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Dynamic and structural properties of glucose oxidase enzyme

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Abstract The catalytic oxidation of β-D-glucose by the enzyme glucose oxidase involves a redox change of the flavin coenzyme. The structure and the dynamics of the two extreme glucose oxidase forms were studied by using infrared absorption spectroscopy of the amide I′ band, tryptophan fluorescence quenching and hydrogen isotopic exchange. The conversion of FAD to $FADH_2$ does not change the amount of α-helix present in the protein outer shell, but reorganises a fraction of random coil to β-sheet structure. The dynamics of the protein interior vary with the redox states of the flavin without affecting the motions of the structural elements near the protein surface. From the structure of glucose oxidase given by X-ray crystallography, these results suggest that the dynamics of the interface between the two monomers are involved in the catalytic mechanism.

Key words *Aspergillus niger* · Glucose oxidase · FTIR · Proton exchange · Secondary structure · Protein dynamics

Abbreviations *FAD* and *$FADH_2$* Flavin Adenine Dinucleotide oxidised and reduced form · *Amide I′ band* Amide I band (1700–1600 cm^{-1}) in heavy water · *H-D* Proton and deuteron · *D_2O* Heavy water · *CaF_2* Calcium fluoride · *FTIR* Fourier transform infrared · *Trp* Tryptophan · *ns* nanosecond (10^{-9} second) · *ps* picosecond (10^{-12} second)

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Introduction

Glucose oxidase (β-D-glucose: oxygen-1-oxidoreductase, EC 1.1.3.4) (GOD) is a homodimeric enzyme (Kriechbaum et al. 1989). Each subunit of this protein contains one coenzyme molecule of flavin adenine dinucleotide (FAD) (Pasur and Kleppe 1964). Each GOD monomer has two distinct domains: one that binds non covalently but very tightly the FAD moiety, and another that binds the β-D-glucose substrate. The first area consists mainly of β-sheets and the second one, 4 α-helices supporting antiparallel β-sheets (Hecht et al. 1993 a). The enzyme catalyses, in the presence of molecular oxygen, the oxidation of β-D-glucose into gluconic acid and hydrogen peroxide. The conversion of β-D-glucose to gluconic acid involves the transfer of two protons and two electrons from the substrate to the flavin moiety.

At neutral pH, the pK of the flavin's N(1) gives a negative charge in its fully reduced form. So, the two redox states of the flavin coenzyme interact differently with the protein. This is enabled by protein-flavin links distributed over 23 H-bonds (Hecht et al. 1993 a), the arrangement of which differs with the redox flavin states (Sanner et al. 1991). These interaction changes between the coenzyme and the protein – which concern the immediate protein environment of the active centre – could also affect other protein domains within GOD. In order to better understand these different protein-flavin interactions, we have attempted to study the relationship between the coenzyme redox state and the structural and dynamic properties of the whole glucose oxidase molecule. Indeed, some reports purport to have demonstrated that protein dynamics are unlikely to make a contribution to catalysis (Kraut 1988), while others strongly believe that dynamics play a fundamental role (Gavish and Yedgar 1995; Lumry 1995). Therefore, in order to specify the relative contribution of sructure and dynamics to the catalytic activity, our investigations used infrared absorption spectroscopy of the amide I′ band, tryptophan fluorescence quenching and hydrogen isotopic exchange on the oxidised and reduced GOD enzymes.

Materials and methods

Protein

Lyophilised glucose oxidase from *Aspergillus niger* (type X-S) was purchased from Sigma and used without further purification since HPLC chromatography gives one band exhibiting a mass around 180 kDa in accordance with previous evaluations (Wellner 1967).

All experiments were carried out at 20 °C in aqueous medium at pH or pD 7 (0.1 M sodium-phosphate buffer). Protein concentrations were determined spectrophotometrically using an absorption coefficient of FAD in GOD $\varepsilon = 2.82\ 10^4\ M^{-1}\ cm^{-1}$ at $\lambda = 450$ nm (Swoboda and Massey 1965). Reduced GOD was obtained after incubation of the oxidised form with an excess of β-D-glucose (100 mM or 10 times the K_m). Because the product of the enzymatic reaction, gluconic acid, produces a parasite infra-red signal, infrared experiments on the reduced GOD form were made after O_2 displacement by bubbling N_2 gas into the solution and addition of a small amounts of dithionite (3.5 or 7 mg per 500 µl).

