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Octyl Glucoside Induced Formation of the Molten Globule-Like State of Glutamate Dehydrogenase[1]

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The interaction between *n*-octyl-β-D-glucopyranoside (octyl glucoside) and bovine liver glutamate dehydrogenase (GDH) was studied using techniques including equilibrium dialysis, UV-spectrophotometry, circular dichroism (CD), fluorescence energy transfer and extrinsic spectrofluorometry in 50 mM sodium phosphate buffer solution (pH 7.6). The equilibrium dialysis experiment showed a higher binding of octyl glucoside to GDH that induces up to 80% enzyme inhibition in 20 mM octyl glucoside solution. The CD study indicated that GDH retains its secondary structure in the presence of octyl glucoside, but loses a degree of its tertiary structure by acquiring a more extended tertiary structure. Measurement of the binding of a hydrophobic fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS), to GDH revealed that the binding of ANS to GDH is increased in the presence of octyl glucoside, a finding that may be interpreted in terms of the increment of surface hydrophobic patch(es) of GDH because of its binding to octyl glucoside. Fluorescence energy transfer studies also showed more binding of the reduced coenzyme (NADH) to GDH and the Lineweaver-Burk plots (with respect to NADH) indicate the existence of substrate inhibition in the presence of octyl glucoside. These observations are aimed at explaining the formation of the molten globule-like structure of GDH, which is induced by a non-ionic detergent such as octyl glucoside.

Key words: bovine liver glutamate dehydrogenase, mild denaturation, molten globule state, octyl glucoside.

The binding of small molecules to proteins is biologically important because this type of interaction (ligand binding) not only alters the structure of proteins but also modulates both the functions and properties of proteins. Among different types of ligand binding, the interaction of detergents with proteins has attracted the attention of a number of investigators (*1–4*)

Generally, among classes of detergents, cationic and anionic detergents are considered to possess denaturing properties (*5, 6*). In contrast to both cationic and anionic detergents, non-ionic detergents lack a denaturing quality, and, as a result, they have been widely used for extracting and purifying membrane proteins without concern over the disruption of innate structures or changes in the physiological functions of proteins (*7–11*).

Indeed many researchers have used octyl glucoside (*12–14*) as a non-ionic detergent in the membrane protein biochemistry field since its first application by Baron and Thompson (*15*). Octyl glucoside, as a non-ionic detergent, is considered to have several advantages over other non-ionic detergents. For example, it is reported that octyl glucoside has (i) a higher solubility power, (ii) no denaturing effect on proteins, (iii) a high critical micelle concentration (CMC), (iv) optical transparency, and (v) no effect on any other assays such as (a) protein assays, (b) rapid removal by dialysis, and (c) no effect on the catalytic activities of enzymes (*13, 16*). In spite of the described advantages of octyl glucoside as a detergent, there are, however, some other reports that emphasize the inability of octyl glucoside to allow solubilized membrane proteins to be prepared in an active form (*17*).

Bovine liver GDH [L-glutamate: NAD-(p)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is composed of six identical subunits (*18*) with a molecular mass of 336,000 Daltons (*19*). This enzyme occurs in the matrix part of mitochondria as a soluble enzyme where it catalyzes the reversible reductive amination of α-ketoglutarate to L-glutamate using ammonia as the source of amine and NAD(H) as the coenzyme (*20*). It has been shown that GDH can associate reversibly with inner mitochondrial membranes (*21*), and that this binding is accompanied by inactivation of the related enzyme (*20*). The binding of GDH to phospholipids is also cited in the literature (*22–26*).

In this study, we have investigated binding and the structural aspects of the interaction between GDH and octyl glucoside by means of physical techniques in order to elucidate the molten globule-like state *via* the interaction between GDH and octyl glucoside as a non-ionic detergent.

MATERIALS AND METHODS

Anthrone, thiourea, octyl glucoside, NADH, ANS, and α-ketoglutarate were obtained from Sigma. NH_4Cl and EDTA

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were purchased from Merck. Visking membrane dialysis tubing (MW cut-off. 10,000–14,000) was obtained from Scientific Instrument Center (SIC, East Leigh, Hampshire, UK). Bovine liver GDH was obtained as a solution in 50% glycerol (Type II) from Boehringer Mannheim. All other materials were of analytical grade, and solutions were prepared in doubly distilled water. Sodium phosphate (50 mM), pH 7.6, was used as a buffer. For all other experiments (except enzyme assays) prior to use, the enzyme solution was dialyzed at 4°C for 24 h with several changes of buffer, then centrifuged for 20 min at 15,000 rpm to remove any precipitate. Enzyme and NADH concentrations were measured spectrophotometrically using E_{280} = 0.97 mg^{-1} ml cm^{-1} (*27*) and E_{340} = 6.22 mM^{-1} cm^{-1} (*28*), respectively. All measurements reported in this paper refer to octyl glucoside concentrations below its CMC.

