Free Flavin Interference in Surface Enhanced Resonance Raman Spectroscopy of Glucose Oxidase

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Abstract: Surface enhanced resonance Raman scattering (SERRS) studies of the enzyme glucose oxidase (GO) adsorbed on a silver electrode were complicated by the presence of free flavin adenine dinucleotide (FAD). The free flavin is present in commercial preparations of the enzyme and is also released from the protein solution upon standing at room temperature. In the latter case, the FAD may be either adventitiously bound and/or produced by dissociation from the active site of the enzyme. In any case, molecular exclusion chromatography at 6 °C does not remove the weakly bound flavin, as shown by fluorescence experiments. A correlation between the increase in SERRS and fluorescence intensity was noted as the protein solution was allowed to stand at room temperature. The weakly bound flavin was found to be effectively removed by either molecular exclusion chromatography at room temperature or extensive dialysis at 6 °C. Following either of these procedures, the stability of GO solutions was significantly improved. The complete removal of free or loosely bound flavin is essential because very low concentrations (10^{-10} M) of FAD are readily detectable by SERRS spectroscopy. Free FAD obscures the spectrum of enzyme-bound FAD, which is extremely weak and even undetectable in some instances by our experimental procedure. However, the presence of adsorbed enzyme was demonstrated even in the absence of SERRS signals by reanodizing the electrode in electrolyte only. Under these conditions a strong SERRS spectrum which closely resembled that produced by free FAD was obtained. Thus, the anodization procedure disrupts the native FAD–enzyme interaction, allowing the FAD to contact the silver surface directly. The relevance of these results to SERRS of proteins in general is discussed.

Surface enhanced resonance Raman scattering (SERRS) spectroscopy has previously been shown to provide vibrational spectra of a number of different types of biologically important molecules adsorbed onto the silver surface. These include cytochromes,[1] hemoglobin,[2] myoglobin,[1] porphyrins,[3] photosynthetic bacterial reaction centers,[4] and membrane preparations from green plants.[5] In each of the systems studied thus far, differences are noted between Raman spectra obtained of the molecules adsorbed at the silver surface as compared to the solution spectra. These differences may result from three possible causes: the proximity of the molecules to the silver surface causing perturbation of the vibrational modes by the strong electromagnetic fields at the surface, the direct chemical interaction of functional groups on the molecules with the metal surface (i.e., complex formation[3,6]), or from alterations in the native structure of proteins near the resonance scattering site.

Enzymes offer an excellent choice for investigating the third possibility because severe distortion of the active site region is expected to result in loss of activity. Glucose oxidase (GO) has been chosen for study by this laboratory[7] and others[8] as it possesses several characteristics making it particularly suitable as a model for interaction of enzymes with the silver surface: it possesses the chromophoric cofactor, flavin adenine dinucleotide (FAD) which allows resonance enhancement. The oxidation state of the flavin is readily monitored by the observed bleaching of the flavin upon reduction. For the native, active enzyme this should allow direct observation of catalytic activity by SERRS. Additionally, a large body of literature exists on the physical and kinetic properties of this enzyme. For these reasons GO appears to be an ideal system for the study of enzyme–surface interactions.

The present work evaluates more critically the suitability of GO as a probe of the enzyme silver interaction on the silver electrode in light of several factors. First, the inhibition of glucose oxidase activity by silver(I) has previously been described[9] as well as the formation and considerable stability of flavin–silver(I) complexes.[10] Second, we will demonstrate in this work the presence of free flavin in the commercial GO preparation most commonly used in this and other laboratories. A fraction of this free flavin appears to be protein bound and is slowly released after routine purification. The presence of this free flavin interferes with the observation of SERRS spectra of the native enzyme. Third, upon removal of this free flavin, as evidenced by low fluorescence emission at 530 nm, the SERRS spectrum was severely attenuated. The implications of these results with respect to SERRS of biomolecules are discussed.

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Experimental Methods

A. Materials. Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4; Type II from *Aspergillis niger*) and flavin adenine dinucleotide were obtained from Sigma Chemical Co. Purification of GO was done either by dialysis (MW cutoff 6000–8000) against deionized water at 6 °C for at least 48 h or by molecular exclusion chromatography with Sephadex G-25 at room temperature or at 6 °C, eluting with 0.1 M Na_2SO_4 or 0.05 M acetate, pH 5. FAD was purified by elution from DEAE-cellulose (Whatman DE-23) with 0.1 M phosphate buffer, pH 7. This purified FAD solution was used as a stock solution for these experiments and was stored at 6 °C in the dark until use. Verification of the purity was performed periodically with thin layer chromatography. This solution was observed to remain pure upon storage over many months.

