Table V. Heavy-Atom Coordinates ($\times 10^4$) and U_{eq} {$^1/_3$ Trace U ($Å^2 \times 10^3$)} for **3**

atom	X	Y	Z	U_{eq}
O(1)	-200 (4)	2124 (2)	1708	58 (1)
O(2)	-2263 (4)	1547 (3)	2495 (6)	70 (1)
O(3)	1309 (4)	782 (2)	2855 (5)	46 (1)
N(1)	-998 (4)	1319 (2)	2854 (6)	43 (1)
N(2)	1232 (4)	1443 (2)	2527 (6)	38 (1)
C(1)	-945 (5)	1261 (3)	4143 (6)	45 (1)
C(2)	-2015 (6)	809 (3)	4517 (8)	58 (1)
C(3)	-3327 (8)	1095 (4)	4174 (9)	85 (2)
C(4)	-3064 (5)	1692 (3)	3459 (7)	53 (1)
C(5)	-2439 (8)	2172 (3)	4232 (8)	66 (2)
C(6)	-1156 (6)	1909 (3)	4689 (7)	52 (1)
C(7)	1964 (6)	427 (3)	1916 (6)	48 (1)
C(8)	3408 (6)	582 (4)	2042 (8)	61 (2)
C(9)	3515 (6)	1313 (4)	2091 (7)	54 (2)
C(10)	2253 (5)	1603 (2)	1660 (6)	40 (1)
C(11)	1869 (5)	1303 (3)	500 (6)	44 (1)
C(12)	1409 (6)	626 (3)	768 (7)	52 (1)
C(13)	-23 (5)	1664 (2)	2315 (6)	38 (1)

least-squares procedure with H-atom positions riding on their bonded heavy atoms. The agreement factors are $R = 0.056$ and $R_w = 0.069$ for 995 observed reflections [$F_o > 1.5\sigma(F_o)$] (out of 1096 measured unique reflections). {$w = 0.3782/[\sigma^2(F_o) + 0.0194F^2]$}. The heavy-atom atomic coordinates and U_{eq} data for **3** appear in Table V. Hydrogen atom coordinates, bond length and bond angle tables, a stereoscopic view, crystal-packing diagram, and anisotropic displacement parameters are included in the supplementary material.

Near-IR spectra were recorded on a Cary 170 using 1-cm quartz cells. Solvents were percolated through a column of activated basic alumina or 4A molecular sieves (propylene carbonate) before use to remove hydroxylic impurities and deaerated with a stream of nitrogen.

ESR and PE spectra and cyclic voltammetry experiments were carried out as previously described.[1] The dynamic ESR program of Heinzer was employed.[16]

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Registry No. 1, 110589-99-6; **3**, 119336-12-8; **3**$^{\bullet+}$, 119336-14-0; **3** monounsaturated, 119336-13-9; **4**$^{\bullet+}$, 110590-21-1; **10**, 59978-63-1; **11**, 61260-90-0; **12**, 71631-54-4; 2-oxa-3-azabicyclo[2.2.2]oct-5-ene hydrochloride, 56239-25-9.

Supplementary Material Available: Stereoviews of the structure of **3** and its crystal packing, as well as tables of its atomic coordinates, atomic displacement factors, and bond lengths and angles, and plots of the PE spectra of **3** and **4** (7 pages). Ordering information is given on any current masthead page.

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Surface-Enhanced Resonance Raman and Electrochemical Investigation of Glucose Oxidase Catalysis at a Silver Electrode

Randall E. Holt and Therese M. Cotton*

Contribution from the Department of Chemistry, University of Nebraska—Lincoln, Lincoln, Nebraska 68588-0304. *Received August 8, 1988. Revised Manuscript Received November 22, 1988*

Abstract: Combined electrochemical and surface-enhanced resonance Raman scattering (SERRS) were used to investigate the non-Nernstian response of a Ag electrode to glucose oxidase (GO) activity. Square-wave voltammetry measurements indicated that AgO_2 was formed from the reaction of H_2O_2, a product of the enzymatic reaction, with the Ag surface. Dissolution of the oxide layer, in turn, generates sufficient Ag ion to form a flavin-Ag^+ complex. The formation of this complex was proven by a comparison of SERRS spectra obtained under a number of different conditions with the resonance Raman (RR) spectrum of the chemically prepared complex. A mechanism incorporating the electrochemical and SERRS results is proposed. The results demonstrate the potential of in situ SERRS for monitoring electrode surface reactions involving biomolecules. Furthermore, the results caution against the use of Ag (or other reactive materials) as electrodes for electrochemical studies or sensor devices involving GO catalysis.

I. Introduction

In the present study, in situ surface-enhanced resonance Raman scattering (SERRS) spectroscopy was used to provide structural information regarding the non-Nernstian, potentiometric response of a Ag electrode to glucose oxidase (GO) activity. The SERRS results, in combination with electrochemical measurements, have suggested a plausible mechanism for the potentiometric behavior that involves several reactions at the electrode surface.

SERRS has already been demonstrated to be a powerful technique for the study of chromophore-containing systems. These include a variety of biologically important molecules, such as heme-containing proteins,[1] porphyrins,[2] and more highly organized systems such as photosynthetic reaction centers[3] and membrane preparations from green plants[4] and photosynthetic bacteria.[5] The strength of SERRS lies in its combined surface and resonance

* Author to whom correspondence should be addressed.

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enhancement. Surface enhancement provides information about the interaction of the chromophore with the metal surface and can also provide information about the distance between the surface and chromophores within organized assemblies such as Langmuir–Blodgett monolayers and multilayers.[6] The resonance enhancement provides information about the environment of the chromophore and reflects changes in the chromophore that may also be manifest in its absorption spectrum (e.g., bleaching of an absorption band or spectral shifts that occur upon interaction with other molecules). Thus, the combination of surface and resonance enhancements makes SERRS particularly useful for probing the environment of a chromophoric cofactor or monitoring changes accompanying its reactions (e.g., metal complexation or reduction/oxidation). An additional advantage is that fluorescence quenching is often observed in molecules adsorbed on metal surfaces, allowing observation of the Raman spectrum not otherwise obtainable.