Equilibrium Dialysis—Equilibrium dialysis experiments were carried out to determine the concentrations of free and related octyl glucoside molecules at 27 and 37°C, respectively. The experiments were performed using dialysis bags as the semipermeable membrane. Visking tubes were boiled three times for 15 min each in EDTA and sodium bicarbonate, and stored in 20% ethanol. Aliquots of GDH solution (2 ml) at a concentration of 0.01 (w/v) were dialyzed against 2 ml aliquots of octyl glucoside solutions at different concentrations At equilibrium, the free concentration of octyl glucoside was assayed using the anthrone method (*29*).

CD Measurements—CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) at protein concentrations of 0.3 and 2.7 mg/ml for far-UV and near-UV, respectively The results are expressed as molar ellipticity, [θ] (deg cm^2 $dmol^{-1}$), based on mean amino acid residue weight (MRW). The molar ellipticity was determined to be $[\theta]_\lambda = (\theta \times 100\ MRW)/(cl)$, where c is the protein concentration in mg/ml, l is the light pathlength in cm, and θ is the measured ellipticity in degrees at a given wavelength. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291}$ = 7,820 deg cm^2 $dmol^{-1}$ (*30*), and with JASCO standard nonhygroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5}$ = 7,910 deg cm^2 $dmol^{-1}$ (*31*). Noise in the data was smoothed using JASCO J-715 software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without peak shape distortion.

GDH Assays—The enzyme activity was determined from the decrement in the absorption at 340 nm following the oxidation of NADH at 27°C using a Shimadzu UV-3100 spectrophotometer with jacketed cell holders. The temperature was regulated by an external thermostated water circulator within ±0.05°C. The assays were carried out in 50 mM sodium phosphate buffer, pH 7.6, in the presence of 5 mM α-ketoglutarate, 50 mM NH_4Cl, 0.2 mM EDTA, and 0.1 mM NADH except where stated otherwise. The enzymatic reaction was started by the addition of 5 μl of diluted enzyme (in 50 mM sodium phosphate buffer, pH 7.6) to 1,040 μl assay mixture with the composition described above. Every experiment was repeated three times

Fluorescence Measurements—Fluorescence intensity measurements were carried out on a Hitachi spectrofluorimeter model MPF-4. The binding of a fluorescent dye, ANS, to GDH was monitored by exciting the ANS at 350 nm and recording the emission spectra in the range of 400–650 nm. In fluorescence energy transfer measurements, GDH was excited at 292 nm (excitation at this wavelength causes the least photodecomposition of the enzyme) and the NADH emission peak was recorded at 452 nm. All slit widths were 10 nm and all experiments were performed at 27°C. The protein concentration in the fluorescence studies was 0.05 mg/ml.

RESULTS AND DISCUSSION

Figure 1 shows the number of octyl glucoside molecules bound per molecule of GDH, $\bar{\nu}$, as a function of the free octyl glucoside concentration, $[S]_{free}$, as measured by equilibrium dialysis at 27 and 37°C. Binding isotherm curves initially rise steeply, reaching plateau-like regions, followed by the appearance of very steep regions. This observation suggests that a degree of saturation is reached during the initial binding as the subsequent binding begins. It is well established that the binding of ionic surfactants to proteins involves the initial binding of the ionic surfactant to charged groups or the opposite sign on the protein, followed by more extensive hydrophobic binding as the surfactant concentration increases (*32, 33*) Surprisingly, we observed that octyl glucoside, a non-ionic surfactant, which has a polar head group and a relatively short hydrophobic tail (8 carbon atoms), binds to a protein in a similar manner as ionic surfactants Of course, it may not be unreasonable to point out that the polar head group of octyl glucoside (*i.e.* the glucoside residue) has four free alcoholic hydroxyl groups and two etheric oxygen atoms. Consequently, octyl glucoside, because of its relatively high polarity, binds easily to both polar and ionic residues of the surface of the protein through dipole-dipole and dipole-ionic interactions. Eventually, preliminary binding is followed by a more extensive interaction of octyl glucoside through hydrophobic interaction by means of its hydrophobic tails, resulting in conformational changes (as will be discussed later).