B. Instrumentation. Fluorescence energy measurements were made on a Perkin-Elmer MPF-44A spectrofluorimeter with excitation at 450 nm and emission measured at 530 nm in 1 cm quartz fluorescence cuvettes, single beam mode.

SERRS spectra were recorded with use of an Ar^+ laser (Coherent, INNOVA 90-5) as the excitation source. Typical laser powers used were 7 mW. The samples were irradiated in the backscattering configuration. The scattered light was collected and focussed onto the slits of a monochromator/spectrograph (Spex, Triplemate 1877). A 1200-g/mm (D^{-1} = 1.4 nm/mm) grating was used in the spectrograph stage with a slit

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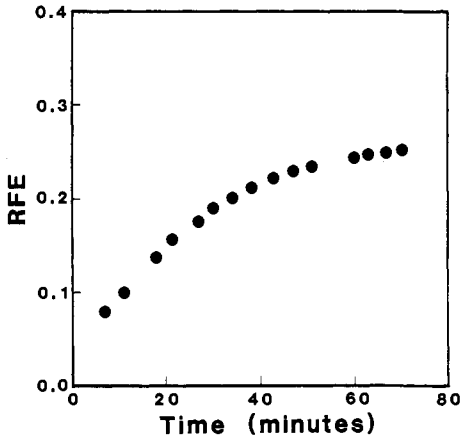

Figure 1. Increase in relative fluorescence energy of purified GO (molecular exchange chromatography, 6 °C) as a function of time standing at room temperature. Details described in text.

width of 0.200 mm; and two 600-g/mm gratings were used in the monochromator stage. An intensified diode array detector (PARC 1420) coupled to a multichannel analyzer (PARC OMA II) was used to accumulate and process the data. Indene was used for frequency calibration of all spectra.

Potential control was done with use of a potentiostat constructed in our laboratory. All potentials were recorded and reported here with respect to the saturated standard calomel electrode (SSCE) reference.

C. Procedures. Enzyme activity assay was performed according to the *o*-dianisidine procedure described in the enzyme lot analysis information provided with the enzyme. All assays were done at room temperature and subsaturating O_2 concentrations. Activities assayed under these conditions typically produced results slightly higher than (within 10%) that reported in the specifications for assay at 35 °C. Protein assay was done with use of the modified Lowry procedure provided as a kit (catalog no. 690-A) by Sigma Diagnostics.

Thin-layer chromatography of flavins was accomplished by a modification of a system described by Kilgour et al.[11] Flavin solutions were spotted on a cellulose plate (Kodak, 13255 Cellulose without Fluourescent Indicator, No. 6064) and developed with the organic phase of a mixture of *n*-butyl alcohol/acetic acid/water (40:10:50). This system was found to give efficient separation of FAD (R_f = 0.10), flavin mononucleotide (FMN, R_f = 0.30), riboflavin (R_f = 0.55), lumiflavin (R_f = 0.60), and lumichrome (R_f = 0.70). Visualization was produced by intrinsic fluorescence of the flavins under UV irradiation. As little as 1 ng of flavin may be detected in this manner.

A 1×10^{-6} M solution of FAD in 0.1 M phosphate buffer, pH 6.5, was used as a fluorescence standard. The relative fluorescence energy (RFE) for FAD under these conditions was observed to be linear with concentration to 1×10^{-9} M. The standard solution RFE was observed to be stable upon storage at room temperature over the course of a single day. All fluorescence measurements were made in phosphate buffer to eliminate any differences arising from the known pH dependence of the fluorescence quantum yield of FAD.[12]

SERRS spectra were recorded in a cell which has been described previously.[13] Silver electrodes were prepared by sealing polycrystalline silver wire into glass tubes with Torr-Seal (Varian Assoc., No. 953-0001). The wire was flattened and polished to a mirror-like finish with 0.3 and 0.05 μm alumina.

