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Tertiary structure of human α_1-acid glycoprotein (orosomucoid). Straightforward fluorescence experiments revealing the presence of a binding pocket

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Abstract—Binding of hemin to α_1-acid glycoprotein has been investigated. Hemin binds to the hydrophobic pocket of hemoproteins. The fluorescent probe 2-(*p*-toluidino)-6-naphthalenesulfonate (TNS) binds to a hydrophobic domain in α_1-acid glycoprotein with a dissociation constant equal to 60 μM. Addition of hemin to an α_1-acid glycoprotein–TNS complex induces the displacement of TNS from its binding site. At saturation (1 hemin for 1 protein) all the TNS has been displaced from its binding site. The dissociation constant of hemin–α_1-acid glycoprotein was found equal to 2 μM. Thus, TNS and hemin bind to the same hydrophobic site: the pocket of α_1-acid glycoprotein. Energy-transfer studies performed between the Trp residues of α_1-acid glycoprotein and hemin indicated that efficiency (E) of Trp fluorescence quenching was equal to 80% and the Förster distance, R_0 at which the efficiency of energy transfer is 50% was calculated to be 26 Å, revealing a very high energy transfer.

Keywords: α_1-Acid glycoprotein; Trp residues; Hemin; Förster energy transfer; Fluorescence intensity quenching

1. Introduction

α_1-Acid glycoprotein (orosomucoid), a small acute-phase glycoprotein ($M_r = 41{,}000$), consists of a chain of 183 amino acids,[1] contains 40% carbohydrate by weight and has up to 16 sialic acid residues (10–14% by weight).[2] Five heteropolysaccharide groups are linked via an *N*-glycosidic bond to the asparaginyl residues of the protein.[3]

α_1-Acid glycoprotein contains three Trp residues, one residue, Trp-160, is at the surface of the protein, and two are considered located in the protein matrix.[2–4] In the absence of any crystallographic data describing the three-dimensional structure of α_1-acid glycoprotein, we performed different fluorescence studies such as anisotropy and fluorescence intensity quenching with the Trp residues as a probe.[5–8]

These studies allowed us to suggest a three-dimensional model for α_1-acid glycoprotein. In this model, the protein would contain a pocket with two main domains, a hydrophobic domain where ligands such as progesterone and TNS can bind and a hydrophilic one formed mainly by the glycosylation site of the protein.[6,8] Although the model we have described is in good agreement with different results obtained by other authors and has been confirmed by different methods,[9–12] it is based on conclusions drawn from dynamics and structural experiments, and none of the experiments performed up to now is straightforward.

TNS binds to α_1-acid glycoprotein with a dissociation constant of 60 μM and a stoichiometry of 1:1. The nature of the binding site is hydrophobic, although contacts with a polar environment (solvent) do exist.[11]

In the present work, binding studies are performed between α_1-acid glycoprotein and hemin, a co-factor

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that binds to the hydrophobic pocket of hemoproteins.[13] If α_1-acid glycoprotein contains a hydrophobic pocket, hemin will bind to this pocket with a well-defined stoichiometry. Also, if TNS and hemin bind to the same binding site, and if the affinity of hemin to α_1-acid glycoprotein is more important than that of TNS, binding of hemin to a protein–TNS complex will remove the TNS from its binding site. In fact, our results showed that hemin binds to α_1-acid glycoprotein at the same binding site of TNS with a stoichiometry of 1:1 and a dissociation constant of 2 μM instead of 60 μM for the TNS–α_1-acid glycoprotein. This work shows for the first time straightforward evidence that supports the presence of a binding pocket in α_1-acid glycoprotein.

