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Study of the Dynamic Structure of Native and Hydrophobized Glucose Oxidase by Time-Domain Dielectric Spectroscopy

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The dynamic structures of native and hydrophobized (by covalent attachment of palmitoyl chains) glucose oxidase were studied by time-domain dielectric spectroscopy (TDDS). Analysis of the dipole correlation function for both types of the enzyme showed that the decay of the correlation function of the macromolecule motion can be presented as a sum of components corresponding to different kinds of protein motion: isotropic rotation of the protein molecule as a whole, anisotropic Brownian tumbling of subunits, and anisotropic intramolecular motion of polar groups and substructures. The slowest relaxation time was found to be longer for the modified enzyme than for the native enzyme. The dielectric strengths for all relaxation processes, as well as the dipole moment and the molecular volume, were also larger for the modified glucose oxidase. The observed differences between various types of the dipole motion for the native and modified glucose oxidase are discussed.

Introduction

Glucose oxidase (GOx) is the most widely used enzyme in the field of biosensors.[1] It catalyzes the oxidation of β-D-glucose by molecular oxygen to D-glucono-1,4-lactone and hydrogen peroxide and has many important applications in the quantitative determination of glucose in body fluids, foodstuffs, beverages, and fermentation liquor.[1,2]

The structure of bacterial GOx from *Aspergillus niger* is well characterized. It is a rigid dimeric glycoprotein of molecular weight 150−160 kDa (583 amino acid residues and high mannose-type carbohydrate up to 16% of the total molecular weight) containing one tightly bound flavin adenin dinucleotide (FAD) per monomer as a cofactor.[3−7] According to the crystal structure data at a resolution of 2.3 Å, the monomeric, partially deglycosylated molecule is a compact spheroid with approximate dimensions 60 Å × 52 Å × 37 Å. The corresponding dimensions of the dimer are 60 Å × 52 Å × 77 Å.[8] Dissociation of the subunits is possible only under denaturing conditions and is accompanied by the loss of the cofactor.[9] The carbohydrate moiety does not contribute significantly to the structure, stability, and activity of GOx.[10]

The efficiency of the GOx-based biosensors is limited mainly by the heterogeneity of the enzyme distribution in the biosensor membrane. Thus, creating an effective, compact, functional analytical device is difficult.[11] Because biosensor membranes or films are composed of organized layers of lipids and enzymes, the attachment of hydrophobic anchors to the enzymes may help to improve the molecular architecture of biosensors.

Magdassi et al.[12−15] have developed a suitable method for the hydrophobization of antibodies and enzymes by covalent attachment of alkyl chains to the protein lysine's amino groups with retention of high biological activity after modification. It has been found that such a modification results in the formation of typical surfactant-like molecules, which spontaneously form colloidal clusters in aqueous solutions and display enhanced surface activity at the air/water interface.[13−18]

Modification of GOx was not accompanied by dissociation of subunits and loss of FAD, and the enzymatic activity measurements have indicated that the modified GOx retained 70% of its specific activity.[15] A very important result is also the decrease of the GOx isoelectric point after covalent attachment of hydrophobic groups.[15] The reason for this effect is a decrease in the number of free positively charged lysine groups with no change in the number of negatively charged groups. This also increases the negative value of the ξ potentials of the modified GOx at any pH value above the isoelectric point, compared with those of the native enzyme. Changes in the net charge of the GOx molecule and in its hydrophobicity may lead to changes in the enzyme structure and its dynamic behavior and thus influence its properties at interfaces and in organized layers.

There are several physical methods that can provide precise information about the structure and dynamic processes that occur in aqueous solutions of proteins. Among these methods, dielectric spectroscopy (DS) allows the study of the relaxation processes of objects in an extremely wide range of characteristic times (10^3−10^{-12} s). Although it does not exhibit the selectivity of NMR and ESR methods, it can offer important and sometimes unique information about the dynamic and structural properties of proteins in solutions. It has been shown that the application

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of time-domain dielectric spectroscopy (TDDS) has been successful in obtaining new information on the structural and dynamic parameters of protein globules in conformational transitions induced by temperature and pH changes. It has been used for the study of myoglobin, lysozyme, RNase, and Binase.[19−32]

In this work, we compare the dynamic behaviors of native and hydrophobized GOx by the TDDS method, and we evaluate the effect of the attachment of hydrophobic groups to the protein molecule on its structure.