Electrolyte solution (0.1 M Na_2SO_4) was degassed by purging with N_2 for at least 10 min. The silver electrode was roughened by an oxidation–reduction cycle (ORC) by stepping the potential from −0.6 to +0.45 and allowing ≈25 mC/cm² of charge to be passed. The potential was then returned to −0.6 V until the Ag^+ was rereduced (i.e., until the current fell below 10 μamps). GO and FAD were adsorbed onto the roughened electrode by placing the electrode into a solution diluted to the appropriate concentration. After allowing 10–20 min for the adsorption to occur, the electrode was removed, rinsed, and replaced in the cell containing only electrolyte solution. Spectra were recorded by signal averaging of 25 scans at 1 scan/s.

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Table I

	RFE[b]	specific activity (μM units/mg of protein ×10⁻²)
dialysis purified GO[a]		
before	0.38	1.5 ± 0.1
after (immed)	0.01	1.7 ± 0.1
after (2 h)	0.02	
column purified GO (RT)[c]		
before	0.60	1.7 ± 0.1
after (immed)	0.03	1.6 ± 0.1
after (3 h)	0.02	

[a] 0.25 g of Sigma Type II/mL, dialysis against H_2O, 48 h, 6 °C. [b] Fluorescence standard (10^{-6} M FAD, pH 6.5) RFE = 1.00. [c] 0.20 g of Sigma Type II/mL, Sephadex G-25, elution with 0.1 M Na_2SO_4.

Results and Discussion

An important conclusion resulting from this work is that commercial GO preparations contain both free flavin as well enzyme bound flavin which is readily released after purification. Although the presence of free flavin in GO preparations is not unexpected, and indeed in column purifications a distinct free flavin band is observed under UV illumination, the release of flavin from the purified enzyme was not anticipated. Figure 1 illustrates the increase in relative fluorescence energy (RFE) after purification of Sigma Type II GO by molecular exclusion chromatography at 6 °C. Although the initial RFE was lowered as expected upon removal of free flavin, it increased with time. By comparison with a purified GO aliquot stored at 6 °C over the period of the experiment, it was determined that the increase in RFE was produced during warming of the enzyme to room temperature and not by a photoprocess. Verification of the release of free flavin was made by rechromatographing the purified GO which exhibited the RFE increase. A distinct free flavin band was again observed.

The possibility that the purification process was denaturing the enzyme was considered. This was found not to be the case, however. When the enzyme was purified by dialysis or molecular exclusion chromatography at room temperature, both purifications resulted in low and stable RFE values (Table I). Also, despite the removal of free flavin by these two procedures, the specific activities were observed to be unchanged, indicating that the enzyme is not significantly denatured during chromatography or dialysis under the conditions used. However, denaturation of as much as 5% of the enzyme would not be observed within the error of the specific activity determination. We cannot exclude the possibility of this mechanism giving rise to the flavin release. Alternatively, the flavin may be bound in an adventitious manner and is slowly released without denaturation of the enzyme. In either case, the free flavin is not effectively removed by chromatography at 6 °C. Whether this flavin is present in other GO preparations or is a characteristic of flavoproteins in general has not been determined. It is significant that the preparation tested here is commonly reported as the source of GO used in resonance Raman and SERRS investigations.

The identity of the flavin impurity routinely observed to be present in the commercial source of GO was determined by thin-layer chromatography. The free flavin band retained on the Sephadex G-25 column was collected and analyzed. Compounds having R_f values matching those of FAD and FMN were observed. The FMN likely arises from photolysis of FAD. Its presence is significant as the fluorescence of FMN has been determined to be 8–10-fold greater than that of FAD.[12]

Through a comparison of the absorbance change by bleaching at 450 nm upon addition of glucose to deoxygenated solutions of GO as received and following purification, it was possible to quantitate the total amount of enzymatically inactive flavin present in the commercial preparation. By this strategy it was observed that 5% of the total flavin in the preparation was not bound to the active site. This value represents both unbound and/or nonspecifically bound flavin and it is expected to depend upon the prior treatment of the GO preparation.

As fluorescence emission precludes observation of solution resonance Raman spectra of GO, the possibility was considered

