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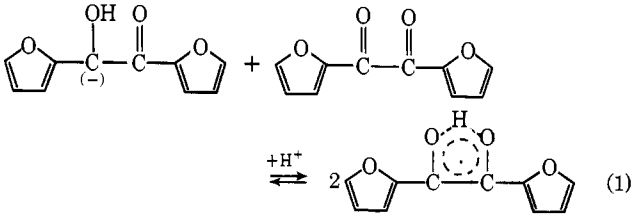
One and Two Electron Transfer Reactions of Glucose Oxidase

Sir:

The mechanisms by which the flavin adenine dinucleotide cofactor (FAD) of glucose oxidase is reduced by glucose and reoxidized by molecular oxygen have received considerable attention.[1-4] Two electron transfer reactions have been considered to be general for the mechanisms of flavoprotein oxidases, dehydrogenases, etc.,[5] because radical intermediates have not been detected.[6] However, we have recently proposed that the interconversion $HC(R_2)OH \rightleftarrows R_2C{=}O$ accompanied by flavin reduction ($Fl_{ox} \rightleftarrows FlH_2$) may well be radical in nature.[7] We report, herein, preliminary results of a study of: (i) the reduction of glucose oxidase (E-FAD) using α-hydroxycarbonyl compounds (I-IV) as glucose analogues and (ii) the oxidation of reduced glucose oxidase ($E\text{-}FADH_2$) employing the nitroxide V as a "model" of $^3O_2$ (I, dihydroxyacetone; II, glyceraldehyde; III, phenacyl alcohol; IV, furoin; V, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl). The glucose oxidase (A-niger) was obtained from Worthington Biochemical Corp. (GOP).

The α-hydroxycarbonyl compounds I-III (0.05 M) were found to reduce E-FAD (2×10^{-5} M) directly to $E\text{-}FADH_2$ (pH 7.3, H_2O, 30 °C, μ = 1.0); no semiquinone (E-FAD·) intermediate could be detected on repetitive scanning (320–600 nm) during the course of reaction. These substrates (I-III) do not reduce 3-methyllumiflavin or FAD at pH 7.0 in aqueous solution so that the enzyme is performing the role of catalyst. Compounds I-III readily reduced the electron defficient 7- and 8-cyano-3,10-dimethylisoalloxazines. In contrast, with furoin (IV) as substrate E-FAD, 7- and 8-cyano-3,10-dimethylisoalloxazine, as well as 3-methyllumiflavin were reduced (pH 7.0). The reaction with the enzyme but not with isoalloxazine was biphasic producing the semiquinone (E-FAD·, λ_{max} 400 nm)[8] as an intermediate. The rate of formation of E-FAD· far exceeded its rate of conversion to $E\text{-}FADH_2$. Since furoin and furil comproportionate (eq 1) at high pH it was crucial to de-



(1)

termine if the observed one electron reduction of E-FAD was merely due to trace concentrations of furil (and, therefore, of semidione radical). The *initial rates* for conversion of oxidized glucose oxidase (2×10^{-5} M) to its radical form in the presence of a constant concentration of furoin (3.9×10^{-4} M) and as a function of added furil (0 to 3.9×10^{-4} M) were determined (pH 7.26). Substituting the equilibrium constant of

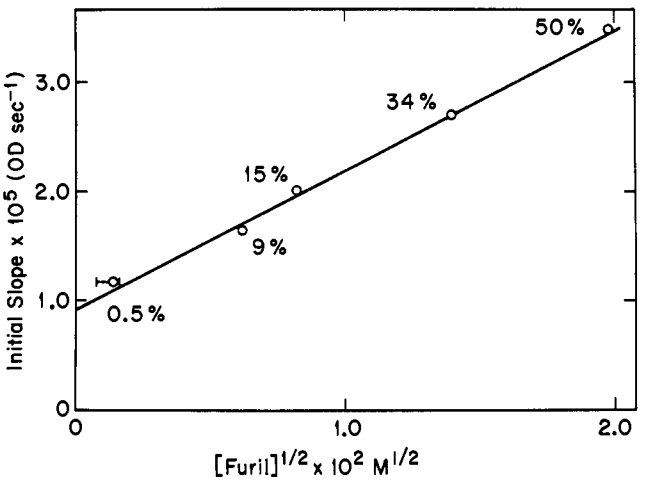


Figure 1. Plot of the initial slope vs. $[furil]^{1/2}$ for the reduction of glucose oxidase (~2×10^{-5} M based on FAD) with furoin (3.9×10^{-4} M) (pH 7.26, 0.063 M phosphate, μ = 1 with KCl, 6% CH_3CN, 30 °C) in the presence of added furil. The percent furil present is indicated in the figure. The horizontal bar is an estimate of the amount of furil present as an impurity.