Experimental Section

Materials. GOx from *Aspergillus niger* (Type VII-S) and palmitic acid hydroxysuccinimide ester were purchased from Sigma Chemical Co. The modification reaction was performed as described previously[15] at a protein-to-ester molar ratio of 1:56. The number of covalently modified lysine residues was determined by the TNBS method,[33,34] which is based on the reaction of trinitrobenzenesulfonic acid (TNBS) with free amino groups of proteins. This number was found to be eight palmitic chains per dimeric GOx molecule. Protein concentrations were determined spectrophotometrically by the Bradford method.[35]

Measurements. Dielectric measurements were performed using the time-domain dielectric spectroscopy system TDS-2 (Dipole TDS Ltd., Jerusalem, Israel) in the frequency range 100 kHz−5 GHz. All measurements were performed in the temperature range 5−40 °C. General principles of TDDS and a detailed description of the setup and procedures of measurement have been described elsewhere.[36] The data treatment was performed directly in the time domain in terms of the macroscopic dipole correlation function (DCF) $\Gamma(t)$

$$\psi(t) \approx \Gamma(t) = \frac{\langle \bar{M}(0)\cdot\bar{M}(t)\rangle}{\langle \bar{M}(0)\cdot\bar{M}(0)\rangle} \qquad (1)$$

where $M(t)$ is the macroscopic fluctuation dipole moment of the sample volume unit, which is equal to the vector sum of all of the molecular dipoles, and the symbol $\langle\ \rangle$ denotes averaging of the ensemble. The velocity and the laws governing the decay function $\Gamma(t)$ are directly related to the structural and kinetic properties of the sample and characterize the macroscopic properties of the system under investigation. The least-squares fitting procedure based on the simplex method was used.

Dipole Correlation Function Analysis

As was mentioned in the previous section, the data analysis was performed in the time domain in terms of the macroscopic DCF, $\Gamma(t)$. Let us discuss the initial principles of the quantitative analysis.

The dielectric spectra of aqueous solutions of globular proteins demonstrate a few clearly marked regions of dispersions: a high-frequency region (γ dispersion), a low-frequency region (β dispersion), and an intermediate broad region with low amplitude (δ dispersion).[20,37,38−41] The first region is due to the relaxation of solvent molecules ($\sim 10^{-11}$ s) and is out of our experimental "time window". The β dispersion at low frequencies ($\sim 10^{-8}$ s) is due to the relaxation of the relatively large protein molecules. The most systematic analysis of the possible polarization mechanisms of this process has been carried out by Grant and co-workers,[38,39,42,43] whose arguments show that, in solutions of small globular proteins, β dispersion is governed by orientational polarization of the macromolecules. The δ dispersion ($\sim 10^{-9}-10^{-10}$ s) has a complicated nature, and it has been shown that at least part of this process (long-time behavior) can be stipulated by intramolecular motion of solute macromolecules (δ_1 process). The short-time behavior can be explained by the hindered rotation of bound water molecules (δ_2 process).[20,38,40,41,44,45]

The frequency range of our spectrometer allows us to study the rotational motion of the macromolecule as a whole (β process), the intramolecular protein mobility (δ_1 process), and the hindered rotation of bound water (δ_2 process), which is at the high-frequency limit of our setup. Because the relaxation scale of bulk and hydration water is outside the experimental time window, we can assume that the total polarization is stipulated only by the protein macromolecule, with the amplitude of polarization described by the Onsager−Oncley theory[46]

$$\Delta\epsilon = \mu^2 Nc/2kT\epsilon_0 M \qquad (2)$$

where M is the molecular weight of protein, N is Avogadro's number, c is the bulk protein concentration, ϵ_0 is the permittivity of free space, k is Boltzman's constant, and T is the absolute temperature. The sample volume unit macroscopic instantaneous dipole moment of the aqueous protein solution in eq 1 is equal to the vector sum of all of the macromolecular dipoles, i.e., $\bar{M}(t) = \sum_i \bar{\mu}_i(t)$, where $\bar{\mu}_i$ is the dipole moment of ith protein molecule. Thus, the nonnormalized function $G(t)$ can be written as follows:

$$G(t) = \sum_i < \bar{\mu}_i(0)\,\bar{\mu}_i(t) > + \sum_{i\neq j} < \bar{\mu}_i(0)\,\bar{\mu}_j(t) > = \Delta\epsilon_{ac}\Gamma_{ac}(t) + \Delta\epsilon_{cc}\Gamma_{cc}(t) \qquad (3)$$

where $\Gamma_{ac}(t)$ and $\Gamma_{cc}(t)$ are auto- and cross-correlation functions of protein motion, respectively. For a dilute aqueous solution of proteins, the cross-correlation term in eq 3 is neglected and the following approximation can be used:

$$G(t) = \Delta\epsilon\Gamma(t) \qquad (4)$$

where $\Delta\epsilon$ is the total amplitude of the sample polarization (see eq 2) and $\Gamma(t)$ is the normalized correlation function of the protein dipole motion in solution, which includes the autocorrelation term only. Note that the exact relationship between the macroscopic and microscopic dipole correlation functions is still an open issue, and in the case of dilute aqueous protein solutions, we can consider it as a molecular correlation function, i.e., it refers to a single macromolecule rather than to the whole system.[38] This assumption is a crude approximation, but it gives quite good results in a DCF analysis of protein dynamics in solution.[26,27,31,32]

To present the correlation function $G(t)$ analytically, a so-called "model-free" approach was used.[26,27] If all atoms of a macromolecule participate in two main independent types of motion (local fast anisotropy rotation and slow Brownian tumbling of the macromolecule as a whole, characterized by the dielectric relaxation time τ_R), the full correlation function can be written as a product of the separate correlation functions. Here, we must consider the protein dipole moment $\bar{\mu}_i$ not as a permanent value over the whole time scale of our observation, but as a vector sum of all of the polar groups and residue dipole moments $\bar{m}_i$ in the protein globule. There are several independent types of internal motions that average this dipole contribution. Because these motions are anisotropic, the dipole correlation function does not decay to zero on the time scale of the relaxation of the protein rotational diffusion as a whole, i.e.

$$\lim_{t \to \tau_R} < \sum_j \bar{m}_j(0) \cdot \sum_j m_j(t) > = \text{constant} \neq 0$$

The correlation function $g_i(t)$ of each type of internal motion can be described as

$$g_i(t) = \Delta\epsilon[(1 - q_i^2) + q_i^2 f_i(t)] \quad (5)$$

where q_i^2 ($0 \leq q^2 \leq 1$) is an anisotropy parameter of motion restriction and is connected with the amplitude parameters in various models. When $q^2 = 1$, the motion is isotropic. In the case with $q^2 = 0$, there is no motion at all. The nature of the anisotropy in intramolecular motion in proteins is stereochemical restriction. It is well-known that the tumbling of the macromolecule as a whole is described by a simple exponential function. In the simplest case, when $f_i(t)$ is also an exponential function, the total correlation function can be written as

$$G(t) = \Delta\epsilon \exp(-t/\tau_R) \prod_{i=1}^{n} [(1 - q_i^2) + q_i^2 \exp(-t/\tau_i)] \quad (6)$$

where τ_i and q_i^2 are the correlation time and the anisotropy parameter, respectively, of the *i*th type of local motion.

If the relaxation times of motions differ significantly, i.e., if $\tau_1 \ll \tau_2 \ll ... \ll \tau_n \ll \tau_R$, then the normalized function $\Gamma(t)$ (see eqs 4 and 6) can be approximated as a sum of *n* components

$$\Gamma(t) = w_1 \exp(-t/\tau_1) + w_2 \exp(-t/\tau_2) + ... + w_n \exp(-t/\tau_n) + w_R \exp(-t/\tau_R) \quad (7)$$

where w_i is the weight of *i*th component, i.e., $w_1 = q_1^2$, $w_2 = (1 - q_1^2)q_2^2$, ..., $w_R = (1 - q_1^2)(1 - q_2^2)...(1 - q_n^2)q_R^2$. The correlation time of macromolecular rotation as a whole, τ_R, can be associated with the volume of the macromolecule by the well-known Debye formula

$$\tau_R = \chi V \eta / kT \quad (8)$$

where V is the volume of the protein globule, η is the viscosity of the solvent, k is Boltzmann's constant, T is the temperature in the Kelvin scale, and χ is a coefficient on the order of 2−3 that characterizes the degree to which the real volume exceeds the apparent volume.[47] Using this relationship, the relative protein volume V/V_0 can be evaluated (V_0 is the apparent volume of protein globule in its native state).