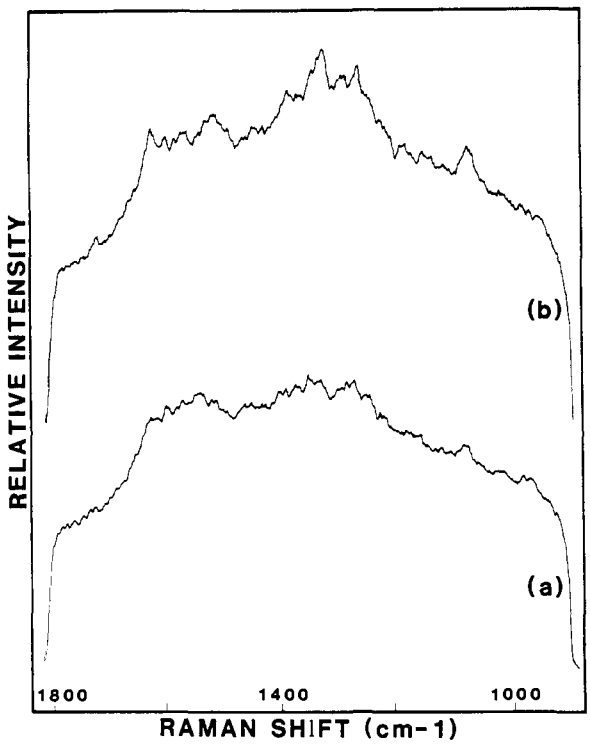

Figure 2. SERRS spectra of FAD adsorbed onto a roughened silver electrode, electrode potential = −300 mV: (a) 6×10^{-10} M adsorbing solution concentration, (b) 6×10^{-9} M adsorbing solution concentration.

that the removal of the flavin which is not bound at the active site might allow observation of this spectrum. However, following purification resulting in low RFE values, the resonance Raman spectrum was still dominated by fluorescence with excitations at 488.0, 457.9, and 514.5 nm. This is contrary to the assertion by Swoboda stating that complete quenching of fluorescence occurs upon binding of FAD to the GO apoprotein.[14] The fluorescence maximum in our purified preparation is observed to be 530 nm, the maximum observed for free FAD. Thus, this residual fluorescence may be due to a very small amount of free flavin released after the purification. The purification methods used in this work significantly lower the amount of free flavin but do not guarantee a GO preparation completely devoid of free flavin at the time of analysis. RFE values show some variability after purification by molecular exclusion chromatography. The lowest and most stable RFE was achieved by purification by dialysis against H_2O for >48 h at 6 °C.

A direct measurement of GO activity on the electrode surface from changes in its SERRS spectrum in the presence of glucose would provide more convincing evidence of enzyme integrity. A decrease in SERRS intensity upon addition of glucose to the oxygen-free system should accompany the bleaching of the FAD absorption spectrum associated with the reductive half-reaction of the active enzyme. Attempts to monitor this process on a Ag electrode have been hampered by the extremely weak spectrum observed for the purified enzyme as well as an uncharacterized shift in the open circuit electrode potential when bulk enzyme is present in solution.[15] Attempts are now underway to utilize Ag sols for this purpose.[16]

The presence of free flavin in GO may be expected to interfere with or mask the SERRS spectrum of GO, especially if the latter is very weak. SERRS on the roughened electrode is extremely sensitive to the presence of FAD as evidenced in Figure 2. At 5×10^{-9} M adsorbing solution concentration the characteristic profile of FAD is unquestionably present; even at 5×10^{-10} M, FAD is still discernable. Removal of the free flavin from the GO