Fluorescence and optical absorption measurements

Optical absorbance measurements were made with a Hewlett Packard 89 500 (Ultra violet/Visible) spectrophotometer. Fluorescence spectra of Trp residues in the GOD enzyme were obtained with a Perkin-Elmer LS-5B spectrofluorometer. The bandwidths used for excitation and emission were 2.5 nm. The solutions were illuminated at the red edge of the Trp residue's absorption ($\lambda_{exc} = 295$ nm), were energy transfer between Trp residues is known not to occur (Weber 1960). In order to eliminate the excitation light scattering from measurements, fluorescence intensities from Trp residues were observed through a Schott cut-off filter (WG 320) passing wavelengths above $\lambda = 310$ nm.

Fluorescence quenching measurements used acrylamide to quench indole fluorescence. Inner acrylamide absorbance filtering was corrected on the experimental fluorescence intensity.

Time-resolved fluorescence measurements

The tryptophan fluorescence decays were measured using the single photocounting technique and the ultra violet light provided by the storage ring of Orsay working at a frequency of 8.33 MHz (super ACO) in the two-bunch mode. The storage ring provides a light pulse with a full width at half maximum of about 500 ps. The excitation wavelength was set to 300 nm with a bandwidth of 6 nm; the emission was selected at 340 nm with a bandwidth of 6 nm and was detected by a Hamamatsu microchannel plate R1564U-06. The apparatus response function was measured with a scattering ludox solution near the emission wavelength. Time resolution was 23.8 ps/channel, with 2048 channels used for decay storage. The corresponding tryptophan lifetimes are multiexponential with the values' distribution determined by the maximum entropy method (Livesey and Brochon 1987).

The Trp fluorescence intensity decay was expressed as:

$$F(t) = \Sigma\ \alpha_i\ \exp(-t/\tau_i)$$

where α_i is the pre-exponential factor and τ_i is the lifetime for the i^{th} emitting component. The fractional contribution (f_i) to the total fluorescence intensity (F) for the i^{th} component (F_i) is related to its corresponding pre-exponential factor as follows:

$$fi = F_i/F = \alpha_i\ \tau_i / \Sigma\ \alpha_i\ \tau_i \qquad (\text{and } \Sigma_i\ f_i = 1)$$

The average lifetimes were obtained by the classical relationship:

$$\tau = \Sigma\ f_i\ \tau_i$$

where f_i and τ_i are the previously defined intensity fraction and lifetime of each fluorescence component, respectively.

Infrared spectroscopy

To study the secondary structure of the oxidised and reduced GOD enzyme, infrared spectra of the amide I′ band were made with a FTIR 10 MX Nicolet.

To eliminate the contribution of atmospheric water vapour in the IR experiments, dry air was continuously blown into the instrument. Samples in D_2O buffer were placed in a temperature-regulated specatype infrared cell with CaF_2 windows and a 100 µm pathlength. The spectral resolution was 2 cm^{-1} which gives peaks within 0.2 cm^{-1}. Solvent contribution was removed by subtracting the buffer's spectrum from that of the protein solution.

Data treatment was performed with software developed in Ottawa (Kauppinen et al. 1981) which allows the contribution of water to the interactively subtracted. The second derivative of the resulting spectrum was used to determine the position and the number of the different components (Dong et al. 1990). The experimental amide I′ band was decomposed into pure Gaussian curves. The area of each component was assumed to be proportional to the amount of the structure type which is assigned to it (Cameron and Moffatt 1987; Surewicz and Mantsch 1988). However, the absolute quantitation of the different types of structures cannot be attained by the direct proportionality of the relative area of the different components (Jackson and Mantsch 1995). Thus, only the ratio of each component's area between reduced and oxidised GOD (Red/Ox) has been used as an index of any rearrangement within the H-bond lattice, which would occur after the flavin redox state change.

