Second Harmonic Generation of Glucose Oxidase at the Air/Water Interface

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ABSTRACT   We present a study of the adsorption of the glucose oxidase enzyme (GOx) at the air/water interface, using the nonlinear optical technique of surface second harmonic generation (SSHG). Resonant SSHG experiments were achieved by probing the π-π^* transition of the flavin adenine dinucleotide (FAD) chromophores embedded in the GOx protein. Because of the subsequent resonance enhancement of the signal, the second harmonic (SH) wave arising from the GOx entities adsorbed at the interface was detectable for protein bulk aqueous concentrations as low as 70 nM. The protein adsorption was followed, and, at high GOx coverage, a change in the orientation of the FAD chromophore was observed, indicating either a rearrangement or a reorientation of the protein at the interface. Inasmuch as GOx is negatively charged at the biological pH of 7, its interactions with charged surfactants were also investigated. As expected, spreading positively charged surfactants onto a partial protein monolayer was found to increase the GOx surface concentration, whereas in the case of negatively charged surfactants, the GOx surface concentration decreased until the SH signal went back to the pure buffer solution response level. With the increasing GOx surface concentration, the rearrangement or reorientation of the protein was also observed.

INTRODUCTION

Many chemical reactions in biological media occurring at membrane surfaces involve proteins as enzymes, membrane receptors, or ion transfer agents. It is therefore of great interest to develop surface-sensitive techniques to investigate the structure of membrane surfaces, for instance to study proteins or phospholipids adsorbed at these interfaces, and to investigate interfacial reaction dynamics. Second harmonic generation (SHG) is a nonlinear optical phenomenon whereby two photons at a fundamental frequency ω are converted into one photon at the harmonic frequency 2ω (Brevet and Girault, 1996). This process is only allowed in noncentrosymmetric media, such as nonlinear optical crystals or interfaces between two centrosymmetric media. As a result, when liquid interfaces are probed, no SH signal arises from the two adjacent bulk solutions, an important advantage of the technique as compared to other optical detection methods, like absorption or fluorescence spectroscopy, in which an overwhelming background signal arises from bulk molecules. The counterpart of this advantage is the very low efficiency of the SHG process. However, if the SH wavelength is tuned to an electronic transition of the interfacial probe molecules, the resonance enhancement of the SH signal partially compensates for this low efficiency.

Owing to its surface specificity, the SHG technique has been widely used to get information on structural and equilibrium properties of bare liquid interfaces (Conboy et al., 1994), orientation and solvation of dye molecules adsorbed at interfaces (Tamburello-Luca et al., 1997), and orientation relaxation (Antoine et al., 1998) or chemical reaction dynamics (Kott et al., 1993). The first study of biological systems using SHG was made by S. Fine and W. P. Hansen (Fine and Hansen, 1971), and it was shown that the presence of constituents with asymmetric structures, such as collagen or keratin, determines the SH activity of a tissue (Freund et al., 1986). The SH activity of polypeptides was first quantified by B. F. Levine and C. G. Bethea for solid samples of α-helix chains by the electric field-induced second harmonic generation (EFISH) technique (Levine and Bethea, 1976), and the measured second-order and third-order nonlinear polarizabilities were found to depend on the number of peptide monomers composing the α-helix. Similar measurements were also performed on proteins, for instance, on bacteriorhodopsin (bR) in dried purple membrane (Huang et al., 1989).

The aim of the work reported here was to attempt detection by resonant SHG of proteins adsorbed at liquid interfaces, as a model of structural proteins embedded in membrane bilayers or antibodies adsorbed on a surface, and to investigate the protein reorientation or structural rearrangement. Indeed, detection of the reorientation of a structural protein would open the way to the detection of channel opening in membranes, for example.

Glucose oxidase (GOx) adsorbed at the air/water interface was chosen as a model protein. This enzyme is responsible for catalysis of the oxidation of glucose by molecular oxygen. It is a globular protein made up of two identical subunits of molecular mass 80 kDa, linked by disulfide bonds (Hecht et al., 1993). In aqueous solutions, each monomer is a compact spheroid of dimensions 60 Å $\times$ 52 Å $\times$ 37 Å. GOx is made of 20 different amino acids and contains one redox coenzyme, flavin adenine dinucleotide (FAD), per monomer. The FAD cofactor is not covalently

bound to the protein and can be released under denaturing conditions, after dissociation of the two subunits.

