enzyme periphery.[2]

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