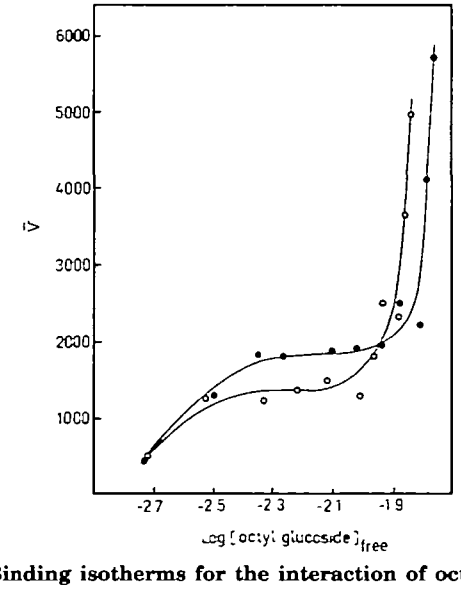


Fig. 1 **Binding isotherms for the interaction of octyl glucoside with bovine liver GDH in 50 mM sodium phosphate buffer, pH 7.6.** The GDH concentration is 0.1 mg/ml. The figure shows Scatchard plots for the binding of octyl glucoside to GDH (●) 27°C and (○) 37°C

The calculation of the Gibbs energy of binding as applied to the entire binding isotherm is based on the Wyman binding potential concept (*34*). The binding potential is calculated from the area under the binding isotherms according to Eq. 1:

$$\pi = RT\int_{\bar{v}_i=0}^{\bar{v}_i} \bar{v}\,d(\ln[S]_{free}) \quad (1)$$

and is related to the apparent binding constant, K_{app}, as follows (*35*):

$$\pi = RT\ln(1+K_{app}[S]_{free}^{\bar{v}_i}) \quad (2)$$

Values of K_{app} were determined by applying of Eqs 1 and 2, and used to determine the values of Gibbs free energy (ΔG):

$$\Delta G = -RT\ln K_{app} \quad (3)$$

The Gibbs free energy of binding per mole of octyl glucoside, $\Delta G_{\bar{v}}$, at any specified $\bar{v}$ can be determined by equation 4.

$$\Delta G_{\bar{v}} = \frac{\Delta G}{\bar{v}} \quad (4)$$

Figure 2 shows the variation of $\Delta G_{\bar{v}}$ as a function of the total concentration of octyl glucoside The shapes of these curves show the initial high energy binding of the ionic or polar sites on the surface of the protein. The gradual decrease in binding energy indicates the characteristic of hydrophobic binding (*36–38*). This decrement is mostly observed for octyl glucoside at the concentrations above 5 mM, indicating that the binding of detergent to GDH in the designated concentration range occurs predominantly through hydrophobic interactions. In addition, there is a considerable temperature dependency of $\Delta G_{\bar{v}}$ because the decrement of binding energy at 37°C is greater than the energy at 27°C. This finding is perhaps due to the hydrophobic nature of the binding, especially in the second half of the profiles.

In order to investigate the effect of such high level binding of octyl glucoside on the catalytic activity of GDH, enzymatic assays were performed in the presence of various concentrations of octyl glucoside (Fig. 3). As shown in this plot, octyl glucoside inhibits GDH, and causes nearly 80% inhibition at 20 mM. This result contradicts the previous reports that octyl glucoside lacks the ability to affect the enzymatic activities of different enzymes (*13, 16*).

The high binding of octyl glucoside to GDH and the resulting inhibition of enzymatic activity led us to investigate the resultant conformational changes in GDH. For this purpose, CD experiments were carried out. The far and near-UV CD spectra of GDH in the presence of various concentrations of octyl glucoside are shown in Figs 4 and 5,