MATERIALS AND METHODS

The light source was delivered by a nanosecond Nd^{3+}-YAG laser-pumped optical parametric oscillator (OPO) (see Fig. 1). In these experiments the idler output ranged from 840 nm to 980 nm, with an energy of 15 mJ for a 5-ns pulse duration and a repetition rate of 10 Hz. The laser beam was polarized by an achromatic half-wave plate and focused onto the air/water interface through the water phase with an angle of incidence of 48.6°. With this angle, the total internal reflection (TIR) configuration is achieved and the SH signal intensity is thereby greatly enhanced. To avoid refraction at the air/cell and cell/water interfaces, we used cylindrical glass cells as sample cells. The SH response reflected from the air/water interface was selected through filters, collected with a lens, polarized, and then focused into a monochromator. The detection was achieved with a boxcar averager fed by a photomultiplier tube, and the signals were sent to a computer for further data analysis.

UV-visible spectra were performed with a standard UV-visible spectrophotometer, and water was purified by reverse osmosis followed by ion exchange (Millipore; Milli-Q SP reagent system) and then distilled twice. GOx from *Aspergillus niger* (Fluka, Buchs, Switzerland), *N-cetyl-N,N,N*-trimethylammonium bromide (CTAB) (Merck, Darmstadt, Germany), and arachidic acid (Fluka) were used as received. The aqueous solutions were buffered at pH 7 (0.1 M KH_2PO_4 + 0.1 M NaOH). All measurements were performed at 20°C.

RESULTS AND DISCUSSION

Glucose oxidase spectroscopy

First we obtained the UV-visible spectrum of the GOx enzyme in the aqueous solution buffered at pH 7 (Badia et al., 1993), to determine a suitable wavelength for resonant SSHG studies. The chromophoric group responsible for the GOx visible absorption properties is the FAD moiety embedded in each protein monomer. Its absorption spectrum in pH 7 buffered aqueous solutions consists of two absorption bands lying at 380 nm and 450 nm (see Fig. 2), with extinction coefficients of 2.136×10^5 M^{-1} m^{-1} and 2.168×10^5 M^{-1} m^{-1} and corresponding to π-π* transitions along the three cycles of the isoalloxazine ring (see the

FIGURE 2   UV-visible spectrum of an aqueous solution of 7.4 μM GOx enzyme in a pH 7 buffered aqueous solution (——) and surface SH spectrum of the enzyme adsorbed at the air/water interface ($\triangle$). ····, A gaussian fit of the SSH spectrum.

FAD structure in Fig. 3). These bands are characteristic of the oxidized form of flavin groups (Bartlett et al., 1997). To determine the GOx nonlinear optical properties at the air/water interface, a SSHG spectrum was performed (see Fig. 2), at a GOx bulk aqueous concentration of 110 nM corresponding to a rather low surface coverage (see below), in a pH 7 buffered solution. Both fundamental and harmonic beams were *p*-polarized (i.e., polarized perpendicular to the interface plane), as this polarization configuration corresponds to the maximum SH intensity. As each protein monomer contains one FAD chromophore, the SH process probes the sum of the two FAD nonlinear activities. This latter FAD activity is determined by the hyperpolarizability tensor of the isoalloxazine ring; the side chain introduces very few perturbations in the optical response. The two monomers face each other in the protein, and the resulting
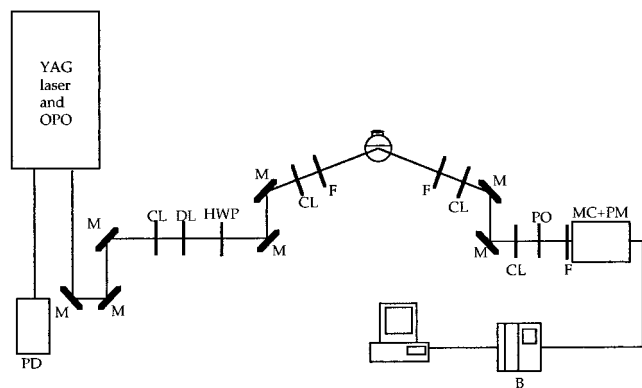