An important question that remains concerning the application of SERRS to complex biological systems pertains to the relevance of the spectra to functional biomolecules. Are the band frequency shifts and intensity changes in SERRS spectra as compared to RR spectra indicative of denaturation of adsorbed biomolecules, or do they result from molecule–surface interactions? In order to answer this question, we undertook a SERRS study of glucose oxidase. Our original goal was to measure the enzyme activity by electrochemical methods as well as by monitoring changes in the surface spectrum. This enzyme was chosen for two major reasons. First, it is fluorescent and, therefore, not easily studied by RR spectroscopy. Thus, it offers a stringent test of the SERRS technique. Second, it is an important enzyme from an analytical viewpoint.

The fluorescence quenching advantage of SERRS is particularly appealing for the study of flavins and flavoproteins. Initial attempts to obtain vibrational spectra of flavins using conventional resonance Raman (RR) methods were unsuccessful because of interference from intense free flavin emission at ca. 530 nm. Several approaches were used to eliminate or reduce fluorescence interference including coherent anti-Stokes Raman scattering (CARS),[7] the addition of a collisional quenching agent,[8] and the judicious selection of excitation and detection wavelengths.[9] When flavins are bound to a protein or complexed with a metal ion, the absorption and fluorescence properties are modified, and often observation of RR spectra without fluorescence interference is allowed. Interaction of the flavin adenine dinucleotide (FAD) with the apoprotein of several flavoproteins has been studied in this manner[10] as has the structure of flavin–metal complexes.[11] Interpretation of the observed vibrational spectra has been facilitated by the study of substituted and isotopically labeled flavins[12] and by normal coordinate analyses.[13]

There have been no previous attempts to monitor enzyme activity directly by SERRS. However, an O_2 electrode was used by Copeland et al.[14] to determine the activity of GO adsorbed on Ag sols following SERRS measurements. Also SERRS spectra of a number of flavoproteins, including GO, were obtained on Ag sols by Lee et al.[15] who concluded that the proteins were denatured. Interference by free flavin in SERRS studies of GO adsorbed on silver surfaces has also been reported.[16] SERRS spectra of free flavins adsorbed on a silver electrode[17] or silver colloids[18] have recently been reported. The spectra are typically very intense, even when excited with very low laser power (<10 mW), and are relatively free of fluorescence.

The second important reason for choosing GO as the subject of our SERRS investigation is its ability to catalyze the oxidation of glucose:

$$\text{glucose} + O_2 \xrightarrow{\text{GO}} \delta\text{-D-gluconolactone} + H_2O_2 \qquad (1)$$

H_2O_2 can be detected electrochemically, and for this reason GO has been used in the construction of enzyme-based electrodes which are selective for glucose. Such sensors have important potential application for the routine clinical assay of glucose, or for the development of an in vivo artificial pancreas. The sensors have been most commonly based upon the amperometric detection of reaction participants (e.g., O_2 or H_2O_2) or upon changes in the potential of an electrode which is sensitive to reactants or products. However, these potential changes have not been well characterized. Wingard et al. have shown a potential dependence upon glucose concentration using platinum, gold, and porous carbon electrodes both in the presence and absence of GO.[19] A potentiometric response was observed for bare platinum in glucose solutions, and this has become the subject of a second paper by this group.[20] The electrochemical approach used to investigate these systems underscores the complicated nature of the mechanism underlying the potentiometric response and the limited interpretation that is possible on the basis of electrochemical data alone.

In the results which follow, the potentiometric response of a silver electrode to glucose, both in the presence and absence of GO, is described. A confident description of the reactions occurring at the electrode during enzyme activity was not possible on the basis of potentiometry alone. However, through combined electrochemical and SERRS analysis, we were able to characterize the surface reactions that are responsible for the potential shift. A mechanism incorporating these results is proposed, and implications regarding the use of silver electrodes for flavoprotein electrochemistry or in GO-based sensors are discussed.

II. Experimental Methods

A. Materials. Flavin adenine dinucleotide, flavin mononucleotide (FMN), and glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4.; Type II from *A. niger*) were purchased from Sigma Chemical Co. FAD and FMN were used as received or were purified by anion exchange chromatography (Whatman DE-23), using pH 7, 0.1 M phosphate buffer as an eluant. GO was used as received.

ACS analytical grade silver nitrate (Mallinckrodt Inc.) and sodium sulfate (Fisher Scientific Co.) were used as received.

B. Instrumentation. All Raman spectra were obtained using the 488.0-nm line of an Ar^+ laser (Coherent, INNOVA 90-5). The laser power was typically 7 mW. The sample was irradiated in the backscattering configuration. The scattered radiation was collected and focused onto the slits of a monochromator/spectrograph (Spex, Triplemate 1877). The spectrograph gratings were 1200 g/mm (D^{-1} = 1.4 nm/mm) or 1800 g/mm (D^{-1} = 0.9 nm/mm) with slit widths of 0.050–0.200 mm. The monochromator stage contained two 600-g/mm gratings. An intensified diode array detector (PARC 1420) coupled to a multichannel analyzer (PARC OMA II) was used to accumulate and process the data.

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Indene was used for frequency calibration of all spectra.

Potential control during the SERRS experiment was accomplished using a potentiostat constructed in this laboratory. All potentials were recorded and are reported here with respect to the silver/silver chloride (Ag/AgCl) reference electrode. Square-wave (SWV) voltammetry was performed using a BAS-100 electrochemical analyzer (Bioanalytical Systems Inc.). SWV parameters used were as follows: square-wave amplitude, 25 mV; frequency, 15 Hz; step potential, 4 mV. Potential measurements were made using a pH meter with an input impedance of $>10^{12}$ ohms (Fisher, Model 610).

Absorption spectra were recorded with an HP 8450A UV-vis spectrophotometer (Hewlett-Packard) using 1-cm cuvettes and H_2O as a reference and blank.

C. Procedures. The flavin-Ag^+ complex was prepared as described below. A sufficient quantity of FAD or FMN was dissolved in H_2O to provide a concentration of 1×10^{-3} M. A 10-fold molar excess of solid $AgNO_3$ was then added to the FAD solution, and the solution was cooled to 0 °C while stirring in subdued light. The color of the solution changed immediately from yellow to orange, concurrent with a drop in the pH of the solution from 5.2 to 2.5. The pH was adjusted to 6.3 by the dropwise addition of NaOH solution using care to avoid formation of insoluble Ag_2O at a more basic pH.