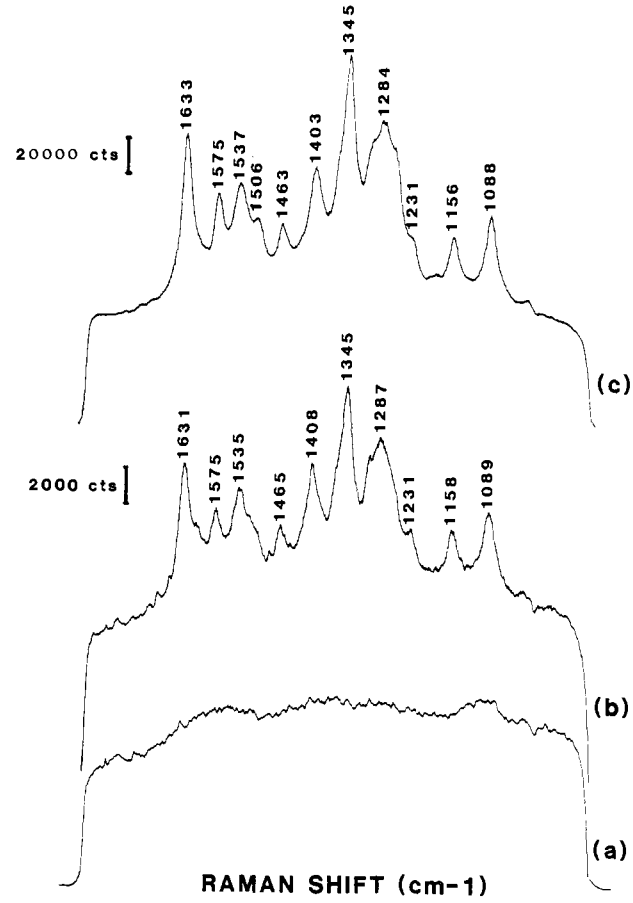

Figure 3. SERRS spectra of GO and FAD preparations on a roughened silver electrode, electrode potential = −300 mV: (a) after adsorption from purified GO solution (dialyzed), [Flavin] = 6×10^{-7} M, (b) electrode in spectrum a after reanodizing, (c) adsorbed FAD, [flavin] = 6×10^{-7} M.

preparation resulted in the inability to observe any SERRS spectrum of GO adsorbed onto the electrode (Figure 3a). The adsorbed GO can be shown to be present, however, by roughening the electrode a second time with only the adsorbed preparation present (no bulk GO present). The flavin spectrum so produced is identical with that of FAD adsorbed from a solution of equal flavin concentration, albeit much weaker in intensity (Figure 3, parts b and c, respectively).

The nature of the species giving rise to the flavin SERRS spectrum after reanodizing the electrode is probably free FAD adsorbed at the electrode surface, and not native GO. Denaturation of the GO most likely occurs during the oxidation step upon interaction of the enzyme with the silver(I) ion produced. Complexation of the silver ion with the protein-bound FAD is expected to occur, as well as a disruption of the protein tertiary structure due to the probable interaction with the locally high concentration of heavy metal ion. These reactions lead to release of the FAD which adsorbs directly onto the electrode surface in the reduction step of the ORC.

Further evidence that the FAD SERRS spectrum which we observe for GO is due to free flavin present in a GO preparation and not the native enzyme was obtained from a correlation of the RFE and the intensity of the SERRS spectrum. Figure 4 compares the SERRS spectra of three GO preparations having different RFE values relative to the RFE of the FAD standard. The lowest RFE GO preparation produces a SERRS spectrum showing no evidence of flavin. As the RFE of the preparation increases, the intensity of the flavin SERRS spectrum also increases. This correlation strongly suggests that the flavin SERRS spectrum observed in these GO preparations adsorbed on the silver electrode arises from free flavin present in the preparation and not the native enzyme. The adsorbed GO gives no detectable SERRS spectrum under the conditions used. These results cannot exclude the

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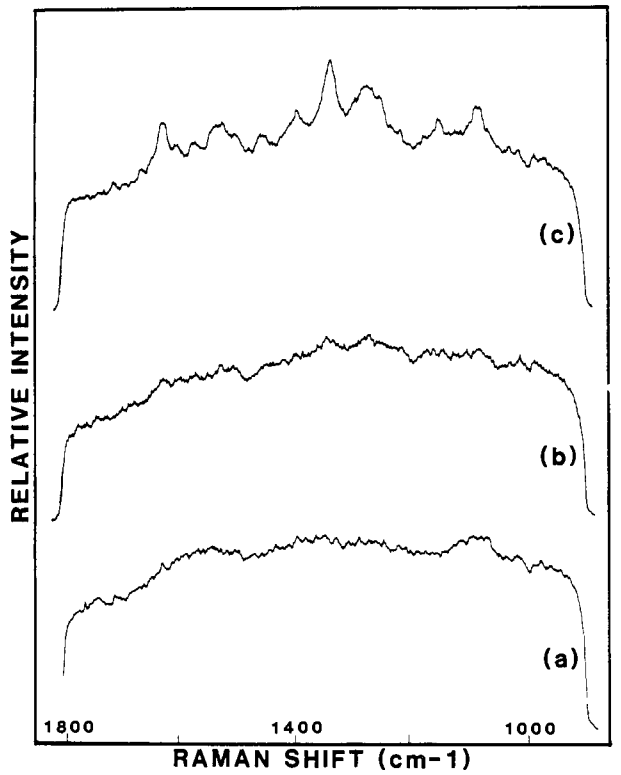

Figure 4. SERRS spectra of GO adsorbed onto the roughened silver electrode from solutions having different corrected relative fluorescence energies (corrected for differences in flavin concentration and normalized to the FAD fluorescence standard), E = −300 mV: (a) corrected RFE = 0.008, (b) corrected RFE = 0.016, (c) corrected RFE = 0.360.

possibility that GO undergoes some form of denaturation upon adsorption onto the electrode. However, it is also evident that the adsorption interaction of GO with the silver electrode in itself does not produce free FAD. This is a very important distinction since it indicates that the source of the free flavin is the original GO preparation.