FIGURE 1   Experimental SHG set-up. PD, Photodiode; M, mirror; CL, converging lens; DL, diverging lens; HWP, half-wave plate; F, filter; PO, polarizer; MC, monochromator; PM, photomultiplier; B, boxcar.
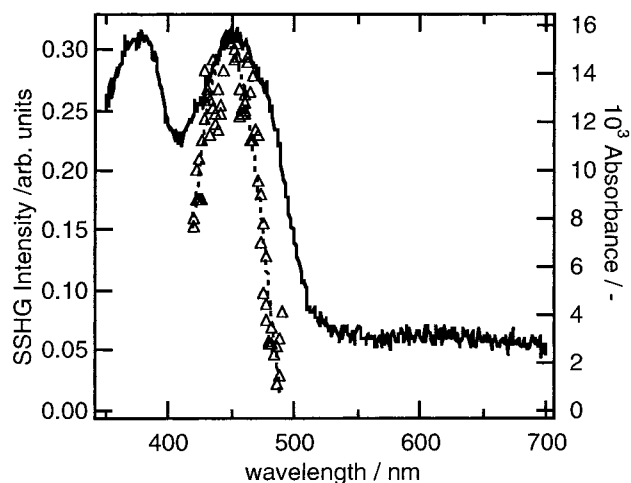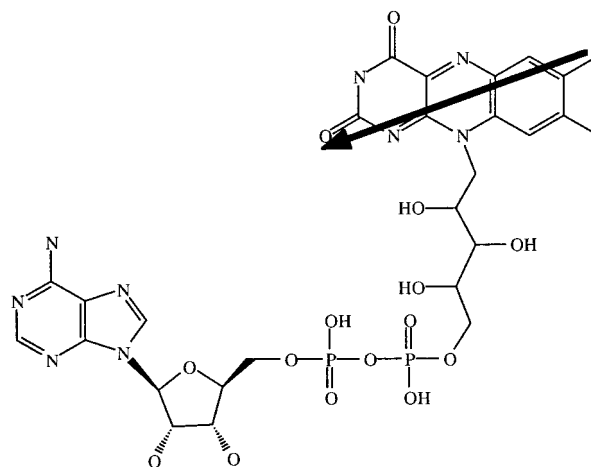

FIGURE 3   Structure of the FAD chromophore. The π-π* transition moment probed by SSHG has an angle of orientation of ~35° with respect to the axis of the three cycles of the isoalloxazine ring.

tensor component is expected to be smaller than each FAD hyperpolarizability tensor element along a direction perpendicular to the protein dimer axis. Inversely, along this axis, the resulting component is expected to be larger. As seen on Fig. 2, the location of the maximum of the SH spectrum, corresponding to the protein adsorbed at the interface, is the same as the location of the maximum of the UV-visible absorption spectrum corresponding to the protein in the bulk aqueous solution. The FAD chromophore has no solvatochromic behavior, as indeed may be seen from the UV-visible spectra of the FAD in bulk aqueous solution or in ethanol. It is thus not possible to gain any information on the possible denaturation of the protein at the interface with a solvatochromic analysis. Finally, it is worth noting the narrowing of the transition band at the interface as a result of the SHG process.

In view of the GOx SH spectrum, the SH wavelength was set at 450 nm for the remaining experiments, to get the resonance enhancement of the SH signal. Furthermore, because the corresponding fundamental light at 900 nm is not absorbed by the GOx proteins, potential damages like photobleaching or photochemical reactions involving the protein were avoided.