SERRS spectra were recorded in a cell similar to that described previously.[21] Silver electrodes were prepared by sealing polycrystalline silver wire into glass tubing using Torr-Seal (Varian Associates, No. 953-0001). The wire was flattened and polished to a mirror-like finish using 0.3- and 0.05-μm alumina.

Electrolyte solutions (0.1 M Na_2SO_4) were degassed for >10 min by purging with N_2 immediately prior to the experiment. The silver electrode was roughened in electrolyte only, by stepping the potential from −0.55 to +0.50 V and allowing the passage of ca. 25 mC/cm^2 electrode area. The potential was then stepped back to −0.55 V to reduce the Ag^+ formed during the anodization. Samples were added directly to the cell or, alternatively, were adsorbed on the anodized silver electrode by removing the electrode from the cell and placing it into the sample solution. In the latter case, the electrode was allowed to remain in contact with the solution at room temperature with occasional mixing for 10–20 min, following which it was removed, rinsed well with H_2O or electrolyte solution, and returned to the cell containing only electrolyte solution. All spectra were recorded by summing 25 scans at 1 scan/s.

Resonance Raman spectra were measured in a flow system to minimize photodegradation during laser irradiation. The system is similar to that described by Benecky et al.[11a] with an internal volume of 2 mL and flow rate of 10 mL/min. A 0.5-mm glass tube was used as the window through which the flowing sample was irradiated and the scattered light was collected. The total circulated volume of the flavin-Ag^+ complex was 4 mL. The solution was filtered through a 0.45-μm membrane filter (Millipore Corp.) and cooled in an ice bath prior to circulation in the flow system. No significant change in the absorption spectra of the flavin-Ag^+ complexes was observed following laser irradiation during the RR experiment.

Potential measurements were made in a cell containing 25 mL of electrolyte solution (0.1 M Na_2SO_4). The solutions were purged with either air or nitrogen presaturated with H_2O for >5 min prior to and during the measurements. The Ag electrode and reference were placed into the cell and aliquots of the desired components were added. Potentials were recorded after an apparent steady-state potential was reached (typically <2 min).

Square-wave voltammetry of the H_2O_2-treated Ag electrode was performed following removal of the electrode from the solution, a brief rinsing by immersion into H_2O, and immediate transfer to a 15-mL electrochemical cell containing 1 M NaOH (previously N_2 purged). The voltammetry was initiated immediately following electrical connection to the BAS-100 to minimize dissolution of Ag_2O that was formed by the treatment. In order to facilitate assignment of the reduction peaks by comparison to previous electrochemical studies of the $Ag/Ag_2O/AgO$ system[22] and to minimize dissolution of the Ag_2O, 0.1 M NaOH was chosen as the electrolyte.

III. Results and Discussion

The initial goal of this research was to utilize SERRS to monitor directly the activity of adsorbed GO. In the oxidized state, the FAD prosthetic group of the enzyme is in resonance with the laser excitation wavelength used in these studies. A strong SERRS spectrum of FAD results, as shown in Figure 1. In contrast, the reduced form of the enzyme is nearly colorless and, hence, no longer in resonance with the laser excitation wavelength. If the adsorbed enzyme is active, its SERRS signal should decrease substantially upon addition of glucose under anaerobic conditions (O_2 reoxidizes the enzyme). Figure 2 shows that changes were indeed observed in the spectrum of the oxidized enzyme under these conditions. However, a rapid shift in electrode potential from its rest potential (typically −100 to +100 mV) to a final value of +200 to +300 mV (versus SSCE) accompanied these spectral shifts. The direction of the potential shift indicates an oxidation

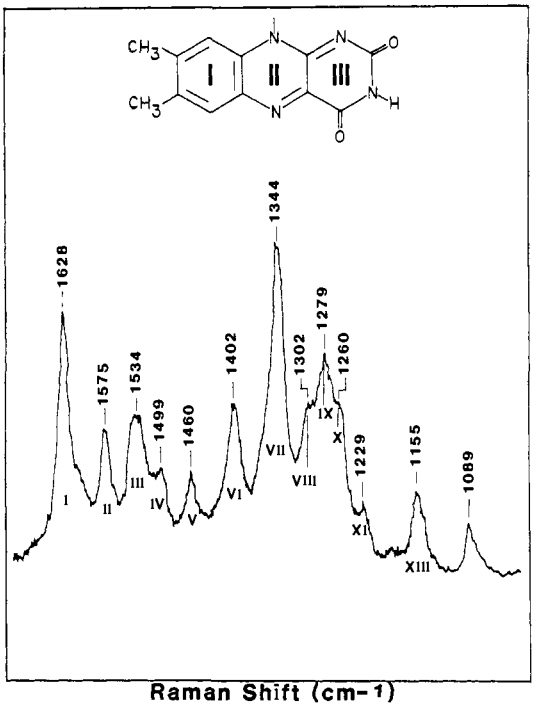

Figure 1. SERRS of FAD adsorbed on a Ag electrode: applied potential = −250 mV, $[FAD]_{ads}$ soln = 1×10^{-7} M, laser power = 7 mW, band-pass = 3 cm^{-1}.