Results and Discussion

A typical dipole correlation function (DCF) for a GOx solution (4.7 mg/ml) in 0.003 M phosphate buffer at 25 °C is presented in Figure 1a. Within the limit of experimental error, the DCF can be described by the sum of four exponential components that correspond to several internal motions and the overall macromolecule tumbling. The optimal number of exponents was obtained by the routine method of sequential subtraction of relaxation components from the DCF.[27] The spectrum of the complex dielectric permittivity is presented in Figure 1b. In both presentations (time and frequency domains), all four components can be clearly observed.

The temperature dependencies of the relaxation times τ_i of these processes for both the native and modified protein solutions are presented in Figure 2. One can see that, for both sample types, the slowest relaxation time, τ_R, demonstrates Arrhenius behavior up to 20−25 °C, with the parameter for the modified protein being greater than that for the native protein.

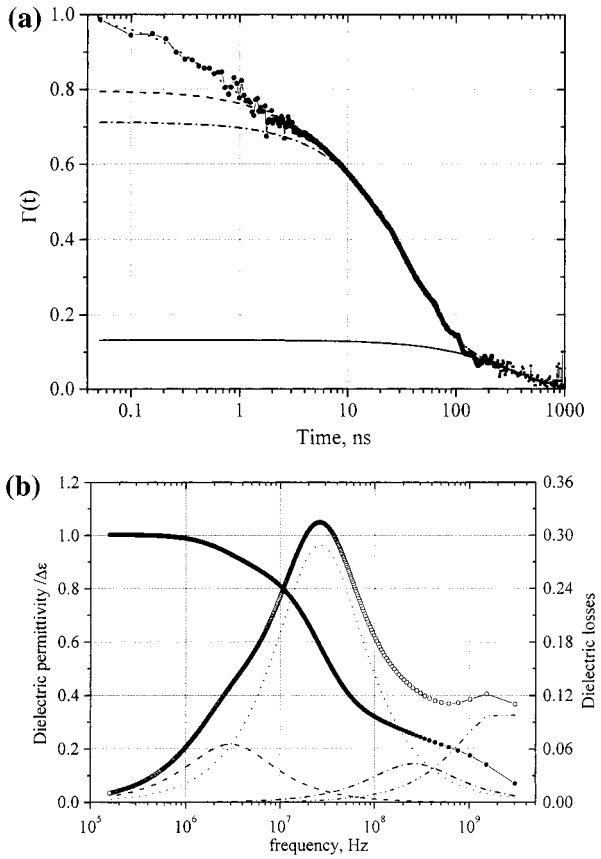

Figure 1. Dipole correlation function (DCF) for modified GOx in solution (4.7 mg/mL) in 0.003 M phosphate buffer, pH 7.0, at 25 °C. (a) Circles represent experimental normalized DCF, dotted line is a fitting curve of all four relaxation processes (see eq 7) with the following parameters: $w_R = 0.12$ and $\tau_R = 338$ ns; $w_3 = 0.528$ and $\tau_3 = 37.6$ ns; $w_2 = 0.076$ and $\tau_2 = 3.86$ ns; and $w_1 = 0.19$ and $\tau_1 = 0.46$ ns. Solid line is a fitting curve of the first (fastest) relaxation process; dash-dotted line is a fitting curve of sum of the first and second processes; and dashed line is a fitting curve of sum of the first, second and third relaxation processes. (b) Spectrum of the dielectric permittivity (●) and losses (○) restored by using the fitting parameters (see above). Here, all components (relaxation processes) of the spectrum are presented.

Further increases in temperature result in an increase in the τ_R value. As for the relaxation times of the other components, they show a complex temperature behavior. It should be mentioned that the τ_3 and τ_2 values for the native protein are greater than those for the modified protein, whereas this tendency is reversed for τ_1.

