Interaction of a series of fluorescent probes with glucose oxidase

Liu Peng, Yegor Domanov, Valeriya Ioffe, Alexander Finashin and Galyna Gorbenko*

Department of Biological and Medical Physics, V.N. Karazin Kharkiv National University, 4 Svobody Sq., 61077 Kharkiv, Ukraine

The fluorescence of a family of cationic styrylpyridinium probes, 4-p-(dimethylaminostyryl)-1-methylpyridinium (DSM), 4-p-(dimethylaminostyryl)-1-hexylpyridinium (DSP-6), and 4-p-(dimethylaminostyryl)-1-dodecylpyridinium (DSP-12) increases dramatically upon association with glucose oxidase (GOx) and exhibits blue shifts (up to 30 nm) of the emission maxima. Enhancement in fluorescence has also been observed for another, neutral, probe (DMC) with a small spectral shift in the presence of GOx. Based on the fluorescent spectroscopy data, binding parameters (number of binding sites and equilibrium dissociation constant or partition coefficient) characterizing the probe-protein interactions have been determined. Binding data obtained with styrylpyridinium probes having hydrophobic tails of different lengths as well as the effective binding observed for neutral DMC suggest that hydrophobic interactions play a predominant rôle in the probes' non-covalent binding to GOx.

Keywords: binding parameters, fluorescent probe-protein interaction, glucose oxidase, polarity

1. INTRODUCTION

Much effort has been devoted to study the mechanism of binding between ligands and macromolecules, particularly between ligands and proteins. Due to its high sensitivity and relative ease of use, fluorescence spectroscopy is recognized to be one of the most suitable techniques to study the interactions of ligands with micro-heterogeneous environments including proteins [1–4]. Two styrylpyridinium derivatives, 4-p-(dimethylaminostyryl)-1-vinylbenzylpyridinium chloride and 4-p-(dimethylaminostyryl)-1-phenethylpyridinium bromide, possessing donor and acceptor groups, have been employed to study the features of the microenvironments of DNA and bovine serum albumin (BSA) because of their environmentally sensitive fluorescence from an intramolecular charge-transfer (ICT) excited state [5]. Mallick and co-workers [4] used the polarity sensitive fluorescence probe 3-acetyl-4-oxo-6,7-dihydro-12H indolo-[2,3-a]quinolizine to determine the polarity of the probe environment in BSA.

This knowledge is of great relevance to a variety of applications, and in particular to protein-based biosensors. Fluorescence has been widely used in sensing applications due to its simplicity and sensitivity. An affinity optical sensor for glucose based on the competitive binding of glucose and fluorescein-labelled dextran to concanavalin A was proposed in [6], and 8-anilino-l-naphthalenesulfonic acid (ANS) binding to apo-glucose oxidase has been helpful in designing a glucose sensor [7]. The development of synthetic glucose probes remains an important goal for biomedical research [8]. Therefore, in order to test the possibility of using non-covalently bound fluorescent probes for signal transduction in GOx-based biosensors we studied the binding mechanism of a family of cationic styrylpyridinium probes, 4-p-(dimethylaminostyryl)-1-methylpyridinium (DSM), 4-p-(dimethylaminostyryl)-1-hexylpyridinium (DSP-6), and 4-p-(dimethylaminostyryl)-1-dodecylpyridinium (DSP-12) and another uncharged probe, 4-dimethylaminochalcone (DMC) to GOx.

Glucose oxidase (GOx) is a widely studied enzyme showing a high degree of specificity for β-D-glucose. The holoenzyme consists of two identical subunits, and every monomer folds into two structural domains, one binding cofactor FAD covalently and the other being involved in substrate binding [9].

2. MATERIALS AND METHODS

2.1. Materials

Glucose oxidase from *Aspergillus niger* (185 units/mg) was purchased from Fluka (Switzerland). DSM, DSP-6, DSP-12, and DMC were obtained from Zonde (Latvia). D-glucose was from Sigma (Germany).