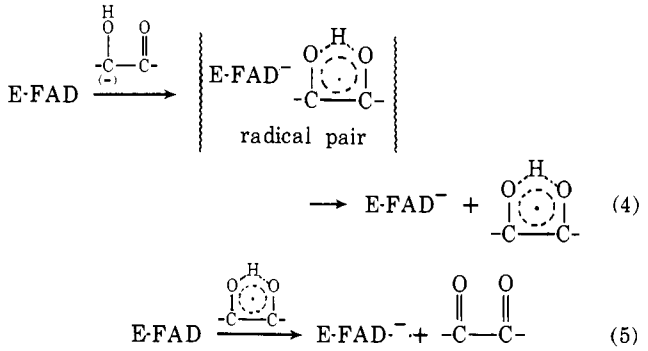
semidione formation (K_e = [semidione]^2/[furil][furoin]) into the appropriate rate expression for one electron reduction of E-FAD by both furoin and semidione (i.e., $k_{obsd} = k_1$[semidione] + k_2[furoin]) provides eq 2.

$$k_{obsd} = k_1 K_e^{1/2}[furil]^{1/2}[furoin]^{1/2} + k_2[furoin] \quad (2)$$

From eq 2, at constant [furoin]:

$$k_{obsd} = k_1 K_e'[furil]^{1/2} + c \quad (3)$$

A plot of initial rate of E-FAD· formation vs. $[furil]^{1/2}$ was found to be linear with a markedly positive intercept (Figure 1). The point on the plot of initial rate vs. $[furil]^{1/2}$ corresponding to the lowest furil concentration represents the maximum concentration of furil impurity present (HPLC analysis) in the furoin sample used. The intercept of the plot of Figure 1 pertains to the rate for one electron transfer (k_2) from furoin to E-FAD. These results support a one electron transfer reaction from both furoin and semidione radical to glucose oxidase (eq 4 and 5). In model reactions of furoin (and



(4)

(5)

benzoin) with oxidized flavins the respective carbanions (enediolate ions) have been shown to be the reactive substrates.[9] The reactions of eq 4 and 5 are proposed. The distinction between furoin (IV) which undergoes a one electron transfer to glucose oxidase, and I, II, and III where E-FAD· intermediate cannot be detected is postulated to reside in the lessened standard free energy of formation ($\Delta G°$) of the semidione radical derived from IV as compared to the $\Delta G°$'s for the radicals formed from I, II, and III. The greater the $\Delta G°$ for -Ċ(OH)CO- formation the closer the free energy content of the radical pair (eq 4) to the $\Delta G^{\ddagger}$ for conversion of E-FAD· to $E\text{-}FADH_2$ (i.e., the lower the free energy barrier for the