The dielectric strengths $\Delta\epsilon_i$ of the native and modified proteins for all relaxation processes are presented in Figure 3. In the case of the modified protein, these parameters are greater for all relaxation components and exhibit complex temperature dependence. Because all of the processes characterize the protein macromolecule, the dipole moment $\bar{\mu}$ of GOx was calculated through the total dielectric strength $\Delta\epsilon$ (see eq 2). The temperature dependencies of the dipole moments are presented in Figure 4. For the native protein, the dipole moment is independent of temperature up to 25 °C and is equal to ∼1470 D. At higher temperatures, this parameter decreases. For the modified protein, the dipole moment is much greater (1900−2600 D). Moreover, one can see that it grows in an essentially linear fashion throughout the entire temperature range under investigation.

An analysis of the TDDS data presented above allows us to draw conclusions concerning the mechanisms of the relaxation

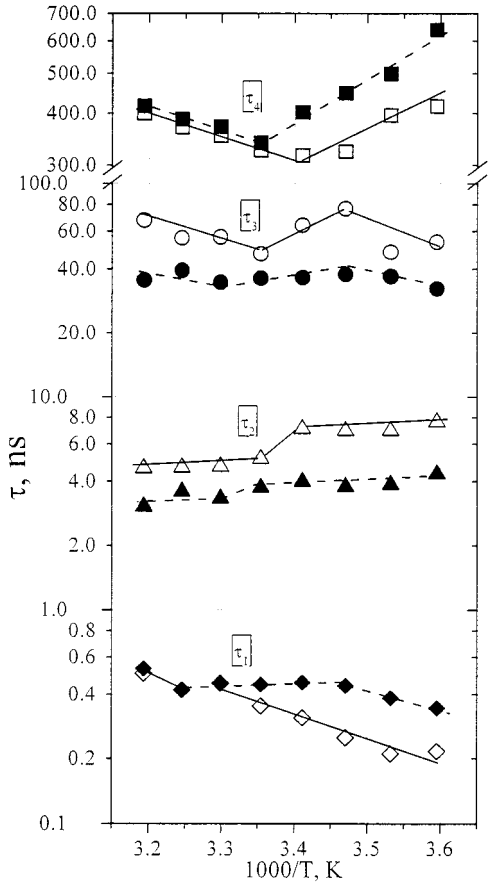

Figure 2. Characteristic times of relaxation processes versus temperature. Open symbols correspond to the native GOx, and solid symbols correspond to the modified GOx. The average standard deviations are 2.6% for τ_4, 4.3% for τ_3, 4.4% for τ_2, and 6.2% for τ_1.

processes. The slowest process can be ascribed to the rotational motion of the GOx macromolecule as a whole. It has been shown[26,27] that, in dilute solutions of proteins, the interprotein long-range dipole−dipole interactions can be neglected, and we can monitor the rotational diffusion of the protein with the characteristic time being proportional to the apparent volume of the globule (see eq 8). An application of this relationship was examined for more than 30 globular proteins.[24,30,47] It was shown that there is a systematic overestimation of experimental values against the theoretical ones by a factor of 2−3. The nature of this overestimation is discussed in detail elsewhere.[38,47,48] The relaxation times calculated by eq 8 using the sizes of the GOx molecule mentioned above were in the range of ∼300 ns, which is in good agreement with the experimental values for native GOx.

The nature of the faster processes is assumed to be related to the intramolecular motion of different protein parts at different size scales. It is well-known that the size of a kinetic unit correlates to its characteristic time mobility.[49,47,50,51] Thus, long relaxation times correspond to the rotation of larger fragments of the macromolecule. Following this hypothesis, we can assume that the third relaxation process can be ascribed to the rotational mobility of two subunits of GOx. Another two fast relaxation processes (1st and 2nd) can be ascribed to the anisotropic rotation of polar and charged groups of different protein structures. The simplest way to present these processes is by the sum of the exponential terms mentioned above. The values of the relaxation times for these motions are in good agreement with the data obtained by dielectric spectroscopy for many other globular proteins.[25,27,31,52] Note that, in general, the fast anisotropic motions in the protein could be described by a more complex correlation function.[53] Thus, the total decay of the correlation function reflects the different types of the protein dipole motion: rotation of the macromolecule as a whole; mobility of the subunits; and anisotropic motion of polar groups, charged groups, and substructures.