2.2. Spectroscopic measurements

The measurements were performed in 5 mM sodium-phosphate buffer, pH 7.4, at room temperature. Glucose oxidase (dimer) concentration was determined spectrophotometrically, using the extinction coefficient $\varepsilon_{450} = 2.8 \times 10^4 M^{-1} cm^{-1}$. Glucose oxidase enzymatic activity

*Corresponding author. E-mail: galyna.p.gorbenko@univer.kharkov.ua

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was determined using an oxygen electrode at 37 °C and pH 7.4. A stock solution of glucose (300 mM) was left to mutarotate overnight. The fluorescent spectra of probes were recorded with a CM 2203 combined spectrofluorometer/spectrophotometer (SOLAR, Belarus). Excitation and emission slit widths were set at 7.5 nm and 5 nm respectively.

3. RESULTS AND DISCUSSION

3.1. DSM, DSP-6 and DSP-12

The fluorescence emission spectra of DSM and DSP-6 are similar in buffer, while that of DSP-12 has very low fluorescence intensity. This may be attributed to the poor water solubility of DSP-12, which carries a long non-polar alkyl chain attached to the pyridinium nitrogen (Fig. 1C), resulting in the formation of probe aggregates (micelles) and fluorescence self-quenching caused by the collisions of the aminostyryl pyridinium fluorophore head groups. However, as shown in Table 1, similar spectral peak positions of the three probes indicate that the electronic transition of the aminostyryl pyridinium chromophore is not perturbed significantly by the length of the alkyl chain [10].

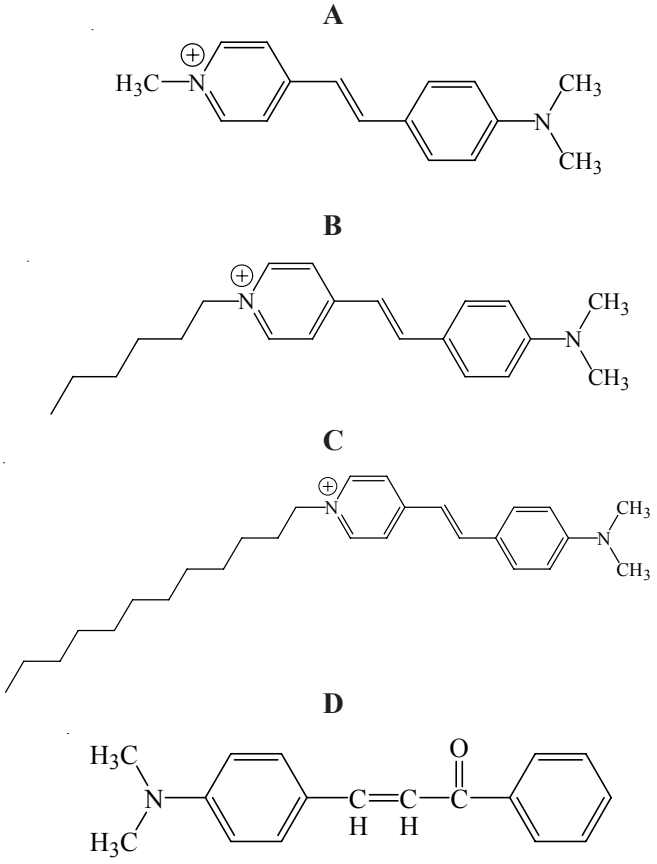

Figure 1. Structures of DSM (A), DSP-6 (B), DSP-12 (C) and DMC (D).

Addition of GOx to the probe in buffer solution leads to enhancement of fluorescence intensity and blue shifts of the emission maxima. The observed effects reflect binding of these probes to the protein, leading to a change of the fluorophore microenvironment. As seen from Table 1, the probe spectral shifts increase in the order DSM → DSP-6 → DSP-12, and are indicative of the polarity decrease. Since it is well known that protein polarity drops from the hydrated shell to the hydrophobic core, this observation suggests that the depth of fluorophore penetration into the protein interior also increases in the above order. This can be rationalized in terms of enhanced hydrophobic interactions between the protein and hydrocarbon chains of increasing length. Due to stronger hydrophobic interactions, derivatives with longer chains are pulled deeper into the protein hydrophobic interior.

Table 1. Fluorescence parameters of DSM, DSP-6 and DSP-12 in buffer and GOx solution.