2. Materials and methods

2.1. Concentrations of biochemical and chemical products

α_1-Acid glycoprotein was purified as already described.[14] The lyophilized protein was dissolved in a 10 mM phosphate–0.143 M NaCl buffer, pH 7. Its concentration was determined spectrophotometrically using an extinction coefficient of 29.7 $mM^{-1}\,cm^{-1}$ at 278 nm.[15]

Potassium 2-(*p*-toluidino)-6-naphthalenesulfonate (TNS) was from Sigma Chemical Co. Its concentration was determined spectrophotometrically using an extinction coefficient of 18.9 $mM^{-1}\,cm^{-1}$ at 317 nm.[16]

Hemin, purchased from Calbiochem, was dissolved in 0.1 N NaOH and then diluted with twice-distilled water. The concentration was calculated by measuring the absorbance at 390 nm, the extinction coefficient at this wavelength being 60.3 $mM^{-1}\,cm^{-1}$.

2.2. Absorbance and fluorescence measurements

Absorbance data were obtained with a Varian DMS-100 S spectrophotometer using 1-cm pathlength cuvettes.

Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectrofluorometer. The bandwidths used for the excitation and the emission were 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Observed fluorescence intensities (either from TNS, hemin or protein tryptophan) were first corrected for the dilution, then corrections were made for the absorption at the excitation and emission wavelengths as already described.[17,18] Finally, fluorescence spectra were corrected for the background intensities of the buffer solution.

2.3. Correction of the fluorescence intensities for the optical densities at the excitation and the emission wavelengths

Titration of α_1-acid glycoprotein with hemin following fluorescence intensity decrease of TNS (Fig. 1) was corrected for the dilution and for absorption at 320 and 442 nm. At the highest concentration of hemin added (4.6 μM), the corrected intensity for dilution was 1.05 times higher than that recorded before correction. Also, at the same concentration of hemin, the intensity after correction for the inner filter effect was 1.33 higher than that obtained before correction.

At the lowest concentration of hemin (0.37 μM), the intensity after correction for the inner filter effect was 1.11 times higher than that obtained before correction.

Titration of α_1-acid glycoprotein with hemin following fluorescence Trp emission (Fig. 3) was corrected for the dilution and for absorption at 280 and 332 nm. At the highest concentration of hemin added (9.5 μM), the corrected intensity for dilution was 1.15 times higher than that recorded before correction. Also, at the same concentration of hemin, the intensity after correction for the inner filter effect was twice that obtained before correction.

At the lowest concentration of hemin (0.74 μM), the intensity after correction for the inner filter effect was 1.2 times higher than that obtained before correction.

All experiments were performed at 20 °C in a 10 mM phosphate buffer–0.143 M NaCl buffer, pH 7.

3. Results

Addition of hemin to a solution of a fixed amount of α_1-acid glycoprotein–TNS complex induces a complete abolishment of TNS fluorescence (λ_{ex} 320 nm and λ_{em}

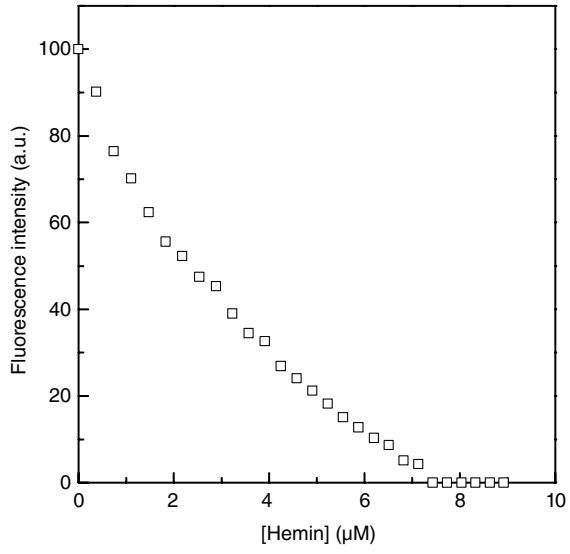

Figure 1. Displacement of TNS (4 μM) bound to 7.5 μM α_1-acid glycoprotein with hemin. λ_{ex}, 320 nm and λ_{em}, 420 nm.

420 nm) by 1 mol hemin/mol α_1-acid glycoprotein (Fig. 1). Thus, TNS and hemin bind to the same binding site on α_1-acid glycoprotein.