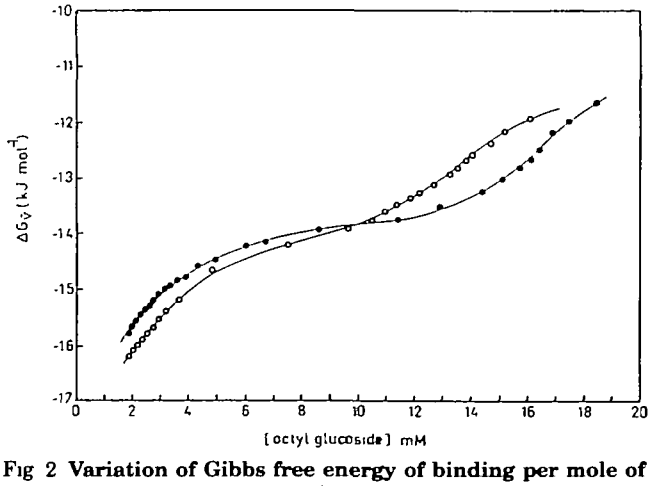


Fig 2 **Variation of Gibbs free energy of binding per mole of octyl glucoside upon interaction with GDH as a function of total concentration of octyl glucoside (●) 27°C and (○) 37°C.** The figure presents the variation of binding enthalpy per mole of octyl glucoside as a function of $\bar{v}$ at 27°C Other conditions are as described in the legend to Fig. 1

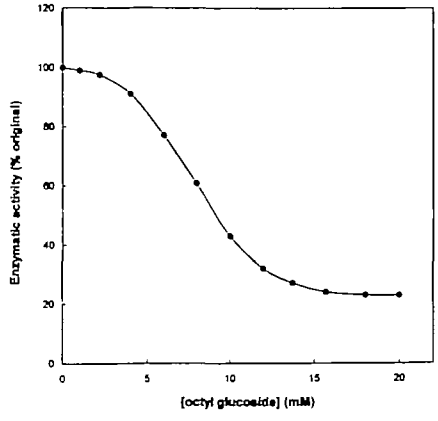


Fig. 3 **Variation of the enzymatic activity of GDH as a percent of the original value in the presence of various concentrations of octyl glucoside.** The assays were carried out in 50 mM sodium phosphate buffer, pH 7 6, in the presence of 5 mM α-ketoglutarate, 50 mM NH_4Cl, 0.2 mM EDTA, and 0 1 mM NADH at 27°C The enzyme concentration in the assay mixture was 0 36 nM

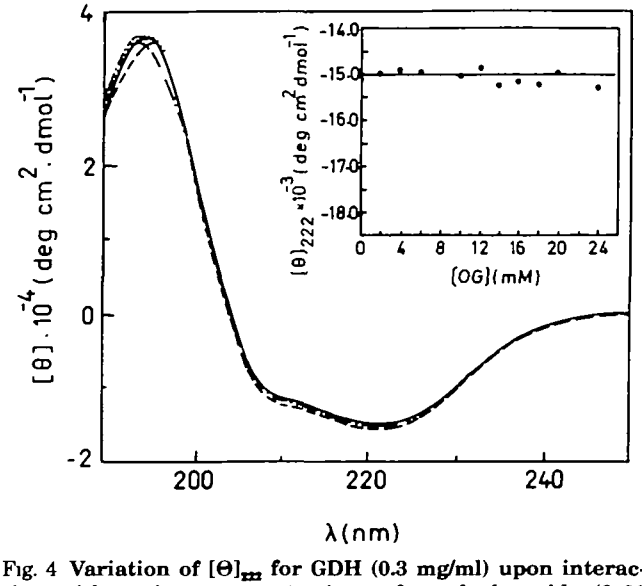
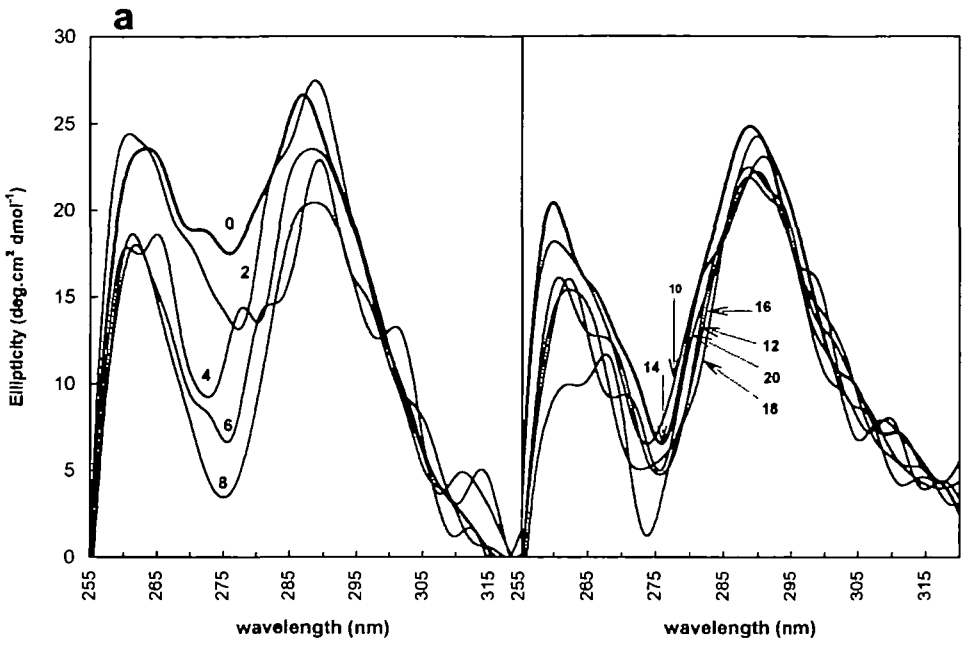