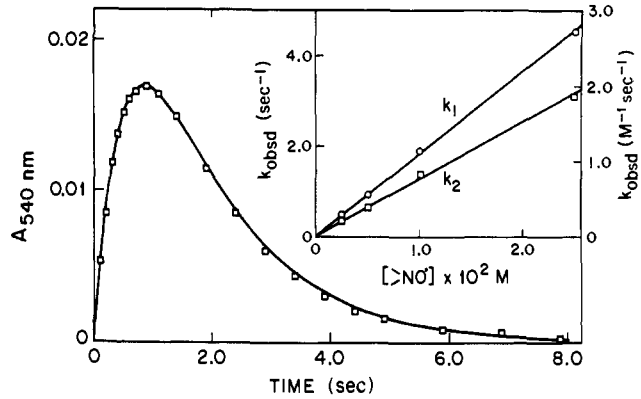


Figure 2. Plot of absorbance (540 nm) vs. time for the reaction of reduced glucose oxidase (~2×10^{-5} M) with the nitroxide V (0.01 M) (pH 6.47, 0.1 M Mops, μ = 1 with KCl, 30 °C). The extinction coefficient for the glucose oxidase radical intermediate was used as a scaling factor to obtain the best fit. The inset represents plots of k_{obsd_1} and k_{obsd_2} vs. [V] (pH 6.47, 0.1 M Mops, μ = 1 with KCl, 30 °C). Second-order constants of appearance (k_1) and disappearance (k_2) of the semiquinone intermediate are obtained from the slopes. The ordinate for k_{obsd_1} is represented by the scale on the left and that for k_{obsd_2} is represented by the scale on the right.

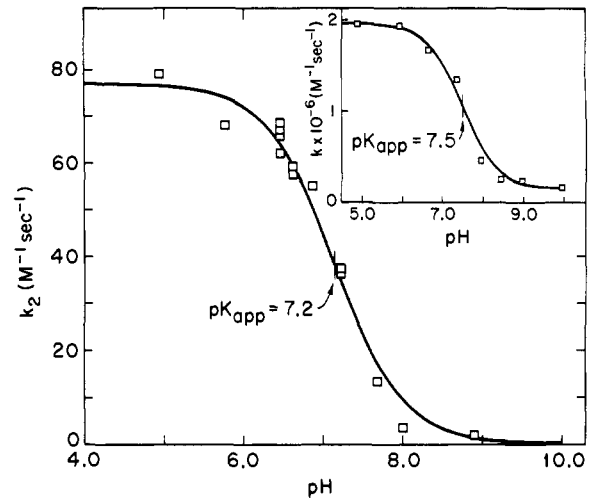


Figure 3. Plot of the second-order rate constants (k_2) vs. pH for the reaction of glucose oxidase radical with nitroxide V (pH 4.95, 0.1 M acetate; pH 5.78, 0.1 M Mes; pH 6.47, 6.63, 0.1 M Mops; pH 7.23, 7.74, 0.1 M phosphate; pH 8.0, 8.9, 0.1 M Tris). The inset is the pH-rate profile for the oxidation of reduced enzyme with oxygen.[4]

second electron transfer and thus the less likely is the radical to be detected). This instability of the radicals formed from I, II, and III may even cause the initial one electron transfer to be rate determining. We have previously shown[10] that the calculated $\Delta G°$'s (standard state 1 M) of formation of radical species by one electron transfer from dihydroflavin to $CH_3COCO_2^-$, CH_3COCO_2H, $CH_3COCO_2C_2H_5$, CH_3CO-$CONH_2$, and CH_2O are less positive than the experimental $\Delta G^{\ddagger}$ for their two electron reduction by dihydroflavin.

The oxidation of reduced glucose oxidase (2×10^{-5} M reduced by 5×10^{-4} M mannose) by the nitroxide V (2.5×10^{-3} to 2.5×10^{-2} M) was followed by stopped flow spectrophotometry at 450 and at 540–560 nm. Formation of E-FAD (increase in A_{450}) did not follow the first-order rate law and a rapid formation and decay of a transient intermediate was observed at 540 nm. The appearance and disappearance of the intermediate (Figure 2) followed the consecutive first-order kinetic scheme of eq 6.

$$\text{E-FADH}_2 \xrightarrow{k_1[>\text{N-O}\cdot]} \text{E-FAD}\cdot \xrightarrow{k_2[>\text{N-O}\cdot]} \text{E-FAD} \qquad (6)$$

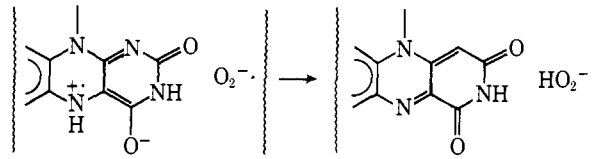
$$k_{obsd_1} = k_1[>\text{N-O}\cdot] > k_{obsd_2} = k_2[>\text{N-O}\cdot]$$