The decrease of TNS fluorescence intensity was analyzed by plotting the ratio of the fluorescence intensities in the absence and presence of hemin as a function of hemin concentration (Fig. 2)[17,18]

$$I_0/I = 1 + K_a\ [\text{hemin}] \quad (1)$$

where K_a is the association constant of the α_1-acid glycoprotein–hemin complex.

The value of K_a was found equal to 0.44 μM^{-1}, that is, a dissociation constant equal to 2.3 μM. Looking closely to Figure 1, one can notice that a 50% decrease in the fluorescence intensity of TNS corresponds to a concentration equal to 2.2 μM. This value is in good agreement with the dissociation constant obtained with Eq. 1 (Fig. 2).

TNS binds to α_1-acid glycoprotein on a hydrophobic site[11] with a dissociation constant of 60 μM. TNS bound to α_1-acid glycoprotein is displaced by hemin as shown by the complete abolishment of its fluorescence (Fig. 1).

Since, hemin is known to bind to the hydrophobic pockets of hemoproteins,[13] one should expect that hemin binds to the only hydrophobic site of α_1-acid glycoprotein, its pocket.

Data of Figure 1 can also be analyzed by plotting the fluorescence intensity as a function of the logarithm of added hemin concentration. This will yield a curve going from 100% to 0% TNS labeling (Fig. 3). At 50% labeling, the corresponding concentration of ligand is called

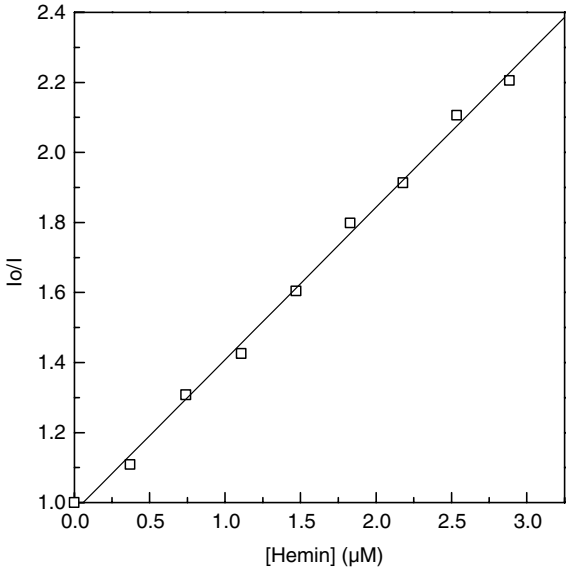

Figure 2. Plot of the fluorescence intensity decrease of TNS bound to α_1-acid glycoprotein by hemin. Data are from Figure 1. I_0 and I are the fluorescence intensities of TNS before and after addition of hemin (see Eq. 1). The dissociation constant obtained for the hemin–α_1-acid glycoprotein is equal to 2.3 μM.

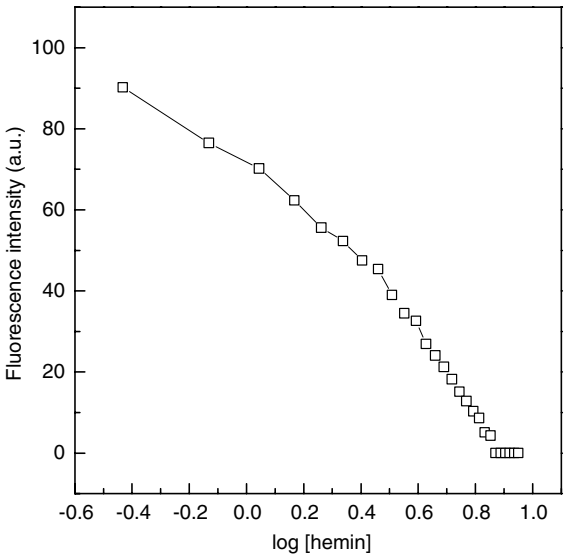

Figure 3. Semi-logarithmic plot of the displacement of TNS bound to α_1-acid glycoprotein by hemin. Data are from Figure 1.

the IC_{50} or 50% inhibitory concentration and is equal to 2.43 μM.