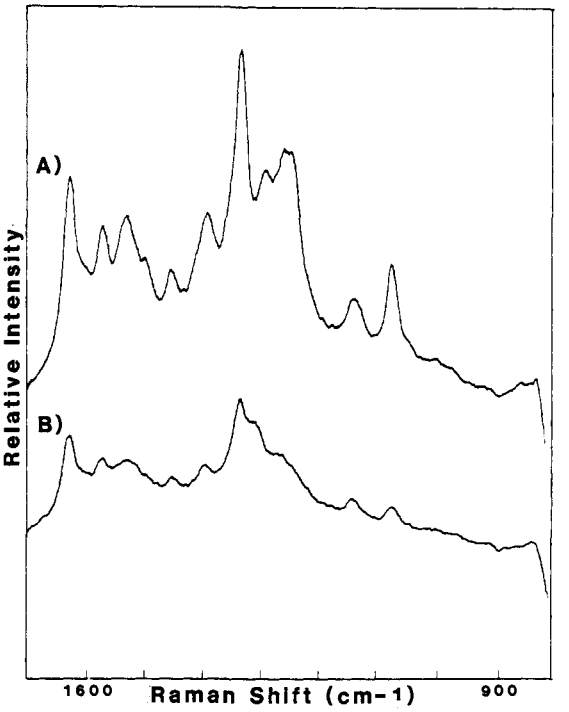

Figure 2. Change in SERRS spectrum of GO upon addition of glucose at open circuit potential: (A) GO only, (B) GO + glucose. [GO] = 2×10^{-6} M, [glucose] = 6×10^{-2} M, laser power = 7 mW, band pass = 11 cm^{-1}.

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Table I. Slope of Ag Electrode Potential versus log Analyte Concentration

system	slope (mV/decade of concn)
1. GO (23 mg/dL) + glucose (4–400 mg/dL) aerated	+105 ± 15
2. glucose (4–400 mg/dL) aerated	−4 ± 2
3. GO (13 mg/dL) + glucose (4–400 mg/dL) deaerated	−1 ± 4
4. GO (0.2–40 mg/dL) aerated + glucose	−21 ± 5
5. GO (absorbed only) + glucose (4–400 mg/dL), aerated	+2 ± 3
6. FAD (1×10^{-6}–1×10^{-4} M)	+4 ± 10
7. glucose (4–400 mg/dL) deaerated	+5 ± 15
8. H_2O_2 (0.13–130 mg/dL)	+33 ± 8

process occurs at the Ag electrode concurrent with, or as a consequence of, enzyme activity. This observation suggests that the potential shift may provide a means of quantitating enzyme activity or the concentration of substrate. To explore this possibility, we have investigated the potentiometric behavior of Ag in several systems containing GO, as described in section A below. In order to understand the nature of this unexpected oxidation process, we have undertaken a study to correlate the changes in the SERRS spectrum with the potential shift and to identify the reactants and products formed at the electrode surface. These results are discussed in section B.

A. Potentiometric Study of GO Activity at the Ag Electrode. In order to correlate the Ag electrode potential with analyte concentration, several different systems were examined, as shown in Table I. The slopes of the concentration versus potential plots were calculated from the difference between the steady-state potentials measured after the first and final additions of analyte. Triplicate measurements were made for each set of conditions, and the average and standard deviation for the slopes are shown in Table I.

System 1 was an aerated solution of GO. Aliquots of glucose, spanning two decades of concentration (4–400 mg/dL or 2×10^{-4} to 2×10^{-2} M) were added to the GO solution. Large positive shifts in potential were observed when the cell contained bulk GO and O_2, conditions under which GO catalysis is expected. The magnitude of the slope measured for the catalytically active system (+105 mV/decade) clearly specifies a non-Nernstian process.

In contrast to the above results, when either GO (system 2) or O_2 (system 3) was absent from solution, no significant potential response was observed as a function of glucose concentration. Also, changing the GO concentration (0.2–20 mg/dL or 1×10^{-8} to 1×10^{-6} M) in aerated solutions of glucose (system 4) had comparatively little effect on the electrode potential. Moreover, no significant potential response was measured for system 5, containing only adsorbed GO (no GO in solution), glucose, and O_2. As will be discussed below, no detectable change in the SERRS spectrum occurred under these conditions, in contrast to the large changes observed when bulk GO was present. These latter results suggest that the adsorbed enzyme is inactive.

Other control experiments were also performed to determine the source of the potential response. No change in electrode potential was observed as a function of FAD (system 6) or glucose (system 7) concentration in solutions lacking GO and O_2. These results may be compared to a previous report that the potential of Pt, Au, or porous carbon electrodes is sensitive to glucose concentration.[18] In this latter study, the response of Pt or Au electrodes to glucose alone (no glucose oxidase was present) was reported with slopes of −17 and −34 mV/decade glucose concentration, respectively. The reactions responsible for these potential changes are not known. For Pt, the involvement of Pt oxides on the electrode surface has been suggested.[19] The behavior of these systems at the electrochemical interface is complex, and the mechanisms responsible for the potential shifts cannot be determined from potentiometric measurements.

The product of the catalytic oxidation of glucose by GO is H_2O_2, which can be monitored potentiometrically. Accordingly, the possiblity that Ag/H_2O_2 response was non-Nernstian was addressed. Although the slope of potential change over the concentration range used suggests that the response is Nernstian (Table I, system 8, no enzyme or substrate) the response was nonlinear. Nevertheless, the large changes in potential that were observed in system 1 do not occur in the presence of H_2O_2 alone. Thus, the formation of H_2O_2 is not responsible for the potentiometric changes that occur when the enzyme is active. Rather, it appears that the involvement of GO and its reaction products severely perturbs the potentiometric response of the catalytic system. Additionally, large standard deviations are associated with these measurements and the responses for systems 1, 4, and 8 were all nonlinear. These factors limit the use of potentiometry for characterization of the electrode process, as well as for quantitation of the reactants.

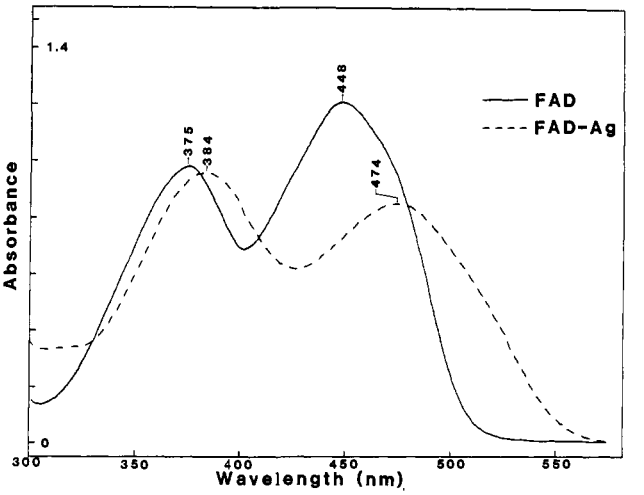

Figure 3. Change in electronic absorption spectrum of FAD upon Ag complexation: (—) 1×10^{-4} M FAD. (---) 1×10^{-4} M FAD–Ag^+.