Although there is a striking similarity between the flavin spectrum produced upon reanodizing the electrode in the presence of adsorbed GO and that of adsorbed FAD, the former displays a marked photosensitivity at open circuit potentials while the FAD spectrum is stable. This photosensitivity is manifested by bleaching of the entire spectrum (Figure 5). The stability of FAD may be due to its ability to form a complex between the isoalloxazine and adenine rings in the molecule. The formation of this complex has been invoked to explain the photostability of FAD in solution and the diminished fluorescence quantum yield of FAD relative to FMN and riboflavin.[12] In the presence of the protein, the FAD may be prevented from forming such a complex at the electrode surface.

The potential for interference in SERRS investigations of other flavoproteins containing noncovalently bound flavin is significant. The reported range of dissociation constants for flavin binding is 10^{-7} to 10^{-10} M.[17] Consideration of the equilibrium concentrations of free flavin shows that these concentrations are above the limits of detection for free flavin reported in this work. Lee et al. have recently concluded that free flavin interference completely masks the SERRS spectra of glucose oxidase and five other flavoproteins in silver colloid preparations, based primarily upon the similarity of the spectra, consideration of steric constraints placed upon the flavins in the intact proteins, and the inability to observe the SERRS spectra when apo-riboflavin binding protein is added to the system.[18] The ability to obtain SERRS spectra of these flavoproteins that is uncomplicated by the presence of free flavin requires observations made before the free flavin

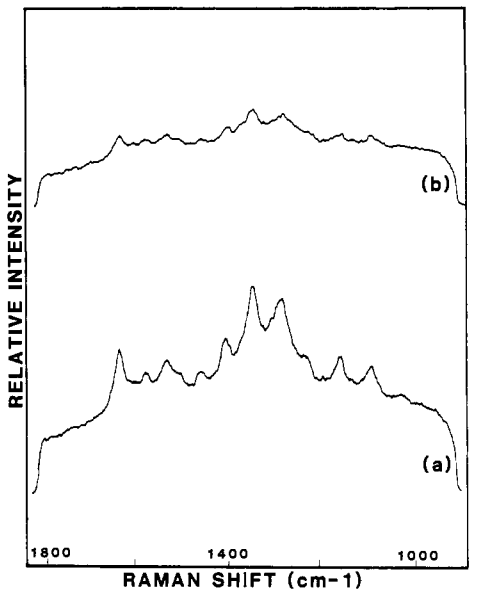

Figure 5. SERRS spectra of purified GO adsorbed onto the rough silver electrode and reroughened by ORC: (a) electrode potential = −300 mV, (b) after 30 s illumination at open circuit potential.

concentration increases as equilibrium is approached. The rate at which equilibrium is achieved is variable for different flavoprotein preparations. Old yellow enzyme ($K_D < 10^{-10}$) has been reported to dissociate with $t_{1/2} \approx 8$ days while the $t_{1/2}$ for flavodoxin is determined to be on the order of 100 min.[17] This variability requires some independant verification of free flavin concentration, such as fluorescence intensity, to ensure the elimination of interference by free flavin in each SERRS investigation.

In light of the results described above we can comment on the mechanism most likely giving rise to the enhancement of flavin on the silver electrode. The great sensitivity of SERRS to free flavin and the insensitivity of SERRS to flavin that is protein bound and positioned at some distance from the electrode surface argue strongly for a chemical enhancement mechanism such as has been advanced by Otto.[19] A chemical mechanism requires direct contact of the adsorbate with the electrode surface and gives rise to estimated enhancement factors of ca. 2 orders of magnitude. One model suggested[20] for the flavin SERRS active species requires formation of a chemisorbed species. Clearly, electromagnetic (EM) enhancement should also be occurring under the conditions used in this study, but it is not sufficient to allow observation of the FAD bound at the protein active site. The magnitude of the EM enhancement is expected to be reduced if the protein matrix prevents the FAD from contacting the electrode directly. Also, the dielectric constant of the protein layer may reduce the enhancement and shift the excitation maximum to the red. This effect has been predicted from theory and demonstrated experimentally on other adsorbates.[21]

Consideration of the results that we have presented shows that the implications for SERRS of GO on the roughened silver surface are great. Future work involving SERRS of GO should include careful consideration of the presence of free flavin and its potential for interference in the SERRS analysis.