SSHG measurements of the GOx protein adsorption isotherm at the air/water interface

The SH response of the GOx protein was monitored as a function of its bulk aqueous concentration, to determine the adsorption isotherm. Indeed, the SH intensity arising from the interface is described by the relation (Brevet, 1997)

$$I^\Omega = \frac{\omega^2}{8\epsilon_0 c^3} \frac{\sqrt{\epsilon_1^\Omega}}{\epsilon_1^\omega(\epsilon_m^\Omega - \epsilon_1^\omega \sin^2\theta_1^\omega)} |\chi|^2 (I^\omega)^2 \quad (1)$$

where I^Ω is the SH intensity in W·cm^{-2}, I^ω is the fundamental intensity in the same units, ϵ_1^ω and ϵ_1^Ω are the relative optical dielectric constants of the aqueous phase at the fundamental and the harmonic frequency, respectively (light propagates through the aqueous solution before reaching the interface), ϵ_m^Ω is the relative optical dielectric constant of the protein monolayer at the harmonic frequency, and θ_1^ω is the angle of incidence of the fundamental beam with respect to the interface normal. ϵ_m^Ω is here taken as the average between the water and the air optical dielectric constants. The quantity χ in Eq. 1 is defined as

$$\chi = a_1 \chi_{s,XZX}^{(2)} \sin 2\gamma \sin \Gamma + (a_2 \chi_{s,XZX}^{(2)} + a_3 \chi_{s,ZXX}^{(2)}$$
$$+ a_4 \chi_{s,ZZZ}^{(2)}) \cos^2\gamma \cos \Gamma + a_5 \chi_{s,ZXX}^{(2)} \sin^2\gamma \cos \Gamma \quad (2)$$

where $a_i, i = 1 \ldots 5$ are coefficients depending on the relative optical dielectric constants of air and the aqueous solution and the angle of incidence θ_1^ω, and γ and Γ are the polarization angles of the fundamental and the harmonic waves, respectively. For a p-polarized wave, γ or $\Gamma = 0$, and the wave is polarized perpendicular to the interface plane. The tensor elements $\chi_{s,XZX}^{(2)}$, $\chi_{s,ZXX}^{(2)}$, and $\chi_{s,ZZZ}^{(2)}$ are the

three nonzero independent elements of the surface susceptibility tensor; they characterize the nonlinear optical response of the interface. In the presence of a SHG active monolayer (and the GOx may be assumed to be so at the frequencies used), the SH signal arising from the bulk solvents is negligible and the susceptibility tensor can be reduced to the one of the adsorbed molecules (Tamburello-Luca et al., 1995). Furthermore, because the GOx proteins are only monitored through their FAD chromophores, the inherent chirality of the surface may be neglected, an assumption that is not necessarily valid outside the 450-nm resonance. The surface susceptibility tensor depends on the number of species adsorbed at the interface, N_s, and on the molecular hyperpolarizability, $\vec{\vec{\beta}}$, of each single unit of the GOx proteins:

$$\vec{\vec{\chi}}_s^{(2)} = \frac{N_s}{\epsilon_0} \langle \vec{\vec{\beta}} \rangle \quad (3)$$

where the $\vec{\vec{\beta}}$ tensor is taken as an ensemble average over all of the possible orientation configurations. The SH response is thus proportional to the square of the number of adsorbed species, N_s, or the surface coverage, θ, and can therefore yield the adsorption isotherm of the protein.

In Fig. 4 is presented the square root of the SH signal intensity, proportional to the number of adsorbed protein, as a function of the GOx bulk aqueous concentration in pH 7 buffered solutions. It has been obtained from the SH response measured at 450 nm, with the fundamental and harmonic beams both p-polarized. The SH signal is increased by a factor of 5 as the GOx bulk concentration increases from zero up to 400 nM. At this bulk aqueous concentration though, the surface coverage cannot be deduced from the experimental data, as it is not possible to determine the SH signal corresponding to the full monolayer coverage. However, at 400 nM we observe protein-protein interactions, a clear indication that we have reached a significant surface coverage. Comparison with surface
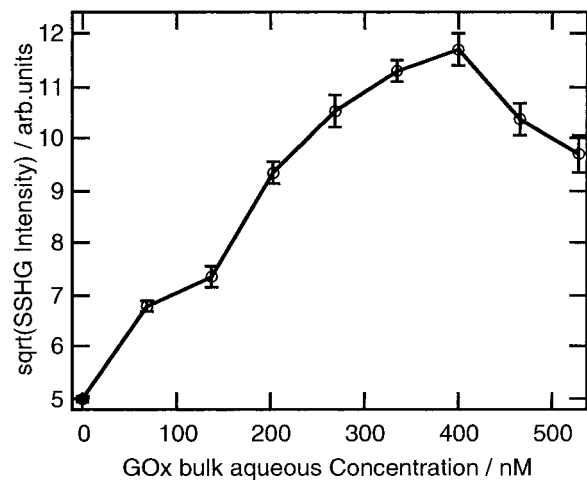