B. SERRS of the GO/Ag Reaction Product. The potentiometric results described above indicate that bulk GO, glucose, and O_2 (i.e., catalytically active enzyme) are essential for producing the positive shifts in Ag electrode potential that were originally observed in conjunction with SERRS measurements. Moreover, the requirements for observing changes in the SERRS spectrum were identical with those determined from the potentiometric measurements; i.e., bulk enzyme, glucose, and O_2 must be present. This suggests a common origin for the potentiometric and spectroscopic effects. From the spectroscopic changes it is apparent that the FAD moiety is modified during enzyme activity. However, based upon the measured open circuit potential, the flavin is not reduced. Rather, it appears more probable that oxidation of the Ag electrode is occurring and Ag^+ is reacting with flavin. Consequently, the possibility that a flavin–Ag^+ complex is formed on the electrode surface was considered. It is well known that Ag^+ forms a stable complex with flavins, and the resonance Raman spectra of FMN and RF–Ag^+ complexes have been reported.[11,23] To test this hypothesis, the FAD–Ag^+ complex was prepared and its SERRS spectrum characterized.

The absorption spectrum of the FAD–Ag^+ is shown in Figure 3. This spectrum is similar to those published for FMN–Ag^+ [11a] and RF–Ag^+ [23] with respect to the shoulder in the 500–550-nm region in the complex and the shift and bleaching of the 450-nm peak of the uncomplexed flavin. Two differences are apparent, however, the 530-nm shoulder is not as distinct nor is the bleaching of the 376-nm peak as pronounced as in the spectrum of FMN–Ag^+ or RF–Ag^+. The possibility was considered that the 10-fold molar excess of Ag^+ added in the synthesis was insufficient to complex completely the flavin. To determine if this was the case, an additional 7-fold molar excess of $AgNO_3$ was added to the

(23) Bessey, O. A.; Lowry, O. H.; Love, R. H. *J. Biol. Chem.* **1949**, *180*, 755–769.
(24) Baarda, I. F.; Metzler, D. E. *Biochim. Biophys. Acta* **1961**, *50*, 463.

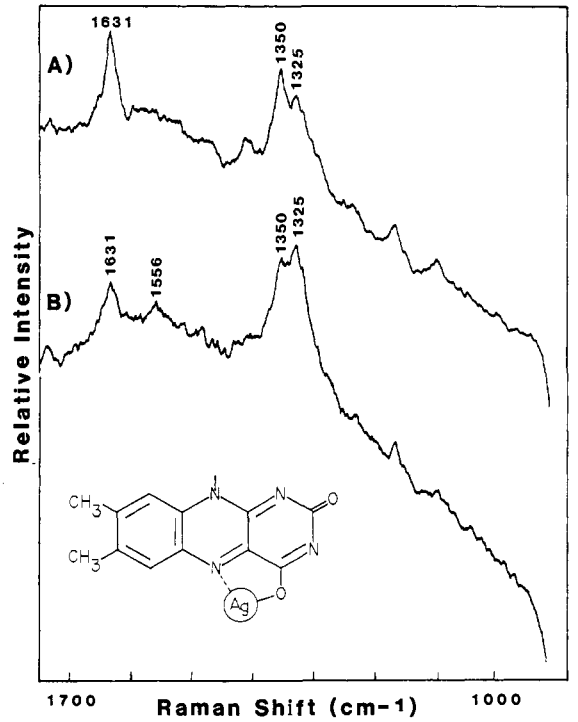

Figure 4. Resonance Raman (flow cell) spectra of chemically generated flavin–Ag^+ complexes: (A) FMN–Ag^+, (B) FAD–Ag^+. Concn = 1×10^{-3} M, laser power = 7 mW, band pass = 11 cm^{-1}.

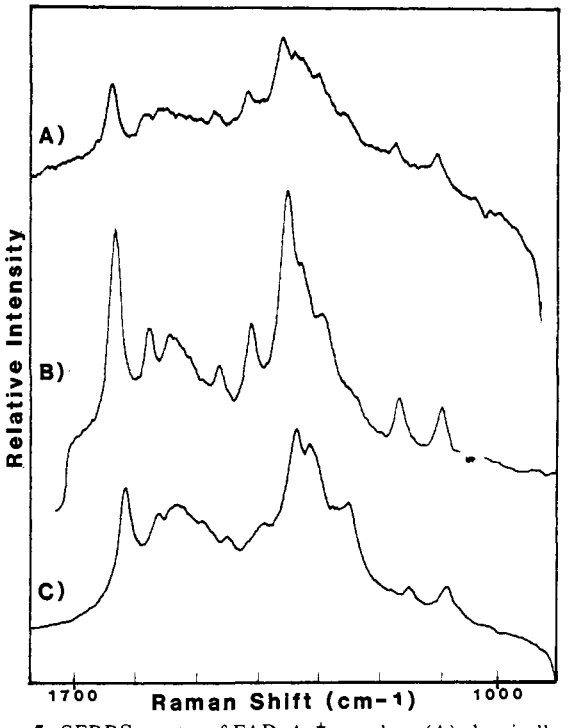

Figure 5. SERRS spectra of FAD–Ag^+ complex: (A) chemically generated, adsorbed from 1×10^{-3} M soln; (B) generated by addition of Ag^+ to FAD adsorbed onto electrode, $[Ag^+] = 1 \times 10^{-5}$ M; (C) electrogenerated (see text). Laser power = 7 mW, band pass = 11 cm^{-1}.