Conclusions

We have demonstrated the presence and gradual release of free flavin from preparations of glucose oxidase, even after purification by accepted methods. The importance of this observation lies in its implications for surface-enhanced resonance Raman spectroscopy of glucose oxidase at the silver electrode and perhaps other flavoproteins as well. The extreme sensitivity of SERRS

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to FAD is demonstrated by the ability to detect FAD from solutions as low as 10^{-10} M. When the amount of free flavin in the GO preparation is reduced by purification (as evidenced by low relative fluorescence of the adsorbing solution), the SERRS spectrum of GO is severely attenuated or nonexistent under our experimental conditions. Additionally, a correlation is observed to exist between the corrected relative fluorescence of the adsorbing solution and the intensity of the resulting flavin SERRS spectrum. This indicates that the SERRS active species adsorbed onto the electrode is actually free flavin. The free flavin is not produced by the adsorption of GO onto the electrode but is released spontaneously from the protein in solution. On the other hand, FAD is released from the enzyme if the electrode anodization is performed in the presence of the enzyme. Because of the extreme sensitivity of SERRS to FAD, consideration of its potential interference should be made in future SERRS investigations of GO or other flavoproteins.

The relevance of these results to SERRS studies of flavoproteins and proteins in general is clear. First, it is essential that highly purified protein preparations be employed. It is apparent that a chemical enhancement mechanism is involved in the SERRS spectrum of FAD and this obscures any long-range enhancement that might be present. The chromophore in the native enzyme is apparently not able to interact directly with the silver, either due to the presence of the protein envelope or because the orientation of the adsorbed protein prevents this. The presence of impurities (especially those which are resonantly enhanced) which adsorbed strongly onto Ag may result in spectra which are artifactual. Second, the adsorption of large biological molecules onto metal electordes does not necessarily produce denaturation or the loss of noncovalently bound cofactors. However, anodization of the electrode in the presence of the adsorbate can produce these effects. Third, SERRS may provide a means for detecting partially denatured protein or free chromophore in other proteins as well. It has already been shown that SERS can be utilized to detect low concentrations of denatured DNA.[22]

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Registry No. FAD, 146-14-5; GO, 9001-37-0.

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Theoretical Study of the Ground- and Excited-State Reactivity of Na + FH. Comparison of SCF-CI and VB Treatments

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Abstract: An exploratory study of the reactions arising in the triatomic system Na, F, H has been achieved. In a first step, a complementary use of MO and VB techniques is made for building up correlation diagrams. Special attention was given to the weakly endothermic reaction Na + FH → NaF + H (2), which is shown to proceed through a "harpooning" mechanism in the ground state (GS). For this reaction, a transition state is found at SCF level, but it does not persist when electron correlation is introduced. In the excited state, the presence of stable exciplexes is shown by SCF-CI calculations. For the lowest excited 3P states of Na, the exciplex energy and geometry are close to that of the SCF GS transition state, thus providing a possible reactive channel which links the Na* + FH system to the GS of NaF + H. A small-size VB calculation has been carried out, including the dominant ionic and covalent structures of the aforementioned system, yielding quasi-diabatic and adiabatic potential energy surfaces. These various types of surfaces are analyzed in detail for the harpooning process (reaction 2, GS). They give a clear numerical insight to this mechanism, which essentially results from the crossing between the covalent Na···FH surface and the ionic one $Na^+ \cdots FH^-$.

The gas-phase reactions of alkali metal atoms with small molecules constitute a vast field of investigation for both experimentalists and theoreticians. In this perspective, Li and Na are good candidates for detailed analysis, and many collisional studies of these elements with diatomic molecules have been achieved. Most of these triatomic systems deal with the metal in its ground state (GS) and a nonpolar molecule. Polar molecules, such as hydrogen halides, have been used only scarcely. The prototype system, Li + FH, has been the subject of experimental[2-4] and theoretical studies, either at the semiempirical[5-10] or ab initio + CI level.[11-14] In the case of Na + HX, (X = F, Cl), only a few studies have appeared[3,15] for the GS of the system, but neither an experimental nor a theoretical study of the excited-state reactivity is available. However, the reactions of Na (GS or excited

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