The reverse steps of k_1 and k_2 are too slow under the experimental conditions to be observed. The pseudo-first-order rate constants for appearance (k_{obsd_1}) and disappearance (k_{obsd_2}) of radical intermediate were determined by computer fitting of A_{540} to the A → B → C consecutive first-order equations.[11] Values of k_1 and k_2 were then determined from plots of k_{obsd_1} and k_{obsd_2} vs. [>N-O·] (see Figure 2 inset). Up to nitroxide concentration of 2.5×10^{-2} M, no saturation of the enzyme was observed. The two-step reaction of >N-O· with E-FADH$_2$ (eq 6) resembles the like reaction of >N-O· with N^5-alkyl-1,5-dihydroflavin which also produces flavin radical as a metastable intermediate.[12] In the cyclic redox process of eq 7 the ratio [E-FAD·]/[E-FADH$_2$] is provided by k_1/k_2. At

$$\text{E-FAD} \xrightarrow[\text{(fast)}]{\text{glucose}} \text{E-FADH}_2 \qquad (7)$$
$$k_2[>\text{N-O}\cdot] \quad k_1[>\text{N-O}\cdot]$$
$$\text{E-FAD}\cdot$$

pH 7.8 ($k_1/k_2 \approx 20$) a solution of glucose (1×10^{-3} M), V (1×10^{-3} M), and enzyme (2×10^{-5} M) exhibited the typical spectrum (λ_{max} 400 nm, 484 nm) of the radical form of the enzyme. The enzyme radical could be obtained in stable form by chromatography on Sephadex G25.[13] The reaction of purified E-FAD· with excess nitroxide was associated with values of k_{obsd} (pH 6.47, 7.74, and 8.00) identical with k_{obsd_2} (eq 6).

The value of k_1 is virtually pH independent (for seven determinations between pH 5 and 9, $k_1 = (1.77 \pm 0.28) \times 10^2$ M^{-1} s^{-1}). However, a plot of k_2 vs. pH fits a theoretical "titration curve" for a 1 H^+ dissociation (Figure 3). A comparison of this pH dependence for one electron transfer from glucose oxidase radical to >N-O· (Figure 3) to that for the $^3O_2$ oxidation of completely reduced glucose oxidase[4] (Figure 3 inset) shows their near identity. The similarity of the pH dependence for >N-O· + E-FAD· → >N-OH + E-FAD and O_2 + E-FADH$_2$ → H_2O_2 + E-FAD suggests that the latter reaction involves the formation of an [E-FAD· O_2^-·] radical pair at steady state which goes on to products in a (partially ?) rate determining one electron transfer reaction. Further, the pK_a for conversion of the (blue) neutral flavin radical species to the (red) flavin radical anion species has been estimated to be 7.4–7.5 for glucose oxidase.[8] This value may be compared to the pK_{app} values determined in the $^3O_2$ oxidation of reduced glucose oxidase and nitroxide oxidation of glucose oxidase radical (pK_{app} 7.5 and 7.2, respectively). One might conclude that the rate determining steps in both the $^3O_2$ and nitroxide reactions involve one electron transfer to O_2^-· from the neutral (blue) radical of enzyme bound FAD (eq 8). It is well appre-



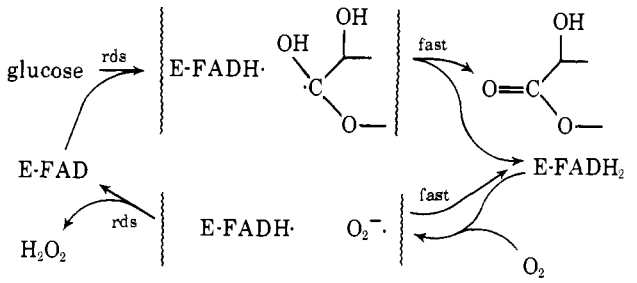
(8)

ciated in model studies that $^3O_2$ reacts much more rapidly with flavin radical anion than with neutral radical.[14,15] The apoprotein of glucose oxidase must play a catalytic role in the oxidation of its bound FADH$_2$. It should be noted that Gibson, Swoboda, and Massey[1] in their investigation of the reduction of $^3O_2$ to H_2O_2 by reduced glucose oxidase could find (by EPR) no radical intermediate. This result was interpreted as evidence against the formation of a radical intermediate. However, a low steady state concentration of a radical pair would also fail to give rise to an EPR signal. Other evidence offered to repudiate a radical mechanism were the findings that the radical forms of flavin oxidases do not react with the nor-

mal substrates of the enzymes.[8,16,17] If a radical pair formed from enzyme bound flavin and substrate were an intermediate then there should be no expectation that enzyme radical would react with substrate.