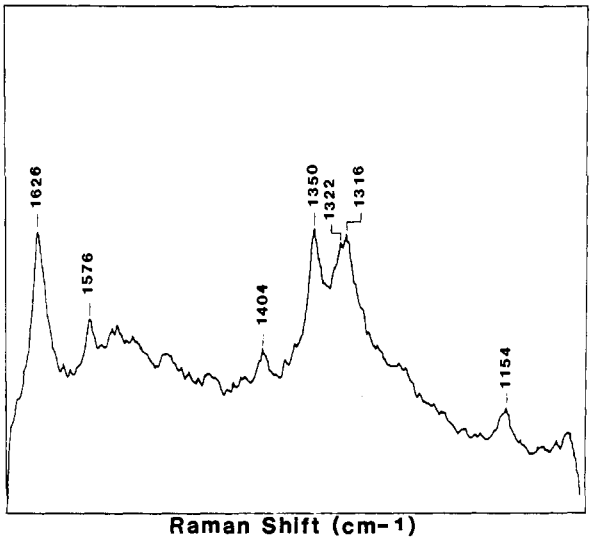

Figure 6. SERRS of FAD adsorbed on a Ag electrode: applied potential = +750 mV, laser power = 7 mW, band pass = 8 cm^{-1}.

$FAD–Ag^+$ solution. No further change in the absorption spectrum was observed, indicating that complexation was complete.

Resonance Raman spectroscopy was used to characterize this complex using a flow system similar to that described by Benecky et al.[11a] for the characterization of the $FMN–Ag^+$ complex. RR spectra of both $FAD–Ag^+$ and $FMN–Ag^+$ are shown in Figure 4. The similarity of these two spectra suggests that the adenine ring of FAD does not significantly affect the nature of the binding interaction between Ag^+ and FAD. Thus, stacking of the adenine and isoalloxazine rings, which was proposed to account for the diminished fluorescence quantum yield of FAD in solution relative to FMN,[23] is either disrupted by, or does not significantly interfere with, the complexation of Ag^+ with the isoalloxazine ring. The intensity of the band at 1325 cm^{-1} was variable relative to the band at 1350 cm^{-1}, however, and increased upon continued laser irradiation. This may be an indication of partial photodecomposition of the complex as discussed further below. Variations in the spectra shown in Figure 4 in this respect are believed to result from differences in irradiation time.

SERRS spectra of the $FAD–Ag^+$ complex were obtained by adsorption of the complex onto the surface of the electrochemically roughened electrode (Figure 5A). Spectra similar to that of the chemically prepared $FAD–Ag^+$ complex were also obtained from FAD obtained on the silver electrode by two other procedures: (1) addition of Ag^+ to the electrolyte in the SERS cell (Figure 5B) and (2) application of a potential sufficiently positive to oxidize the silver electrode (Figure 5C). Spectra obtained by all three procedures show the same differences relative to the spectrum of uncomplexed FAD (Figure 1): a new intense peak is present at 1320–1328 cm^{-1}, the intensity of bands VI, IX, and X (see Figure 1 for band numbering) is decreased relative to other bands in the spectrum, and the overall spectral intensity is decreased. Some bands observed in Figure 5 may be identified with the presence of free flavin on the electrode in addition to the complex. Consideration of the association constant for the riboflavin–Ag complex (ca. 6×10^7 M^{-1}[23]), predicts that a low concentration of free flavin should exist concurrently with the complex. The observation of small amounts of free flavin is not surprising based upon the sensitivity of SERRS to free flavin as noted previously.[16] When a potential of +750 mV was applied to the electrode (Figure 6), the equilibrium shifted toward formation of the complex as a result of the high Ag^+ concentration. The free flavin bands disappeared and the resulting SERRS spectrum is very similar to the RR spectra of the flavin–Ag^+ complexes (compare Figures 6 and 4). To obtain the solution RR spectra shown in Figure 4, a 10-fold excess of Ag^+ was used, forcing the equilibrium away from the free flavin and effectively quenching its fluorescence which would otherwise obscure or completely mask the RR spectra. The similarity of the three types of surface spectra under conditions whereby the $FAD–Ag^+$ complex is formed, as well as the similarity of the GO SERRS spectrum to the RR spectrum of the $FAD–Ag^+$ complex, verify that we are indeed observing the formation of the $FAD–Ag^+$ complex on the electrode surface during enzymatic oxidation of glucose. The similarity between Figures 4 and 6 also suggests that the enhancement mechanism includes both surface and resonance contributions.

Slight differences in both the flow cell and SERRS spectra may originate from the photosensitivity of the flavin–Ag^+ complexes.

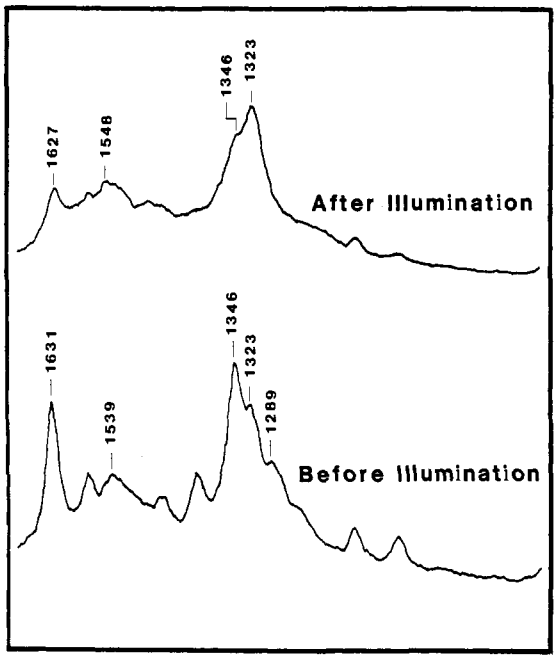

Figure 7. In situ photodegradation of the FAD–Ag^+ complex (formed by addition of 1×10^{-3} M Ag^+ to FAD adsorbed on a Ag electrode). SERRS of complex taken before and after 9 min of continuous irradiation in the laser beam: laser power = 7 mW, band pass = 11 cm^{-1}.

Photosensitivity of the complexes has been previously noted,[11a] but details of the spectral consequences were not presented. Absorption spectra of the FAD–Ag^+ and FMN–Ag^+ solutions used in our flow cell experiments were taken both before and after irradiation and showed no shifts in absorption bands or changes in relative intensities following laser irradiation. However, radiation-dependent changes in the SERRS spectrum of FAD–Ag^+ were observed, as manifest most clearly by the change in the relative intensities of the 1350- and 1325-cm^{-1} bands and the bleaching of all bands as shown in Figure 7. These changes are not due to an irreversible change in the isoalloxazine component of FAD. Reduction of the complex at the electrode by application of a potential less than ca. +100 mV regenerates the characteristic oxidized flavin spectrum. The spectrum observed in Figure 7 after illumination is very similar to the FAD–Ag^+ RR spectrum in Figure 4. This suggests that the illumination shifts the equilibrium of FAD adsorbed at the Ag surface toward formation of the Ag^+ complex.