Hydrogen-Deuterium exchange

In native proteins, slow hydrogen isotope exchange in the amide bond and in the side chain is mostly controlled by the protein conformational dynamics (Gregory and Rosen-

berg 1986). So, the rates of H-D exchange were observed over 48 hours on the FTIR apparatus,with a 50 μm pathlength, and an acquisition time of 8 min for each point. The starting time of each experiment was the instant when the phosphate D_2O buffer at pD 7 (425 μl) was added to the protein in phosphate light water buffer at pH 7 (75 μl). The dissolution of protein in buffered water before the deuteration experiments eliminates all contributions of protein ionisation variations and structural dynamics change, which can be produced by the interactions of soluble proteins with their solvent (Gregory 1995). The use of a water-D_2O mixture means the solvent does not contain 100% deuterons, but is in fact 14% hydrogenated. So the rates observed in the paper are not the absolute rates, but only a relative comparison between the oxidised and the reduced form. In the experiments reported here, the finite time for the dilution and the initial rate of deuteration mean the data are inaccurate during the first 10 min (Blout et al. 1961). The extent and the kinetics of the proton exchanges were obtained from the intensities of the amide II and the amide I′ bands (amide II = 1549.5 cm^{-1}, amide I′ = 1650 – 1654 cm^{-1}), by taking the ratio of one to the other (amide II/amide I′), and following it over time (Blout et al. 1961). For each acquisition, the values of amide II and I′ bands were obtained by subtracting the composite buffer's spectrum (water mixed with D_2O) from that of the protein solution. This gives a flat baseline for the resulting protein spectrum, although the position of the amide I′ shifts from 1650 to 1654 cm^{-1}.

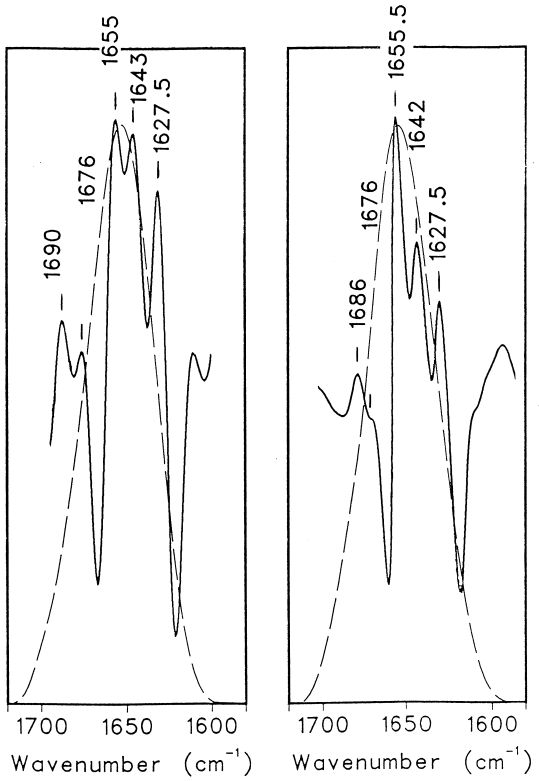

Fig. 1 Experimental and second derivative of the amide I′ band of oxidised and reduced GOD species. Spectra were made at 20 °C on 27.5 mg/ml enzyme solubilised in D_2O buffer (pD = 7). *Right:* oxidised GOD spectrum (- - -) with its second derivative (–). *Left:* reduced GOD spectrum (- - -) with its second derivative (–). See Table 1 for the assignement of each component

Results

Secondary structural organisation of oxidised and reduced GOD forms

The amide I′ band is the most useful for studying protein secondary structure (Byler and Susi 1986). Figure 1 displays the experimental amide I′ bands of oxidised and reduced GOD forms, and their second derivatives. The peak positions in the second derivative give the structural nature of the various components (Yang et al. 1985; Byler and Susi 1986).

Since the assumption of equal molar absortivities of different secondary structures may introduce a misinterpretation of the FTIR data (Jackson and Mantsch 1995), we have only measured the relative area variation for a single component.Table 1 gives the relative amounts for each component between the reduced and oxidised GOD, with their wavenumber in the IR spectra. It appears that the oxidised and reduced forms of GOD have the same amount of α-helix. However, the FAD – > $FADH_2$ change is associated with a conversion of a fraction of labile (1686 cm^{-1}) to more constrained (1676 cm^{-1}) β-turns. For the β-sheets, their wavenumber does not shift between the reduced and the oxidised form, indicating that the H bond's strength is the same in the two GOD forms. However, the integrated area of the β-sheet absorption is 33% greater in the reduced

Table 1 Area ratio of reduced GOD/oxidised GOD of IR amide I′ band components

Components (cm^{-1})	1627.5	1643*–1642	1655–1655.5	1676	1690–1686
Assignement	β-sheet	random	α-helix	β-turn	
				constrained	labile
Area ratio red/oxi	1.33	0.86	1	3	0.2