Let us discuss the temperature dependencies of the relaxation parameters. The slow-process relaxation time demonstrates Arrhenius behavior up to 20 °C for native GOx and up to 25 °C for modified GOx (See Figure 2). In this temperature range, both proteins rotate as rigid kinetic units in a viscous medium with similar activation energies of ∼24 kJ/mol for the transformed protein and ∼18 kJ/mol for the native protein. This thermal behavior is in good agreement with the results typical for various globular protein solutions.[25,31,42,43,52]

At higher temperatures (>20−25 °C), a significant change in the temperature dependence of the characteristic time for the slow process is observed. Such non-Arrhenius behavior was detected for other proteins and was ascribed to a conformational transition in the macromolecules.[26,30−32] This transition is usually accompanied by an increase in the protein molecule volume and by alterations in the intramolecular motion parameters. The temperature dependence of the relative volume for GOx is presented in Figure 5. At temperatures below 20−25 °C, the volume of modified GOx is about 1.4 times greater than that of the native protein. This result is expected, as the modification of the protein by the covalent attachment of eight long alkyl chains should lead to an increase in the protein molecular volume. At temperatures higher than the transition temperature, the volume increases (1.5−2 times) for both proteins. Such volume increases were monitored for various proteins by different physical methods. For example, the radius of Ribonuclease A (pH 5.7) at and above the transition temperature, as obtained by SAXS, increased by 30−60%.[54] Also, a 1.6-times increase in the lysozyme volume (pH 1.2−2.3) was detected by light scattering during the conformation temperature transition.[55]

The GOx temperature transition is also reflected in the dielectric strength behavior for all of the processes (see Figure 3) that occur because of the respective change in the protein dipole moment (see Figure 4). For the native macromolecule, this parameter is equal to ∼1470 D and does not change up to 20−25 °C. Thus, there are no noticeable conformational changes in its structure, i.e., the protein is in its native state. For the modified protein, the linear temperature increase in the dipole moment can be explained by an aggregation process, caused by the hydrophobic interactions of the attached alkyl chains. Spontaneous formation of aggregates of modified GOx in aqueous solutions with a mean diameter of 30 nm has been previously demonstrated by dynamic light scattering.[15]

The analysis of protein internal motion can be carried out in terms of the anisotropy parameters. As mentioned above, this parameter, q_i^2, is an amplitude of polarization averaged by the i type of motion. Because two fast relaxation processes were ascribed to intramolecular mobility, the part of polarization averaged by this motion can be calculated from the equation

$$q_{\mathrm{loc}}^2 = (\Delta\epsilon_1 + \Delta\epsilon_2)/(\Delta\epsilon_1 + \Delta\epsilon_2 + \Delta\epsilon_3 + \Delta\epsilon_4) \qquad (9)$$

where $\Delta\epsilon_1 = \Delta\epsilon\, w_1$ and $\Delta\epsilon_2 = \Delta\epsilon\, w_2$ are the dielectric strengths of the fast relaxation processes of intramolecular motions and the denominator is the full amplitude of polarization $\Delta\epsilon$ (see eqs 6 and 7), i.e., $\Delta\epsilon = \Delta\epsilon_1 + \Delta\epsilon_2 + \Delta\epsilon_3 + \Delta\epsilon_4$.

Figure 6 presents the temperature dependence of q_{loc}^2 for native and modified GOx. One can see that this parameter has