Fig. 4 **Variation of $[\Theta]_{222}$ for GDH (0.3 mg/ml) upon interaction with various concentrations of octyl glucoside (0–20 mM) in 50 mM sodium phosphate buffer, at pH 7.6.** It can be seen that increasing the detergent concentration does not cause any significant alterations in the α-helix content of the protein in the concentration range of 0–20 mM octyl glucoside. Inset: Far-UV CD spectra of GDH (0 3 mg/ml) at various concentrations of octyl glucoside (0–20 mM) in 50 mM sodium phosphate buffer, pH 7.6. The inset shows no changes in the secondary structure upon treatment with various concentrations of octyl glucoside.



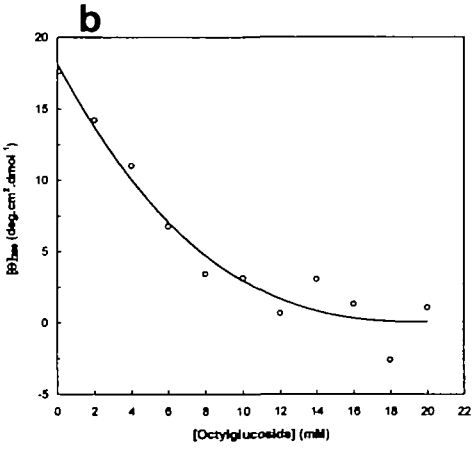


Fig 5. **a: Near-UV CD ellipticity of GDH (2.75 mg/ml) at 280 nm ($[\theta]_{280}$) *vs.* various concentrations of octyl glucoside between 0–20 mM in 50 mM sodium phosphate buffer, pH 7.6. b: Decrease in the near-UV CD ellipticity ($[\theta]_{280}$) of GDH (2.75 mg/ml) with increasing concentrations of octyl glucoside (0–20 mM).** The figure emphasizes the disappearance of some tertiary structure of GDH due to octyl glucoside binding

respectively. In Fig. 4, the apparent shapes of all peaks are identical and the peaks of the α-helix secondary structure show two minima at 208 and 222 nm, and one maximum at 195 nm. These profiles indicate that the extent of α-helix is the same in all spectra. CD spectra in the far UV region (inset in Fig. 4) show no changes in the secondary structure upon treatment with various concentrations of octyl glucoside. The local structure and environment of the side chains of the aromatic amino acids may be judged from their near-UV CD spectra. Each subunit of bovine liver GDH has 18 tyrosine, 23 phenylalanine, and 3 tryptophan residues (*39*). Because of these residues, GDH has characteristic peaks in the near-UV spectra appearing in the 275–282, 255–270, and 290–305 nm regions, respectively. As observed in Fig. 5, a and b, increases in the octyl glucoside concentration cause a decrease in the ellipticity at 280 nm, which reflects a loss of tertiary structure of the native state of the protein (*40*). It is inferred that tyrosine residues in the octyl glucoside-incubated samples, are in different conformational states as observed by differences between $[\theta]_{280}$ of the samples. Since the negative ellipiticity at 280 nm has been ascribed to free tyrosine in the solvent (*41*), the change in the positive ellipticity at 280 nm to negative values represents greater flexibility of the GDH structure in the presence of octyl glucoside.