Probe	λ_{max} / nm [a]		$\frac{F_{max}(GOx)}{F_{max}(buffer)}$ [b]
	buffer	GOx	
DSM	617	610	1.6
DSP-6	619	605	2.4
DSP-12	620	599	14.2

[a] Position of fluorescence maximum.
[b] Ratio of maximum fluorescence intensities.

The fluorescence intensity enhancements of the probes DSM, DSP-6 and DSP-12, observed upon their binding to GOx, are plotted in Fig. 2 as functions of probe (panel A) or GOx (panel B) concentration. The concentration dependences of the fluorescence intensity increase can be used to calculate the number of binding sites and equilibrium dissociation constant.

The fluorescence intensity increase ΔF was assumed to be proportional to the concentration of the protein-bound probe Z_b, i.e.

$$\Delta F = a\, Z_b \qquad (1)$$

where a is the molar fluorescence (proportional to the quantum yield of the bound probe). In the case of GOx titration with probe (direct titration), ΔF is given by:

$$\Delta F_i = F_i - F_i^{(0)} \qquad (2)$$

and in the case of probe titration with GOx (inverse titration):

$$\Delta F = F_i r_{Z_i} - F_0 \qquad (3)$$

where F_i and $F_i^{(0)}$ are the fluorescence intensities of the probe after the ith titration step in GOx solution and in buffer respectively, F_0 is the initial fluorescence intensity

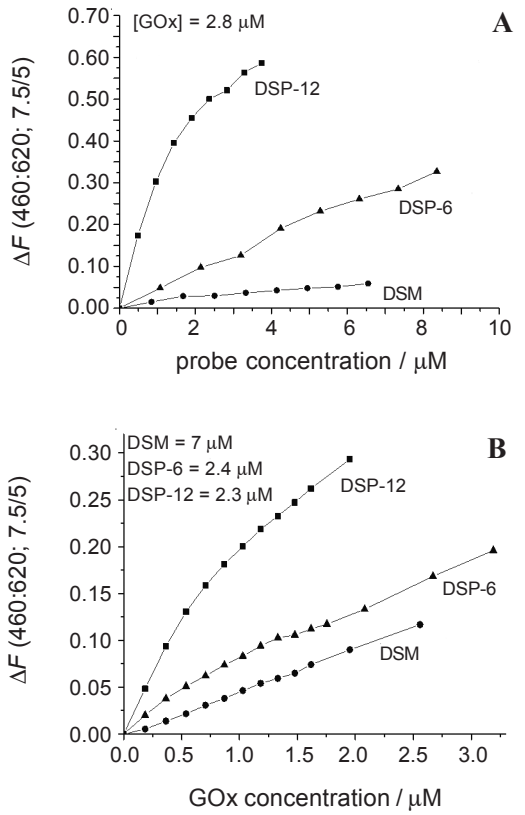

Figure 2. Fluorescence intensity of DSM, DSP-6, and DSP-12 as a function of the concentrations of probes (A) and GOx (B) in buffer, pH = 7.4.

prior to titration, and r_{z_i} denotes the probe dilution coefficient after the *i*th titration step.

It is assumed that the probe molecules noncompetitively and simultaneously bind to the GOx molecules and that there exists one type of binding site on the GOx molecule. Based on this simplest model, the dissociation constant K_d can be expressed by

$$K_d = \frac{(Z_i - Z_{b_i})(P_i n - Z_{b_i})}{Z_{b_i}} \quad (4)$$

where P_i and Z_i denote the concentrations of GOx and probe, respectively, after the *i*th titration, and n is the number of binding sites on the GOx molecule. Rearrangement of eqn (4) results in:

$$Z_{b_i} = \frac{Z_i + P_i n + K_d \pm \sqrt{(Z_i + P_i n + K_d)^2 - 4 Z_i P_i n}}{2}. \quad (5)$$

The Z_{b_i} value obtained by taking the positive square root yields $Z_{b_i} > P_i$, therefore

$$Z_{b_i} = \frac{Z_i + P_i n + K_d - \sqrt{(Z_i + P_i n + K_d)^2 - 4 Z_i P_i n}}{2}. \quad (6)$$