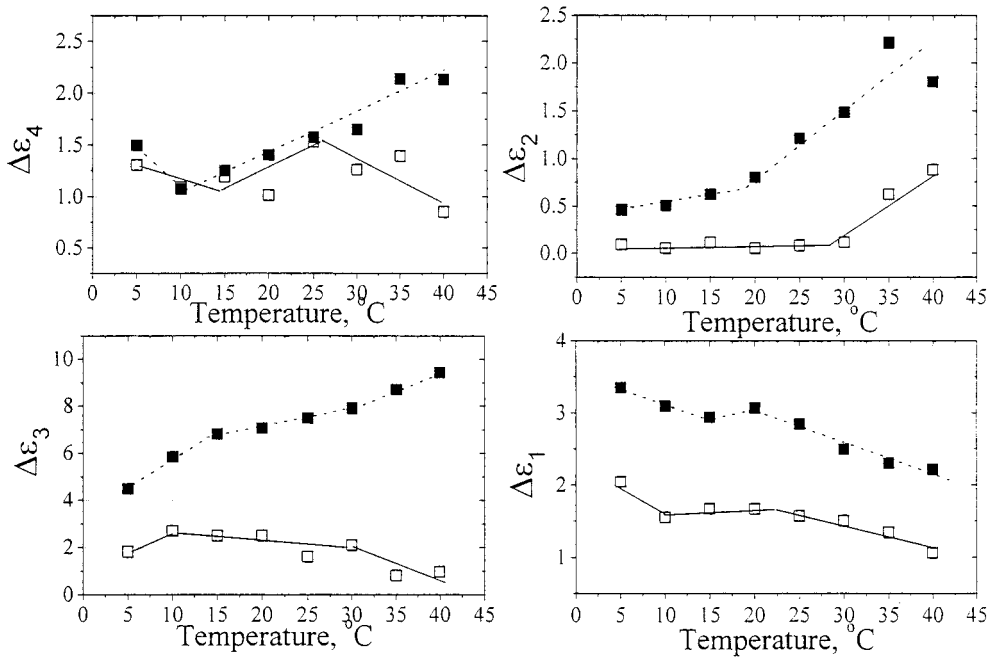

Figure 3. Temperature dependencies of dielectric strength (□, native GOx; ■, modified GOx). The average standard deviations are 12% for $\Delta\epsilon_4$, 4.2% for $\Delta\epsilon_3$, 8.9% for $\Delta\epsilon_2$, and 4.0% for $\Delta\epsilon_1$.

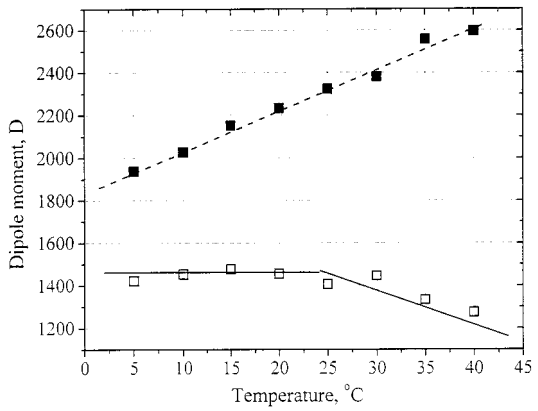

Figure 4. Dipole moment of GOx molecules versus temperature (□, native GOx; ■, modified GOx).

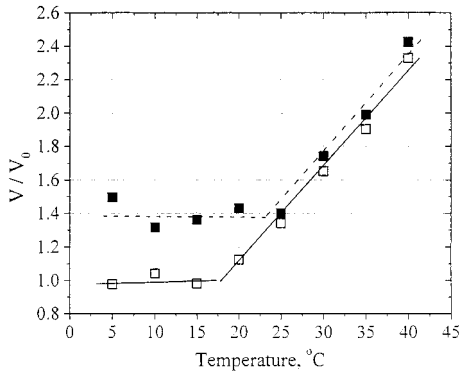

Figure 5. Relative change in GOx molecular volume (□, native GOx; ■, modified GOx).

approximately the same value for both the native and the modified protein up to ~30 °C. Thus, the chemical modification, i.e., the attachment of eight alkyl chains, does not change the intramolecular motions significantly. Moreover, it can be assumed that the attachment of the hydrophobic chains took place mainly at the surface of the macromolecule. Indeed, most of the lysine groups of the GOx molecule, which could

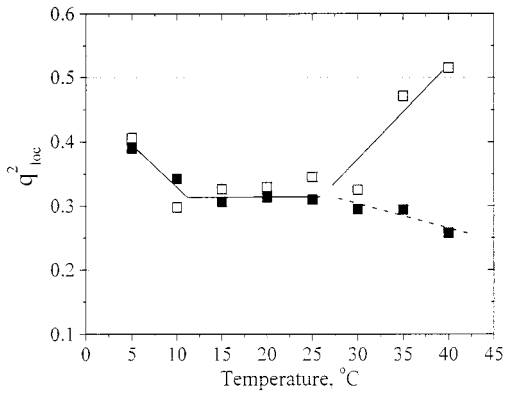

Figure 6. Anisotropy parameters for all local motions, q^2_{loc}. (□, native GOx; ■, modified GOx).

participate in the covalent attachment of alkyl groups, are located at the surface.[8,56,57] After the temperature transition (>30 °C), the behavior of q^2_{loc} for the native and modified proteins is different. The amplitude of the intramolecular motions in the native GOx increases because of a conformational transition in the protein molecule. For the modified protein, the parameter q^2_{loc} decreases slowly. Such a decrease corresponds to more anisotropic (i.e., more restricted) intramolecular motion. This most likely means that the protein modification leads to a more rigid structure at higher temperatures.