It seems that GDH retains its secondary structure in the presence of octyl glucoside but loses a degree of tertiary structure by acquiring a more extended tertiary structure. The situation described above may coincide with a definition of the molten globule state: a "compact globule with native-like secondary structure with slowly fluctuating tertiary structure" (*42, 43*). Thus, it seems that octyl glucoside induces the molten globule-like structure in bovine liver GDH. The molten globule is an intermediate state between the native and fully unfolded states of proteins. In fact, this finding is observed for most proteins during their acid-induced unfolding stages (*42, 44*), during urea or guanidine hydrochloride denaturation (*45*), in the course of thermal denaturation (*43*), as well as in the transient intermediates that are formed during protein folding *in vivo* (*46*). However, we could find no other reports in the literature addressing the induction of a molten globule state by a non-ionic detergent. The absence of a rigid tertiary structure in the molten globule state results in some internal nonpolar groups becoming exposed to the solvent, thus making the surface of the protein more hydrophobic than in the native state (*47*).

Accordingly, the molten globule state, because of its increased surface hydrophobicity, binds nonpolar molecules in solution more strongly than the native protein. The binding of the hydrophobic fluorescence probe, ANS, to the molten globule state is an easy test with which to prove the manifestation of the described state. Since this probe binds to solvent-accessible clusters of nonpolar groups in the native state (*48*), it can also bind more strongly to the molten globule (*49, 50*).

The binding of ANS to GDH in the presence of various concentrations of octyl glucoside was investigated by spectrofluorimetry, as illustrated in Fig. 6. It is clear that the interaction between ANS and the native state of GDH is

accompanied by fluorescence enhancement as well as by a considerable blue shift in λ_{max}. It is known that GDH has at least one hydrophobic patch, which serves as a part of its binding site for the reduced nicotinamide ring (*20*). Thus, it seems that ANS binds to this hydrophobic patch in the native state of the protein (as judged by the fluorescence enhancement), and the presence of octyl glucoside induces conformational changes in the GDH tertiary structure, which extends its hydrophobic patch(es); consequently, ANS binding is promoted (Fig. 6). In addition, in order to investigate the probable dissociation of GDH into its subunits, polyacrylamide gel electrophoresis was carried out in the presence of various concentrations of octyl glucoside, but no dissociation was found (data not shown).

It may be reasonable to assume that if the NADH binding site of GDH becomes extended because of its interaction with octyl glucoside, then more binding of NADH with its binding site in the GDH structure will occur in the presence of octyl glucoside molecules. To explore the effect of octyl glucoside on the NADH binding properties of GDH, fluorescence energy transfer experiments were carried out by exciting GDH and recording the NADH fluorescence emission spectra. It is known that the emission of NADH increases upon binding to proteins. Figure 7 shows the effect of octyl glucoside molecules on the binding of NADH to GDH. As shown in these profiles, the fluorescence intensity of NADH increases at a higher concentration of NADH and ultimately reaches a plateau-like region, which reflects the saturation of the NADH binding site Octyl glucoside shifts the fluorescence intensity to higher values, and more binding of NADH to GDH in the presence of octyl glucoside molecules is clearly shown.

In order to clarify the inhibition pattern of GDH upon interaction with octyl glucoside molecules, the rate of the enzymatic reaction at various (the more hydrophobic) substrate concentrations was measured. Figure 8 shows the double reciprocal plots of the enzymatic rate at various concentrations of NADH. As is clear in this figure, octyl glucoside induces an extense substrate inhibition of GDH. It is well established that there are two binding sites for NADH on each GDH subunit (*51*), and that NADH has different binding affinities for each site (*52, 53*). The reduced coenzyme binds first to site I (active site), and at higher concentrations of NADH (greater than 0.1 mM), it binds to site II (non-active or regulatory site) (*53, 54*). It seems that the concentration does not exceed 0.1 mM for the enzymatic assays at various concentrations of reduced coenzyme. The

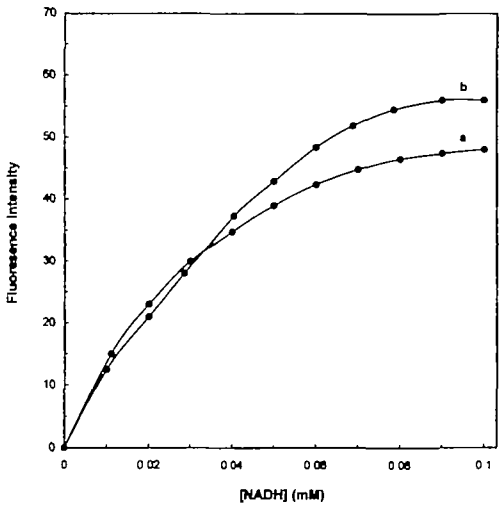


Fig 7 **Variation of fluorescence intensity with NADH concentration in the absence of octyl glucoside (a) and in the presence of 20 mM octyl glucoside (b).** The fluorescence energy transfer studies were carried out by exciting the enzyme at 292 nm and recording NADH emission at the maximum emission value. The GDH concentration was 0 05 mg/ml. All experiments were done in 50 mM sodium phosphate buffer, pH 7.6 at 27°C