In the case of direct titration, substitution of Z_i and ΔF_i by x and y, respectively, and combination of eqns (2) and (6) yields:

$$y = 0.5a\left[x + nP_0\left(1 - \frac{x}{Z_{conc}}\right) + K_d - \sqrt{\left(x + nP_0\left(1 - \frac{x}{Z_{conc}}\right) + K_d\right)^2 - 4xnP_0\left(1 - \frac{x}{Z_{conc}}\right)}\right] \quad (7)$$

where Z_{conc} is the concentration of the probe stock solution, P_0 denotes the initial concentration of GOx before beginning the titration with probes, and $r_{p_i} = 1 - \frac{x}{Z_{conc}}$ is the protein dilution coefficient after the *i*th titration step.

Analogously in the case of the inverse titration, by substituting P_i and ΔF_i by x and y respectively, and combining eqns (3) and (6) one obtains:

$$y = \frac{a}{2}\frac{P_G}{P_G - x}\left[Z_0\frac{P_G - x}{P_G} + xn + K_d - \sqrt{\left(Z_0\frac{P_G - x}{P_G} + xn + K_d\right)^2 - 4Z_0\frac{P_G - x}{P_G}xn}\right] \quad (8)$$

where P_G is the concentration of the GOx stock solution, Z_0 denotes the initial probe concentration before beginning the titration with GOx, and $r_{z_i} = \frac{P_G}{P_G - x}$ is the probe dilution coefficient after the *i*th titration step.

The probe-GOx binding parameters for DSM, DSP-6 and DSP-12 were determined by simultaneous fitting to the direct and reverse experimental titration data (Table 2).

Table 2. Probe-GOx binding parameters.

System	a / μM^{-1}	n	K_d / μM^{-1}	$\chi_{sq}(a, n, K_d)$ / 10^{-4} [a]
DSM	0.01	2.88	2.60	1.8
DSP-6	0.08	1.91	1.19	4.3
DSP-12	0.50	0.52	0.69	16.9

[a] Root mean square deviation.

As seen in Table 2, the values of the molar fluorescence *a* increase in the sequence DSM < DSP-6 < DSP-12. The explanation for this finding seems to be as follows. The main path of non-radiative deactivation of styrylpyridinium derivatives is known to involve rotation around the ethylenic double bond in the excited state associated with trans-cis photoisomerization [11, 12]. Restrictions imposed by the increased viscosity of the microenvironment of the protein-bound probe inhibit its internal motion around the ethylenic bond in the excited state, resulting in a decrease of the rate of non-radiative deactivation and an increase of the quantum yield [5]. The alkyl tails of DSP-6 or DSP-12 penetrating in the hydrophobic core of the GOx molecule interact with the protein hydrophobically, so the

probe rotations will be suppressed; moreover, the longer the alkyl chains, the stronger will they be immobilized.

The dissociation constants reveal a moderate increase in the probe affinity to GOx as the length of the hydrophobic tail increases, which further supports the conclusion about the stronger interaction of the more hydrophobic probes. On the other hand, the values for the effective binding stoichiometry (n) show a rather unexpected dependence on the length of the anchoring alkyl chain. Given that under our experimental conditions the GOx molecule is a dimer [9], one would reasonably expect values of n divisible by 2. While the values for DSM and DSP-6 are quite close to 2 (taking into account the uncertainties associated with the cross-correlation of the fitting parameters), the corresponding estimate for DSP-12 is less than unity (0.52). This fact can be explained by the strongly amphiphilic nature of DSP-12, which has a charged hydrophilic fluorophore moiety and a long hydrophobic tail. Thus, in aqueous solution these molecules will aggregate into micelles if their concentration is sufficiently high. During the titration the monomeric probe concentration will increase only up to the critical micelle concentration (CMC), while further addition of the probe will be buffered by micelle formation [13]. It can be presumed that the CMC for DSP-12 is lower than the concentration needed for complete saturation of all the binding sites available on GOx and it is manifested in the apparent decrease of the binding stoichiometry.