FIGURE 4 GOx adsorption isotherm at the air/water interface as obtained by SSHG measurements.

pressure measurements of GOx adsorbed at the air/water interface (Rosilio et al., 1997) further shows that our experiments may correspond to a nonequilibrated system. Indeed, in the conventional Langmuir-Blodgett trough of the experiments reported in the work of Rosilio et al. (1997), the surface coverage saturation already occurs for a bulk aqueous concentration of 100 nM at equilibrium, whereas in this work the observed saturation concentration is clearly above 400 nM. In the former work, this equilibrium was only obtained after a rather long time, which is incompatible with our experimental conditions, chiefly because of the difficulty of ensuring laser stability for such a long time. The surface pressure isotherm obtained in transient conditions (after 100 min) from the work of Rosilio et al. (1997) shows, however, a saturation at 400 nM.

At a GOx bulk aqueous concentration range similar to the one used to obtain this isotherm, the FAD chromophore free in solution exhibits a very weak surface activity. No SH signal could thus be measured at FAD aqueous concentrations lower than 4 μM. Therefore, it may be assumed that the GOx proteins are not denatured upon adsorption at the air/water interface. The denaturation process of the protein would indeed lead to the release of the FAD moiety, which would then likely desorb back into the bulk aqueous solution at the concentrations used. Whether adsorbed or desorbed, the net result would be a drop in the SH response from the interface at constant light polarization.

The decrease of the SH signal above the bulk concentration of 400 nM is therefore due to a rearrangement and/or a reorientation of the proteins at the air/water interface, as revealed through the reorientation of the FAD chromophore. This is seen from the change in the polarization of the SH signal owing to the reorientation of the FAD π-π^* transition dipole moment at the interface. In a more general manner, and from a polarization analysis of the SH response, the orientation of the transition dipole moment of the chromophore can be calculated using Eqs. 1–3. To do so, the relative ratios of the three hyperpolarizability tensor components must be assumed beforehand. In the present case, the orientation of the FAD π-π^* transition dipole inside the GOx protein adsorbed at the air/water interface can be determined. This dipole moment lies along the long axis of the isoalloxazine ring of the FAD chromophore, and we may reduce the hyperpolarizability tensor to the two elements taken along this dipole moment axis at the harmonic frequency. At a protein bulk aqueous concentration of 300 nM, and assuming a Dirac distribution of this angle of orientation, an angle of orientation of 35° has been determined from the dependence of the SH intensity on the fundamental wave polarization angle, for the p-polarized and s-polarized harmonic wave (see Fig. 5). This angle between the transition moment direction and the surface normal characterizes the distribution but will not lead to a global protein orientation because it is not clear how labile is the FAD moiety inside its pocket within the protein. However, any change in the transition moment angle of orientation will be associated with a global protein reorien-
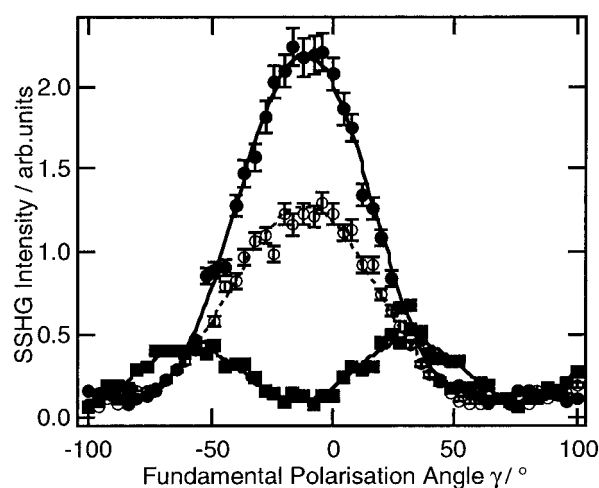