* The first wavenumber is from the reduced form, the second correspond to the oxidised one
All data result from measurements at 20 °C in D_2O buffer. Area precision of each individual component was ≤ ± 0.5%

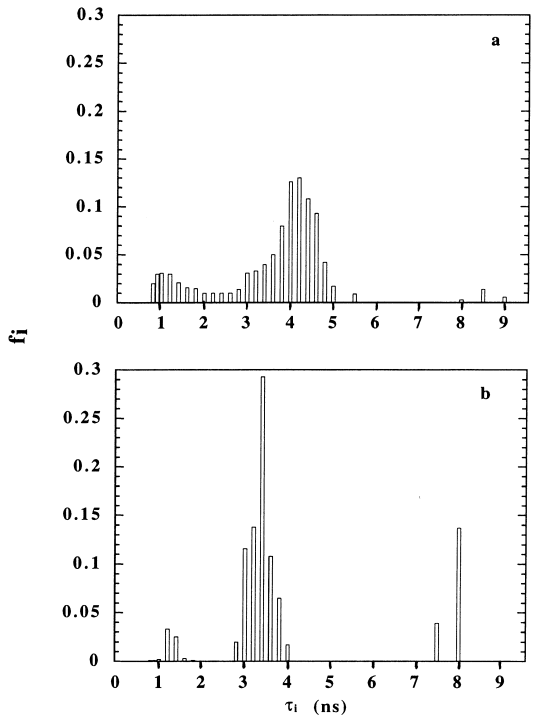

Fig. 2 Lifetimes distribution of tryptophan fluorescence from reduced (**a**) and oxidised (**b**) glucose oxidase. f_i gives the intensity fraction associated at each fluorescence lifetime τ_i

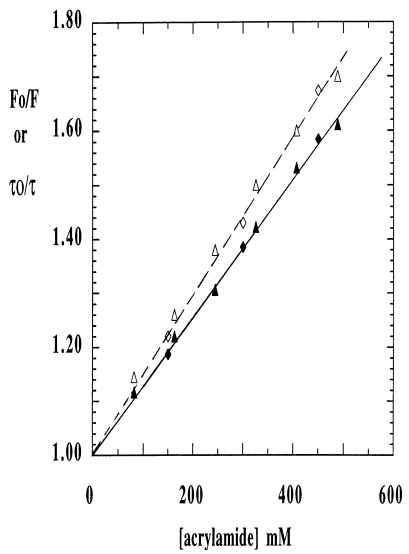

Fig. 3 Stern Volmer representation of the fluorescence quenching by acrylamide of the Trp residues from the two forms of glucose oxidase. τ_0 and F_0, τ and F are the lifetimes and the fluorescence intensities in the absence and in the presence of quencher, respectively. Data from oxidised, (F_0/F (△), τ_0/τ (◇)) and reduced protein (Fo/F (▲) $\tau o/\tau$ (◆))

form, indicating a substantial change in the proportion of β-sheet structures. The fraction of the random coil is decreased from the oxidised to the reduced form. The H bond populations evolve as if a fraction of random coil from the oxidised GOD had become β-sheet by reduction of FAD. Therefore, the coenzyme redox states act on the protein secondary structural organisation.

Fluorescence decay time and fluorescence quenching experiments

The Trp fluorescence decay time measurements range from 0.8 ns to 9 ns (Figure 2). The broad distribution of Trp fluorescence lifetimes reveals the different Trp-flavin energy transfers (Gibson et al. 1964). The distribution change in the Trp emission between the oxidised and the reduced enzyme only results from the optical density (O. D.) change in the visible domain of the co-enzyme, FAD or $FADH_2$. Data give an average lifetime of 4.1 ns for the oxidised GOD form and 3.6 ns for the reduced one.

X-ray crystallography on GOD has shown that the Trp residues are close to the surface (Hecht et al. 1993 b). However, the peak positions of the fluorescence emission from the two GOD forms show that the Trp residues are not in direct contact with the solvent (wavelengths of maximum fluorescence emission are at $\lambda_{em} = 338$ nm and $\lambda_{em} = 335$ nm for the reduced and oxidised forms, respectively). The Trp residues occupy positions protected by the glycosylation of the protein (O'Malley and Weaver 1972), seemingly hidden from the perturbing effect of water or other solutes.