The results of this study establish one electron transfer mechanisms for reduction of glucose oxidase by furoin and its reoxidation by nitroxide (model substrates for glucose and O_2, respectively). On the basis of these results, the partial mechanism of Scheme I does not appear to be unreasonable for the glucose reduction of glucose oxidase and O_2 oxidation of reduced glucose oxidase.

Scheme I



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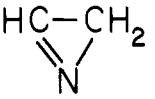
Preparation, Dipole Moment, and Quadrupole Coupling Constants of 2*H*-Azirine

Sir:

Derivatives of 2H-azirine



have been prepared by pyrolysis[1] and photolysis[1,2] of substituted vinyl azides, but the unsubstituted compound was not previously known. We have prepared 2*H*-azirine by flash vacuum pyrolysis and have characterized it by its pure rotational spectrum.

Vinyl azide, prepared by the method of Wiley and Moffat,[3] was passed through a 10 cm length of 12 mm heated quartz tubing. The products were immediately pumped through the absorption cell of a Stark-modulated microwave spectrometer. The pressure was estimated to be between 10 and 100 mT and the Stark cell temperature was at either room temperature or the temperature of dry ice. The intensity of the rotational spectrum of vinyl azide was seen to decrease as the quartz tube was heated above 200 °C and new absorption lines appeared. At 400 °C the vinyl azide lines had virtually disappeared and the new lines were of maximum strength.

The frequencies of the new lines were compared to those previously observed and tabulated in the National Bureau of Standards Microwave Spectral Tables.[4] A series of lines due to acetonitrile was assigned, but other strong lines could not be assigned to any known compound.

On the basis of resolved Stark effects, the transitions in Table I were assigned to a species with rotational constants and moments of inertia listed in Table II. This table also lists the rotational constants calculated for 2*H*-azirine based on the optimized structure calculated by Lathan et al.[5] using ab initio methods with the STO-3G basis set.

All of the assigned lines were found to be multiplets when observed under the best resolution available. The splittings were measured and attributed to the effect of a single nitrogen nucleus in the molecule with quadrupole coupling constants χ_{aa} = 1.04 MHz, χ_{bb} = −3.43 MHz, and χ_{cc} = 2.39 MHz.

Measurement of the Stark effects of four of the transitions gave the following values of the principal axes components of the dipole moment (in D): μ_a = 0.54 ± 0.02 and μ_b = 2.07 ± 0.04. The total dipole moment is thus 2.14 ± 0.05 D. Ab initio calculations[5] using the 6-31G basis set with the STO-3G optimized geeometry gave a dipole moment value of 2.56 D.

Some rotational transitions of the compound with ^{15}N and ^{13}C isotopes in natural abundance have been assigned and

Table I. Microwave Spectrum of 2*H*-Azirine[a]

Transition	Obsd frequency (MHz)	Calcd frequency (MHz)
$1_{01} \leftarrow 0_{00}$	37 288.52	37 288.53
$1_{10} \leftarrow 1_{01}$	20 550.63	20 550.62
$2_{11} \leftarrow 2_{12}$	21 476.73	21 476.73
$2_{11} \leftarrow 2_{02}$	29 902.88	29 903.49
$3_{13} \leftarrow 2_{20}$	36 067.20	36 068.55
$4_{22} \leftarrow 4_{23}$	25 045.93	25 048.87
$6_{33} \leftarrow 6_{34}$	25 326.90	25 335.75
$8_{44} \leftarrow 8_{45}$	23 586.67	23 603.95
$10_{55} \leftarrow 10_{56}$	20 756.66	20 783.78

[a] These frequencies have been corrected for quadrupole splittings.

Table II. Rotational Constants (MHz) and Moments of Inertia (amu Å^2) of 2*H*-Azirine

	Exptl	STO-3G
A	35615.43	35005
B	22223.72	22678
C	15064.81	15152
I_a	14.18989	14.437
I_b	22.74052	22.285
I_c	33.54699	33.354
$I_c - I_a - I_b$	−3.38342	−3.368