FIGURE 5   GOx polarization curves for GOx bulk concentrations of 300 nM (●, harmonic light p-polarized) and 550 nM (○, harmonic light p-polarized). Normalization was achieved on the polarization curves with the harmonic light s-polarized (■).

tation or rearrangement, as long as it is assumed that the chromophore reorientation can only stem from a protein reorientation or rearrangement. The origin of this protein configuration change is difficult to retrieve, however, but because the process occurs at high surface coverage, it is highly likely that it is induced by protein-protein interactions. In Fig. 5 is shown the change in the SH signal polarization curves as the GOx bulk aqueous concentration increases from 300 nM to 550 nM. The data are normalized with respect to the two s-polarized curves, and thus only the 300 nM s-polarized curve is presented. It is clearly seen from this figure that the SH signal measured with both the fundamental and the harmonic light beams p-polarized decreases as a function of the bulk aqueous GOx concentration, hence explaining the decrease observed in the experimental isotherm. From the analysis of these polarization curves, the transition moment angle of orientation with respect to the surface normal has been found to increase as the GOx bulk concentration increases from 300 nM to higher concentrations, approaching the full monolayer coverage. This corresponds to a flattening of the angle of orientation of the isoalloxazine ring toward the interface. This reorientation phenomenon may be related to the mechanism of the GOx adsorption at the air/water interface as described by Rosilio et al. (1997). In their work, the adsorption mechanism is split into three steps: diffusion, penetration of the proteins into the monolayer, and structural rearrangement of the monolayer, with the relative importance of the third step increasing at high GOx bulk aqueous concentrations.

Interaction of GOx with charged surfactants probed by SSHG

At pH 7, the GOx isoelectric point being 4.2, the GOx protein surface is covered with negative charges. It is there-

fore of interest to study the protein interaction with positively and negatively charged surfactants. From a structural point of view, the current model of a neutral surfactant monolayer interacting with a GOx monolayer at the air/water interface describes the GOx as a globular protein with the surfactants sitting on and between the proteins (Okahata et al., 1988; Sriyudthsak et al., 1988). This configuration is consistent with atomic force microscopy measurements of Langmuir-Blodgett films of GOx mixed with behenic acid and deposited on graphite (Fiol et al., 1992). In the case of charged surfactants, such a configuration is expected to induce large electrostatic interactions affecting the adsorption properties of GOx and possibly its own conformation. The SH response from the protein was thus monitored as a function of the surfactant surface coverage at the air/water interface. CTAB was used as a positively charged surfactant and arachidic acid as a negatively charged one. These two compounds present an alkyl chain with 16 and 19 carbons, respectively. In the case of CTAB, because an increase in the negatively charged GOx surface concentration was expected, the initial GOx bulk aqueous concentration was set at 200 nM, corresponding to half the value of the isotherm maximum. A steep increase in the SH intensity was observed, as seen in Fig. 6, and the GOx surface concentration was found to be approximately doubled as soon as the CTAB surface coverage reached ~10%. As in previous experiments, the SH wavelength was set at 450 nm, the fundamental and the harmonic beams both being p-polarized and the aqueous solution buffered at pH 7. If the initial GOx bulk aqueous concentration is set at 400 nM, only a slight increase in the SH signal is observed independently of the CTAB coverage. This observation indicates that the coverage obtained at the GOx bulk aqueous concentration of 400 nM is close to the full monolayer coverage. At this stage, it is worth noting that a nonnegligible background SH signal was measured when CTAB was spread on the inter-