Usually, stabilization of an enzyme structure results in an increase in its thermostability. We compared the enzymatic activity of native and modified GOx at several temperatures. It has previously been shown[15] that modification of GOx by *N*-hydroxysuccinimide is not accompanied by the dissociation of the subunits or by the loss of the cofactor (FAD). GOx retains 50−60% of its original specific activity after attachment of eight palmitic chains. Partial loss of activity can be attributed to the changes in the microenvironment of the active centers (two lysine groups are involved in the FAD-binding domain[8]). The possibility that some of the enzyme molecules that self-assembled into aggregates are "switched off" also cannot be ruled out.

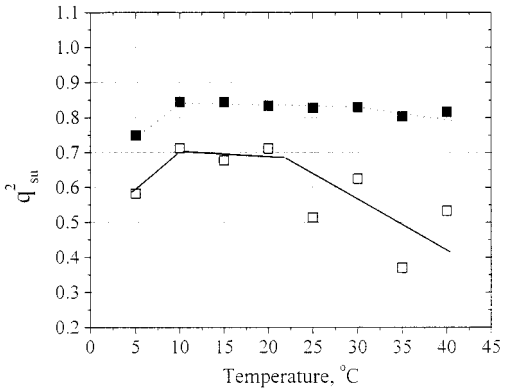

Figure 7. Anisotropy parameter of subunit motions, q^2_{su}. (□, native GOx; ■, modified GOx).

GOx is unstable at temperatures in excess of 40−50 °C.[1,10] We found that the enzymatic activity of modified GOx is more sensitive to heating: after incubation at 40 °C for 1 h, it decreases by 17%, compared with 5% for the activity of the native enzyme (incubation at 60 °C results in a total loss of activity for both forms of the enzyme). A contradiction between these data and the temperature dependence of the factor q^2_{loc} allows us to assume that the observed decrease in enzymatic efficiency is most likely determined by changes in the active site of the enzyme and is not connected with the rigidity of the protein globule as a whole.

Let us discuss the behavior of the subunits in the GOx molecule. In Figure 7, the temperature dependence of the anisotropy parameter of subunit motion, q^2_{su}, are presented. The physical significance of this parameter is that it is the part of the polarization that can be averaged by subunit motion. The time scale of this motion is greater than that for the internal local motions (processes 1 and 2); therefore, these intramolecular motions are not included in the consideration, and parameter q^2_{su} is calculated by the equation

$$q^2_{su} = (\Delta\epsilon_3)/(\Delta\epsilon_3 + \Delta\epsilon_4) \qquad (10)$$

An analysis of the q^2_{su} parameter behavior shows that, in the native protein, the linkage between subunits is more rigid, and the amplitude of motion is less than that for the modified protein. Thus, it can be concluded that the hydrophobic modification results in an increase in the freedom of motion of the subunits but does not change the amplitude of the intramolecular motion, at least in the temperature range below the transition temperature (see Figure 6). As mentioned above, two lysine groups are involved in the FAD-binding moieties, which are situated near the dimer interface. Attachment of long alkyl chains to these groups (or even to one of them) may lead to the formation of a less tightly packed quaternary structure and, therefore, to an increase in the freedom of motion of the subunits. In addition, the modified GOx molecule is more negatively charged than the native protein, because a smaller number of free positively charged lysine groups is present after modification while the number of negatively charged groups does not change.[15] Such an increase in net negative charge on each subunit may result in a weakening of the contacts between them.

At temperatures higher than the transition temperature (20−25 °C), the parameter q^2_{su} decreases considerably for native GOx and negligibly for the modified enzyme. Thus, for native GOx, the transition leads to a decrease in the amplitude of the motion of the subunits. Such an unexpected effect can be explained by formation of additional hydrophobic contacts, which restrict the motion of the subunits against each other. For the modified protein, the q^2_{su} value is practically constant. This can be explained by the presence of long alkyl chains at the surface of the dimeric protein molecule, as these chains form multiple hydrophobic bonds that determine the relative invariability of the quaternary structure of modified GOx across the whole temperature range.

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