Acrylamide molecules are able to penetrate the carbohydrate envelope and to diffuse within the protein surface so as to reach the quenchable Trp residues during the 4.1 ns (oxidised GOD) and 3.6 ns (reduced GOD) of the averaged fluorescence lifetimes. The relative fluorescence efficiency, F_0/F is linearly related to the molar concentration of acrylamide quencher [Q]. Independent measurements of fluorescence lifetime τ upon quenching show that the quenching plots F_0/F and τ_0/τ are parallel (Figure 3). In other words, dark complexes do not contribute appreciably to the emission. This demonstrates that only structural fluctuations must be taken into consideration.

The rate constant, k, of the bimolecular encounter – which reflects the random spreading of the quencher molecules – was obtained by the Stern-Volmer equation (Stern and Volmer 1919):

$$F_0/F = 1+KQ = \tau_0/\tau = 1 + k\tau_0 Q$$

where K is the Stern-Volmer constant and τ_0 and τ are the averaged unquenched and quenched lifetimes, respectively; Q is the quencher acrylamide concentration. Table 2 gives the experimental values obtained for K and $k = K/\tau_0$. The value of k is the same for the two GOD forms.

The bimolecular kinetic constant, k, represents the average of the details of quencher migration into the highly-organised macromolecular structure, close to the protein surface. For these reasons, this rate constant is a simple indication of the possible changes of the acrylamide migration induced by the fluctuations of the protein surface. Thus, the redox states of the active site (FAD or $FADH_2$) do not alter these structural fluctuations.

Table 2 Data showing the dynamics of the protein outer shell from the two forms of glucose oxidase at pH 7

	Fluorescence quenching			Proton exchange	
	$K^{(1)}$ (M^{-1})	$\tau_0^{(2)}$ (ns)	$k^{(3)}$ (M^{-1} s^{-1})	$A^{(4)}$	$\tau^{(5)}$ (hour)
Oxidised GOD	1.4	4.1	$3.4\ 10^8$	0.049	2.5
Reduced GOD	1.25	3.6	$3.4\ 10^8$	0.049	2.5

(1) K: Stern-Volmer constant
(2) τ_0: average fluorescence lifetime of Trp residues
(3) k: bimolecular kinetic constant of the Trp fluorescence quenching by acrylamide
(4) A: pre-exponential factor from the time evolution of the absorbance ratio amide II′/amide I′ in the first exchange period
(5) τ: relaxation time of the H-D exchange in this first period

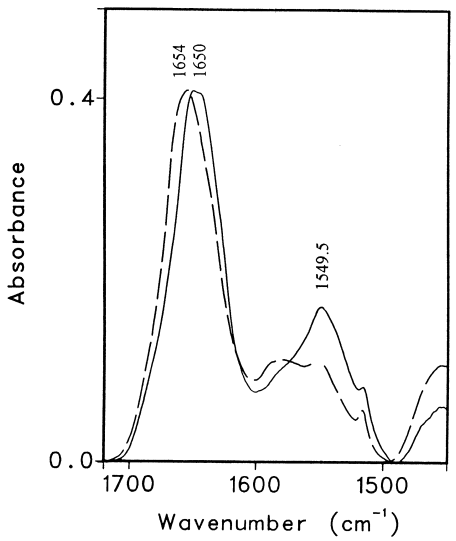

Fig. 4 Infrared spectra of oxidised GOD solubilised in H_2O buffer, and then mixed with a D_2O buffer. – Spectrum of amide II and amide I′ bands 10 min after the instant when the mixing was made. - - - Same spectrum 30 hours after the mixing. Data obtained with 425 µl of D_2O pD 7 (buffered by 0.1 M phosphate salt) added to 75 µl (216.5 mg/ml) of sample solubilised in the aqueous pH 7 (0.1 M phosphate buffer)

Proton-exchange kinetics

Bulk H-D exchange of proteins is the sum of a large number of independent processes. Their constants span over several orders of magnitude. At pH 7 and 20 °C, the average intrinsic relaxation time for amide protons is around 0.5 second. In proteins, amide protons often have, in addition, protection factors (Hughson et al. 1990) which depend on hydrogen bond strength and protein motion (Hvidt and Nielsen 1966; Abaturov et al. 1976). The great number of peptide groups, implicated in protein isotopic exchange, gives a statistical picture of these relaxations (Gregory and Rosenberg 1986; Kim 1986).