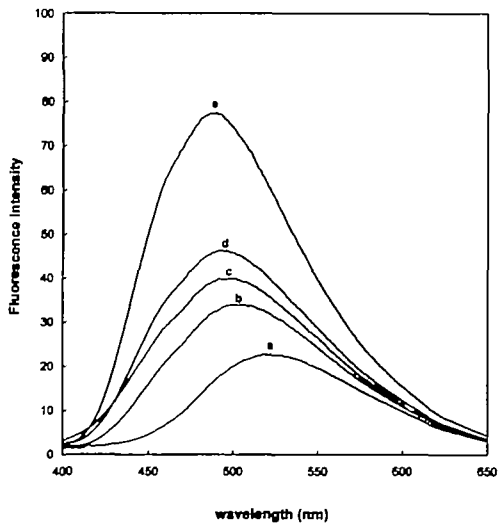


Fig. 6. **The fluorescence emission spectra of: (a) ANS; (b) ANS + GDH; (c) ANS + GDH + 5 mM octyl glucoside; (d) ANS + GDH + 10 mM octyl glucoside; and (e) ANS + GDH + 20 mM octyl glucoside.** The concentrations of ANS and GDH are 50 μM and 0 05 mg/ml, respectively The excitation wavelength was 350 nm All experiments were done in 50 mM sodium phosphate buffer, pH 7 6, at 27°C

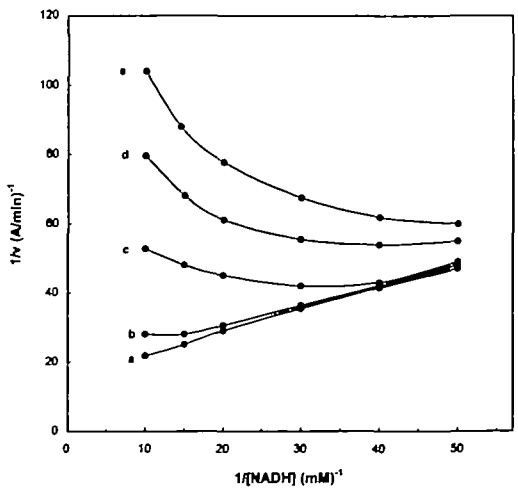


Fig. 8 **Double reciprocal plots of the reaction catalyzed by GDH using various concentrations of NADH in the presence of various concentrations of octyl glucoside: (a) 0 mM; (b) 5 mM; (c) 10 mM; (d) 15 mM; and (e) 20 mM.** The assays were carried out in 50 mM sodium phosphate buffer, pH 7.6, in the presence of 5 mM α-ketoglutarate, 50 mM NH_4Cl, and 0.2 mM EDTA at 27°C The enzyme concentration in the assay mixture was 0 36 nM

presence of octyl glucoside (as judged by ANS binding results) induces conformational changes in the tertiary structure of GDH, which lead to the extension of hydrophobic patch(es) on the surface of the enzyme. Therefore, NADH binds easily to its regulatory site, which is located on the surface of hydrophobic patch(es), even at concentrations below the threshold value (0.1 mM). It is important to note that octyl glucoside exhibits a rather weak substrate inhibitory power at a concentration of 5 mM, whereas its inhibition quality increases extensively at concentrations of 10, 15, and 20 mM.

In conclusion, it appears that octyl glucoside molecules that bind to GDH lower the dielectric constant of water where the concealed or semi-concealed hydrophobic clusters of amino acid side chains are located, allowing them to become exposed partly to the solvent, resulting in an increase in the surface hydrophobicity of GDH. Such a condition coincides with the molten globule or the compact denatured state, in which the secondary structure is retained but with a flexible tertiary structure. Therefore, it is reasonable to state that octyl glucoside is a mild denaturant detergent instead of a non-denaturing one, at least in the case of GDH.

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