face in the absence of GOx. This additional contribution to the SH signal may arise from either a third-order contribution due to the charging of the interface or from changes in the quadrupolar contribution due to the restructuring of the water phase in the presence of the charged monolayer. This background contribution, however, was always much smaller than the SH signal arising from GOx molecules. Furthermore, it should be noted that the addition of the surfactant counterion bromide to the solution has a negligible effect on the ionic strength of the solution. Reorientation of the proteins after the electrostatic interactions with CTAB was also investigated by the polarization analysis. The change in the angle of orientation of the π-π^* transition moment was investigated through the evolution of the ratio of the SH signal in two different polarization configurations: when both the fundamental and the harmonic beams are p-polarized and when the fundamental beam is 45°-polarized and the harmonic beam is s-polarized. As the CTAB monolayer was spread onto the interface, it was observed that this ratio decreased significantly, indicating a flattening of the isoalloxazine ring angle of orientation toward the surface plane. In the presence of the CTAB surfactant monolayer, the protein monolayer thus reorganized in a fashion similar to that observed during the isotherm reorganization. The change in the isoalloxazine ring angle of orientation is smaller, however, in the presence of CTAB. Unfortunately, this rearrangement within the protein cannot be directly associated with a particular conformational rearrangement.

In the case of arachidic acid, the GOx surface coverage was initially set at the maximum we observed, corresponding to a bulk aqueous concentration of 400 nM. When the negatively charged surfactant was spread onto the protein monolayer, the GOx surface concentration was drastically reduced, as indicated by the decrease in the interfacial SH response intensity, until it reached the pure buffer SH response level. In the meantime, the SH intensity ratio representative of the FAD transition moment angle of orientation decreased from its value in the absence of surfactants to the value corresponding to the pure buffer solution. This depolarization behavior of the SH light is thus expected and corresponds to the behavior for desorbing GOx proteins away from the air/water interface back into the bulk aqueous solution. In this case, no information on the protein rearrangements may be retrieved.
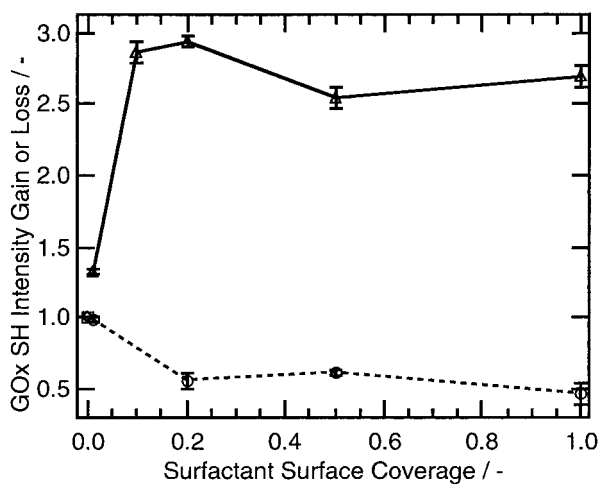
CONCLUSIONS

Detection of the GOx protein adsorbed at the air/water interface has been achieved by SSHG measurements using the resonance enhancement of the π-π^* transition band of the FAD chromophore embedded in each of the GOx protein monomers. The protein adsorption isotherm has been determined, although complete equilibrium may not have been reached. Saturation is occurring at a bulk aqueous concentration higher than 400 nM. Rearrangement of the

FIGURE 6 Gain and loss of the GOx SH intensity as either positively charged surfactants (CTAB, △) or negatively charged surfactants (arachidic acid, ○) are spread onto the air/water interface.

GOx enzyme upon adsorption at the interface has been shown through the depolarization of the SH signal. Indeed, at a GOx surface coverage sufficient to allow interactions between the proteins, a reorientation of the FAD transition moment has been observed, indicating either a reorientation of the enzyme itself or an internal rearrangement involving the reorientation of the FAD chromophore inside the protein. Interaction of the negatively charged GOx enzyme with positively and negatively charged surfactants has also been observed. The positively charged CTAB surfactant has been shown to increase the GOx surface concentration, provided that the protein initial interfacial concentration was less than the full monolayer surface coverage. Furthermore, spreading of the CTAB surfactants onto the GOx monolayer induced changes in the FAD transition moment angle of orientation similar to that already observed during the adsorption process. On the opposite side, the negatively charged surfactant arachidic acid was shown to repel GOx proteins from the air/water interface back into the bulk aqueous solution. In light of these experiments, surface SHG is demonstrated to be a convenient surface analytical technique for the study of proteins at interfaces, and this work may pave the way for future studies in which surface specificity is a necessary requirement, as in ligand-receptor recognition reactions at membranes.