The reference measurement of the infrared spectrum was performed during the initial rapid exchange (10 min), presumably due to the deuteration of the most exposed random regions (Blout et al. 1961). The reference infrared spectrum, of the oxidised GOD form (10 min incubation), and the spectrum, after partial hydrogen-deuterium exchange (close to 30 hours of incubation) are presented after subtraction of their solvent (mixture of D_2O, H_2O and HOD) (Figure 4).

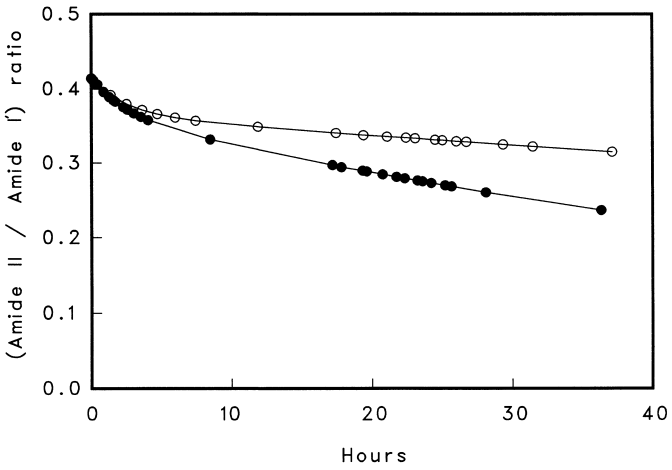

Fig. 5 Comparative deuterium-hydrogen exchanges of the two GOD forms observed over a 48-hour period. Data from oxidised (●) and (○) reduced GOD were obtained by measurements at 20 °C on 32.5 mg/ml of enzyme. The observed rates are not absolute, but only relative to each other (see Materials and methods)

Figure 5 shows the relative rates of H-D exchange between the oxidised and reduced GOD. The evolution of the amide II/amide I′ ratio over time was fitted using non linear regression, giving randomised residuals. On the basis of the experimental observation, the existence of two different types of amide groups in the GOD protein can be postulated, and we have:

$$\text{amide II/amide I}' = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2).$$

This finding was entirely predictable. All proteins with any significant proportion of hydrogen-bonded structure show this behaviour, with unordered segments exchanging rapidly followed by buried and/or strongly hydrogen-bonded protons.

Amide protons with protection factors smaller than 10^5 were detectable in the first 6 hour period. So, experiments in this time period only detect the weaker hydrogen bonds in regions with high structural fluctuations. It is important to note that both GOD forms display the same temporal evolution:

$$\text{amide II/amide I}' = A_1\exp(-t/\tau_1) = 0.049\ \exp(-t/2.5\ \text{hours}).$$

This exchange which occurs in the time interval 10 min to 6 hours is due to the deuteration of the outer protein region. Thus, fluctuations of the protein surface are identical for the oxidised and reduced GOD forms (Table 2).

The exchange observed over longer time periods principally concerns the amide protons deeply embedded inside the protein. Highly protected amide protons (protection factor larger than 10^6) can be regarded as indicating the conformational dynamics of the protein core. This do-

main, resistant to H-D exchange, is most likely hydrophobic and highly protected from the solvent.

The temporal evolution amide II/amide $I' = A_2 \exp(-t/\tau_2)$ gives the same extent $A_2 = 0.365$ for both GOD forms, but different rates τ_2. Remarkably, the dynamics of the protein core, where the dimer interface is located, are highly dependent on the redox state of the flavin group. It is clear that the dimer interface fluctuates more quickly in the oxidised form ($\tau_2 = 83$ hours), than in the reduced form ($\tau_2 = 250$ hours). The finding that the core of the two protein forms have different exchange kinetics is not surprising, if we consider the conversion of some unordered polypeptide chains to β-sheets in this region, as demonstrated by the IR study.