In conclusion, a comparison of the spectrum presented in Figure 2B to those shown for the FAD–Ag^+ complex (Figure 5A–C) demonstrates that FAD–Ag^+ is produced at the electrode surface when GO activity is expressed in the cell. Under these conditions, complex formation occurs between free flavin present in the GO preparation[16,17] and Ag^+.

C. Origin of Ag^+ and the Mechanism of FAD–Ag^+ Complex Formation. The involvement of Ag^+ at the electrode during enzyme catalysis is established from the electrochemical and SERRS experiments described above, but the origin of the Ag^+ is not clear. Consideration of the products of the catalytic reaction:

$$\text{glucose} + O_2 \xrightarrow{GO} H_2O_2 + \delta\text{-D-gluconolactone}$$

suggests that H_2O_2, a strong oxidizing agent, may subsequently oxidize the Ag electrode. This reaction has been reported in the literature[25] to involve the hydroperoxy anion, resulting in formation of a layer of Ag_2O on the Ag surface:

$$H_2O_2 + OH^- \rightarrow HO_2^- + H_2O$$

$$2Ag + HO_2^- \rightarrow Ag_2O + OH^-$$

(25) Bagg, J. *Aust. J. Chem.* **1962**, *15*, 201–210.

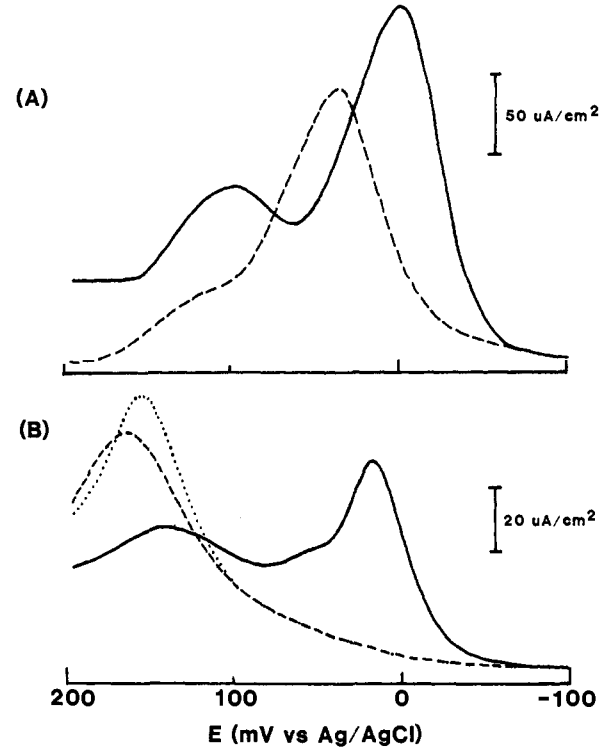

Figure 8. Square-wave voltammetry of the Ag_2O/Ag electrode: (A) produced by reaction of the electrode in 33 mM H_2O_2 (in 0.1 M Na_2SO_4) for 5 min (—), produced by anodization of the electrode at +500 mV for 1 s in 1.0 M NaOH (---); (B) bare Ag electrode control (no prior treatment, ---), electrode treated in 0.1 M Na_2SO_4 solution containing GO ([GO] = 9 mg/dL) and glucose ([gluc] = 0.1 mg/dL) in absence of O_2 (···), and following oxygenation of the solution (—). Voltammetric conditions are described in the text.

The steady-state accumulation of Ag_2O further reacts to decompose HO_2^-:

$$Ag_2O + HO_2^- \rightarrow 2Ag + OH^- + O_2$$

Although no Ag^+ is formed directly in the above sequence of reactions, it is produced by hydrolysis of Ag_2O:

$$Ag_2O + H_2O \rightarrow 2Ag^+ + 2OH^-$$

The K_{sp} for Ag_2O is 9.5×10^{-5} M based upon its reported solubility.[26] From this sequence of reactions, sufficient Ag^+ may be produced to complex with FAD present near the electrode. Additionally, the photolysis of Ag_2O to form Ag^+ has been noted,[27] and this may also be an important source of Ag^+ in the SERRS experiments.

Several observations support the formation of Ag_2O under the experimental conditions used in this study. In the catalytic GO system, as well as in H_2O_2 solutions, a change in appearance of the electrode surface to a dull gray color occurred concurrently with the positive shift in potential. This is consistent with the formation of a layer of Ag_2O on the surface. Ag_2O is reportedly not detectable by Raman spectroscopy[22c] but has been successfully identified by electrochemical methods.[22] Thus, we have used square-wave voltammetry to verify the formation of Ag_2O in the active GO/Ag electrode system. Figure 8A illustrates voltammograms obtained immediately following anodization of the Ag electrode in NaOH (dashed line) and after reacting the electrode with 0.33 M H_2O_2. In both cases, a peak near +40 or +4 mV (versus Ag/AgCl), respectively, arise from reduction of Ag_2O, as shown by comparison with previous electrochemical investigations of the Ag/Ag_2O/AgO system.[22] When the Ag electrode was placed in contact with GO and glucose in the absence of

(26) *Solubilities of Inorganic and Organic Compounds*; Stephen, H., Stephen, T., Eds.; Macmillan Co.: New York, 1963; Vol. 1, Pt. 1; pp 197.
(27) *The Merck Index*, 8th ed., Merck and Co.: Rahway, NJ, 1968; pp 948.