REFERENCES

Antoine, R., A. A. Tamburello-Luca, P. Hébert, P. F. Brevet, and H. H. Girault. 1998. Picosecond dynamics of eosin B at the air/water interface by time resolved SHG: orientational randomization and rotational relaxation. *Chem. Phys. Lett.* 288:138–146.

Badia, A., R. Carlini, A. Fernandez, F. Battaglini, S. R. Mikkelsen, and A. M. English. 1993. Intramolecular electron-transfer rates in ferrocene-derivatized glucose oxidase. *J. Am. Chem. Soc.* 115:7053–7060.

Bartlett, P. N., S. Booth, D. J. Caruana, J. D. Kilburn, and C. Santamaria. 1997. Modification of glucose oxidase by the covalent attachment of a tetrathiafulvalene derivative. *Anal. Chem.* 69:734–742.

Brevet, P. F. 1997. Surface Second Harmonic Generation. Presses polytechniques et universitaires romandes, Lausanne.

Brevet, P. F., and H. H. Girault. 1996. Second harmonic generation at liquid/liquid interfaces. *In* Liquid-Liquid Interfaces: Theory and Methods. A. G. Volkov and D. W. Deamer, editors. CRC Press, Boca Raton, FL. 103–137.

Conboy, J. C., J. L. Daschbach, and G. L. Richmond. 1994. Total internal reflection second harmonic generation—probing the alkane water interface. *Appl. Phys. A.* 59:623–629.

Fine, S., and W. P. Hansen. 1971. Optical second harmonic generation in biological systems. *Appl. Optics.* 10:2350–2353.

Fiol, C., S. Alexandre, N. Delpire, and J. M. Valleton. 1992. Molecular resolution images of enzyme-containing Langmuir-Blodgett films. *Thin Solid Films.* 215:88–93.

Freund, I., M. Deutsch, and A. Sprecher. 1986. Connective tissue polarity: optical second harmonic microscopy, crossed-beam summation, and small-angle scattering in rat-tail tendon. *Biophys. J.* 50:693–712.

Hecht, H. J., H. M. Kalisz, J. Hendle, R. D. Schmid, and D. Schomburg. 1993. Crystal structure of glucose oxidase from *Aspergillus niger* refined at 2.3 Å resolution. *J. Mol. Biol.* 229:153–172.

Huang, J. Y., Z. Chen, and A. Lewis. 1989. Second harmonic generation in purple membrane-poly(vinyl alcohol) films: probing the dipolar characteristics of the bacteriorhodopsin chromophore in bR570 and M412. *J. Phys. Chem.* 93:3314–3320.

Kott, K. L., D. A. Higgins, R. J. McMahon, and R. M. Corn. 1993. Observation of photoinduced electron transfer at liquid-liquid interface by optical second harmonic generation. *J. Am. Chem. Soc.* 115:5342–5343.

Levine, B. F., and C. G. Bethea. 1976. Second order hyperpolarisability of a polypeptide α-helix: poly-γ-benzyl-L-glutamate. *J. Chem. Phys.* 65:1989–1993.

Okahata, Y., T. Tsuruta, K. Ijiro, and K. Ariga. 1988. Langmuir-Blodgett films of an enzyme-lipid complex for sensor membranes. *Langmuir.* 4:1373–1375.

Rosilio, V., M. M. Boissonnade, J. Y. Zhang, L. Jiang, and A. Baszkin. 1997. Penetration of glucose oxidase into organized phospholipid monolayers spread at the solution/air interface. *Langmuir.* 13:4669–4675.

Sriyudthsak, M., H. Yamagishi, and T. Moriizumi. 1988. Enzyme-immobilized Langmuir-Blodgett film for a biosensor. *Thin Solid Films.* 160:463–469.

Tamburello-Luca, A. A., P. Hébert, R. Antoine, P. F. Brevet, and H. H. Girault. 1997. Optical surface second harmonic generation study of the two acid/base equlibria of eosin B at the air/water interface. *Langmuir.* 13:4428–4434.

Tamburello-Luca, A. A., P. Hébert, P. F. Brevet, and H. H. Girault. 1995. Surface second harmonic generation at air/solvent solvent/solvent interfaces. *J. Chem. Soc. Faraday Trans.* 91:1763–1768.