Since the binding stoichiometries for DSM and DSP-12 suggest that there is one binding site per GOx monomer, it was of interest to find possible locations on the GOx surface that might constitute a favourable environment for the hydrophobic binding. Using the GOx crystal structure [14; PDB entry 1CF3] and MDL Chime visualization software, the best candidate was found to be the hydrophobic cavity formed by residues Val_{125}, Phe_{126}, Ile_{394}, Met_{524}, and Met_{528}, which is sufficiently far away from the dimer interface to avoid steric hindrance. Close proximity of the negatively charged Glu_{527} provides an additional possibility for the electrostatic interaction with the pyridinium ion of the probes. Interestingly, activity assays performed in the presence of the fluorescence probes studied here have shown that the enzymatic activity is affected by the probe binding. Specifically, DSP-12, DSP-6, and DMC (the probe discussed in the next subsection) were found to activate GOx by 42%, 31%, and 27%, respectively, while DSM inhibited it by 8%. The effects of fluorophore binding on GOx enzymatic activity is also consistent with the proposed tentative binding site, since this site is located relatively close to the active site of the enzyme.

3.2. DMC

DMC is an uncharged probe that is very sensitive to its environment [15]. For DMC, the binding model described above seems to be inappropriate; the binding mechanism of this probe can be well described as a partition equilibrium, in which there is no concept of binding site on the macromolecules, which are considered to constitute a separate phase.

The partition coefficient (K) of a probe distributed between a water phase and a macromolecule phase is defined as

$$K = \frac{Z_b / V_p}{Z_f / V_w} = \frac{Z_b V_w}{Z_f V_p} \tag{9}$$

where Z_b and Z_f denote the probe concentrations in the protein and water phases respectively, and V_p and V_w denote the volumes of the protein and water phases respectively. For simplicity we assume that the value of V_w is 1 dm^3, and since $V_p \ll V_w$, we obtain

$$V_p = M V_s P_i \tag{10}$$

where M denotes the relative molecular weight of GOx, 160 kDa [9], and V_s is the partial volume of the GOx molecule (1.03 cm^3/g).

In the case of direct titration, combining eqns (1), (9) and (10) yields

$$\Delta F_i = \frac{a K M V_s Z_i P_0 \left(1 - \dfrac{Z_i}{Z_{conc}}\right)}{1 + K M V_s P_0 \left(1 - \dfrac{Z_i}{Z_{conc}}\right)}. \tag{11}$$

In the case of the inverse titration, combining eqns (2), (9) and (10) yields

$$\Delta F_i = \frac{a \dfrac{P_G}{P_G - P_i} K M V_s Z_0 P_i}{1 + K M V_s P_i}. \tag{12}$$

The DMC-GOx binding parameters were found to be $a = 0.11\ \mu M^{-1}$ and $K = 6290$ ($\chi_{sq}(a, K) = 7.04 \times 10^{-5}$) by simultaneously fitting the direct and inverse experimental titration data.

Following light absorption, an electron in the DMC molecule is transferred from the aniline ring to the carbonyl group [16]. This leads to an increase of the probe dipole moment, so that the spectral peak position of DMC becomes sensitive to the polarity of its surroundings. The fluorescence intensity increase and the spectral blue shift of DMC are weakly related, similarly to DSM, but contrary to DSP-6 or DSP-12. This suggests that the DMC molecules are distributed in the hydration shell of the protein, where the polarity is close to that of the water phase.

4. CONCLUSIONS

Pronounced changes in the fluorescence intensities and spectral maxima of a series of cationic styrylpyridinium probes in the presence of GOx suggest that the enzyme readily binds the probes via non-covalent interactions. This has been used to evaluate the binding parameters involved in the probe-GOx interactions, and to determine the nature of the interaction and the probe location in the GOx molecule. Both the affinity of styrylpyridinium probes for GOx and the depth of fluorophore penetration into the protein globule were found to increase with increasing length of the alkyl chain attached to the pyridinium nitrogen. Together with the fact that uncharged DMC also binds GOx quite effectively, these findings reveal the primary rôle of hydrophobic interactions in the probes' non-covalent binding to GOx, rather than electrostatic interactions between the cationic probes and the negatively charged surface of GOx.

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