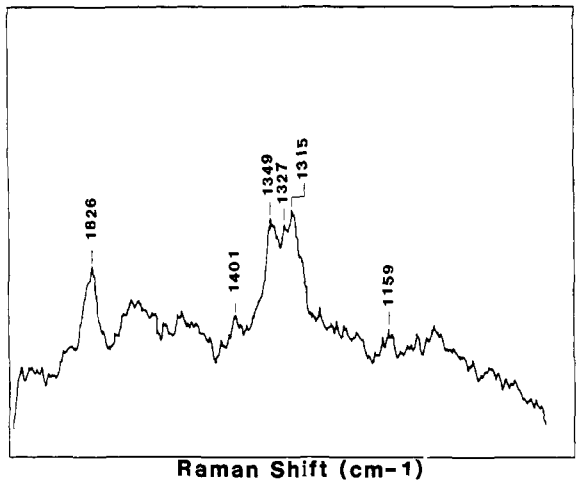

Figure 9. SERRS of FAD adsorbed onto a Ag_2O/Ag electrode: laser power = 7 mW, band pass = 11 cm^{-1}.

oxygen, a voltammogram identical with that of the clean electrode control (Figure 8B) was observed. Upon oxygenation of the mixture, however, a reduction peak at +20 mV was apparent. This peak is attributed to reduction of Ag_2O that is formed by reaction of the electrode with H_2O_2. The latter is a product of the reoxidation of the reduced enzyme by O_2. Small differences in peak potentials observed for the reduction of Ag_2O produced under different conditions (Figure 8) are most likely due to differences in thickness and composition of the oxide layer. For example, anodization in 1 M NaOH produces AgO in addition to Ag_2O. The presence of GO, glucose, and the reaction products in the oxide layer may also affect the observed peak potential. No attempt was made to control the thickness of the oxide layer formed during the various pretreatments. In the presence of the H_2O_2 or active enzyme, the thickness of the oxide layer is dependent upon the H_2O_2 concentration. In the case of the anodization pretreatment, the thickness is determined by the charge passed during the oxidation step and the rate of solubilization of the oxide in basic solution.

In the above system, Ag^+ is formed either concurrently with, or following the formation of Ag_2O via the reaction of H_2O_2 with the Ag electrode. Evidence that the FAD–Ag^+ complex is formed under these conditions is provided in Figure 9. In this experiment, a smooth Ag electrode was oxidized in the presence of 1 M NaOH. The potential was scanned from −200 to +490 $mV_{,}$ allowing approximately 160 mC to pass during the formation of Ag_2O on the electrode surface. The electrode was then rinsed with H_2O and placed into a solution of FAD (1×10^{-6} M) for 10 min, rinsed again, and placed into the cell. The resulting SERRS spectrum is clearly that of the FAD–Ag^+ complex, as shown by comparison with Figures 4 and 5. The presence of the oxide layer does not preclude observation of the SERRS spectrum of the complex.

The implications of our results with respect to the use of silver electrodes in glucose sensors are considerable. The primary problem in potentiometric measurements will arise from passivation of the electrode surface by Ag_2O as a result of its reaction with H_2O_2. This reaction perturbs the potential response in a nonreproducible fashion. In amperometric measurements of H_2O_2, the formation of Ag_2O is prevented by the continuous application of a potential more negative than ca. 0 mV versus SCE. However, open circuiting the electrode in the presence of the H_2O_2 would result in oxidation of the Ag electrode. Several enzyme-based amperometric sensors have been described which use Ag electrodes in their construction and involve the production of H_2O_2.[28] Corrosion of the Ag may be a significant factor in reducing the usable lifetime of these sensors.

Another problem that results from the oxidation of the electrode is the inhibition of GO activity by Ag^+, as described previously.[29] The rate of this inhibition is dependent upon the rate of dissociation of Ag_2O to form Ag^+, whether by hydrolysis or photolysis as described above. The ability of flavins to form stable complexes in aqueous media with other metal ions, such as Cu^+ and Fe^{2+},[30] may also preclude the use of these metals in systems involving H_2O_2 production.

IV. Conclusions

The experimental results presented above demonstrate that the open circuit potential of a Ag electrode is strongly dependent upon the catalytic activity of GO in an electrochemical cell. In the presence of O_2 and bulk glucose, a positive shift in electrode potential is observed when glucose is added to the cell. The magnitude of the shift is dependent upon glucose concentration in a non-Nernstian fashion. A plausible mechanism for this potential shift is proposed based upon the combined electrochemical and SERRS results. The SERRS spectrum observed at the shifted potential was identified as a flavin–Ag^+ complex, by comparison with the spectrum of the chemically prepared FAD–Ag^+ complex. Thus, the mechanism invokes the oxidation of the silver surface by H_2O_2, a product of the catalytic oxidation of glucose by GO. The reaction of H_2O_2 with Ag is known to produce Ag_2O, which causes the positive shift in the open circuit electrode potential. Adsorbed FAD reacts with Ag^+ in equilibrium with Ag_2O. The presence of Ag_2O on the Ag electrode does not interfere with the observation of the SERRS spectrum of the complex as a result of the large resonance contribution to the enhancement mechanism. No shift in cell potential or change in the FAD SERRS spectrum occurs when only adsorbed GO is present on the Ag electrode and glucose is added to the solution. This observation may suggest that the adsorbed enzyme is denatured by its interaction with the Ag surface. However, we cannot rule out the possibility that the amount of H_2O_2 produced under these conditions is too small to affect the electrode potential.

The utility of combining in situ SERRS measurements with electrochemical control of the electrode potential for characterizing the flavin–Ag^+ Raman spectrum has also been demonstrated. The ability to control the Ag potential is an advantage that solid electrodes possess over Ag sols, another popular SERRS substrate. A correlation of the effects of potential on the spectrum has provided valuable insight into the complex changes that occur in the flavin spectrum upon interaction with Ag.[31] The sensitivity of the SERRS spectrum of FAD to potential is undoubtedly important in silver colloids studies as well. However, the potential at the silver colloid–electrolyte interface is difficult to control and is determined by the redox potential of the surrounding electrolyte (i.e., the relative concentrations of Ag^+ to reductant). Further work investigating the potential dependent shifts in flavin bands on the Ag electrode is currently underway. The results should provide insight regarding the variable potential of the silver colloid.

This work demonstrates the considerable potential of SERRS for providing insight into the surface interactions and reactions of complex biomolecules at the solid electrode/electrolyte interface.

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