Discussion

The present work seems to reveal that the flavin redox change FAD – > $FADH_2$ is directly linked to a small secondary structural reorganisation of the GOD enzyme. The study shows that the flavin redox states affect principally the β-sheets content of the protein. X-ray diffraction on the oxidised GOD crystal has demonstrated the existence of two important domains in the distribution of the secondary structures. The α-helices are mainly located in the protein shell and, the β-sheets are close to the subunits' interface, which itself forms one side of the active site. On the basis of the highly plausible assumption that the loss of β-sheet structure between the reduced and the oxidised GOD concerns the latter domain, it transpires that the coenzyme redox state should modulate the interfacial constraints between the subunits of the dimer. Thus, the transition of a small amount of random coil to β-sheet could be – in fact – a quaternary and tertiary reorganisation of a select few flexible segments at the monomer-monomer contact area. This particular quaternary contact could, also, more or less constrain the turns of the protein. This interpretation is in good agreement with crystallographic data on the loop region at residues 43, 238, 256–260 and 340 (Hecht et al. 1993 a). IR corroborates this with there being higher strains on the β-turns of the reduced GOD. The labile interface contact, which alternates between two extreme positions, could serve as a control switch for the access of the specific substrate to the catalytic site (Havsteen 1989). This structural feature would be similar to that, which as been identified in the trypsin family (Shotton and Watson 1970; Freer et al. 1970). On the contrary, the α-helix structure, which occupies a position close to the protein surface, is not affected by the redox states of the coenzyme. So, the structural perturbations brought about by the flavin redox state do not concern the surface vicinity, and seem to be essentially localised over the interface between the monomers. Motions in the GOD enzyme should be coupled to these structural domains. The flexibility of the two proteic domains is reflected in the H-D exchange. It was found, that the protein surface in the two GOD forms requires the same time for proton exchange, whereas the amide hydrogens embedded in the protein interior follow a different pattern according to the flavin redox state. So, the H-D exchanges – which mainly represent the conformational dynamics – seem to demonstrate that the two domains in the GOD enzyme (surface and core) have specific dynamics. One of these domains responds to the flavin redox state by an adjustment of its secondary structure (β-sheet) and by a change in its modes of vibration. The protein's conformational change at the monomer-monomer contact is reversible; when it takes its reverse course, it restores the original vibration mode of the enzyme interior, without affecting the protein surface. Fluorescence quenching experiments of Trp residues embedded in the protein outer shell confirm the proton exchange information over the 6 first hours. Both observations show that the motions of the structural elements near the protein surface are not intimately involved in the catalytic mechanism. The high content of α-helix supporting the antiparallel β-sheets (Hecht et al. 1993 a) should create sufficient constraints to avoid the internal changes and any perturbations reaching the protein surface. The small shift of the α-helix wavenumber (1655.5 – 1655 cm^{-1}) with the flavin redox states supports this assumption. From these considerations, the α-helices distributed around the surface are expected to dampen any modifications of both the structure and the dynamics of the molecular surface during the catalytic process. An alternative explanation to the absence of structural and dynamic changes of the protein surface could be the S-S bridge (Hecht et al. 1993 a) and the carbohydrate envelope, which would improve the surface rigidity. Indeed, the GOD enzyme is glycosylated with a carbohydrate content of 16% (O'Malley and Weaver 1972). Although glycosylation is only held responsible for protein stability, it could substantially restrict the mobility of the external protein layer (Kohen et al. 1997).

In other words, the conformational and dynamic changes linked to the redox states of the flavin are essentially restricted to the protein contact between the two monomers, and do not spread through the entire protein. The perturbation is likely to be caused by the substrate, which, upon specific binding to the enzyme, modifies the subunits' interface and hence the vibration modes near the catalytic site. Local conformational and dynamic changes indicate a lack of propagation; it therefore becomes possible to describe the properties of this incomplete transmission in terms of a percolation model (Deutscher et al. 1983). Although the enzyme has two identical subunits, the enzymatic activity requires their assocation. A tightly associated substructure is formed by fusing surfaces from both monomers. This generates forces able to provide the specific fluctuations for the enzymatic mechanism (Lumry 1995). So, the active entity is the dimer. This supports previous studies on GOD's dissociation into monomers (Jones et al. 1982) suggesting that the GOD monomer cannot be the active enzyme.

In summary, the data suggest that, the subunits' interface in the oxidised enzyme is slack and supple allowing a great flexibility in this region. The strains on the β-turns of each monomer are low. Reduction of the flavin creates

a few H-bonds between the random segments of the interface and strengthens the contact between the two monomers. The stiffness of the contact does not allow for large fluctuations of this domain and has repercussions on the turns. So the redox states of the flavin group are directly linked to the organisation of the subunits contact which manage the structure and the dynamics of the enzyme. In this way, the specific motions in the enzyme complex would assist the coenzyme's role in the proton transfer by reducing the entropic barriers (Young and Post 1996), so as to obtain the stereospecific conformations required for the enzymatic catalysis.

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