Cheng and Prusoff[19] derived an equation allowing one to calculate the dissociation constant (K_i) of the inhibitor–protein complex:

$$K_i = \frac{IC_{50}}{1 + L/K_d} \quad (2)$$

where in our case K_i is the dissociation constant of the hemin–α_1-acid glycoprotein complex, L the concentration of TNS (4 μM) and K_d (= 60 μM) the dissociation constant of TNS–α_1-acid glycoprotein.[11] Eq. 2 yields a value for K_i equal to 2.27 μM, a value in good agreement with that obtained from Figure 1 (K_d = 2.2 μM) or from Figure 2 (K_d = 2.3 μM).

Addition of hemin to a solution of 7.5 μM α_1-acid glycoprotein induces a decrease of the fluorescence intensities of the Trp residues (λ_{ex} = 280 nm and λ_{em} = 332 nm) (Fig. 4). Binding constant of the α_1-acid glycoprotein–hemin complex was analyzed using Eqs. (3) and (4):[11]

$$Flu = \frac{Flu_0 \times (L_0 - L_b) + Flu_1 \times L_b}{L_0} \quad (3)$$

where Flu is the observed fluorescence, Flu_0 and Flu_1 are the fluorescence of free and bound hemin, respectively, L_0 and L_b are the concentrations of total and bound hemin.

The intensity decrease is clearly hyperbolic and therefore a mathematical binding analysis can be performed using the following quadratic equation obtained from the definition of the equilibrium constant:

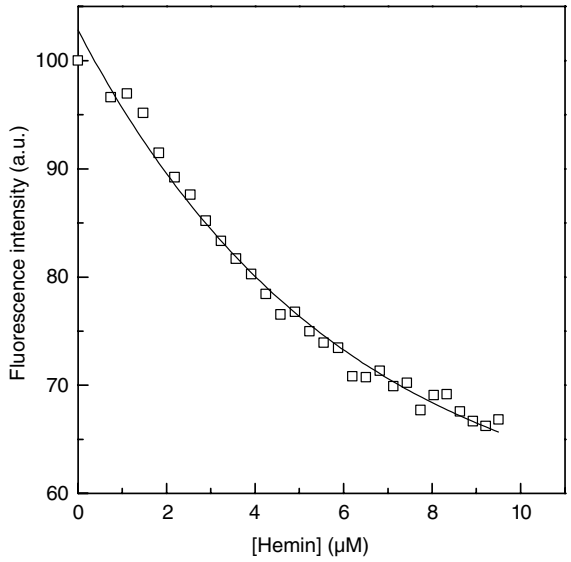

Figure 4. Titration curve of 7.5 μM of α_1-acid glycoprotein with hemin. λ_{ex}, 280 nm and λ_{em} 332 nm.

$$L_b = 0.5[(nP_0 + L_0 + K_d) - \{(P_0 + L_0 + K_d)^2 - 4nP_0L_0\}^{1/2}] \quad (4)$$

where P_0 is the protein concentration. The parameter Flu_1 was found equal to 57. The dissociation constant K_d of the α_1-acid glycoprotein–hemin complex determined from Eq. 4 is equal to 0.67 ± 0.67 μM. This value is different from that (2.3 μM) determined from Figures 1–3. The small value of K_d with its important relative uncertainty, means that the concept of calculating K_d from Eq. 4 is not correct. The reason for this comes from the fact that the decrease of the fluorescence intensity of the Trp residues in presence of hemin is the result of a high Förster energy transfer from the Trp residues to the hemin, and not only because of binding of hemin to the protein such as when TNS displacement was followed (Fig. 1) (see also Discussion).

From the overlap of the emission spectrum of Trp residues in α_1-acid glycoprotein and absorption spectrum of hemin in presence of α_1-acid glycoprotein (Fig. 5), we have calculated the overlap integral J[17,18,20]

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 \, d\lambda}{\int_0^\infty F_D(\lambda) \, d\lambda} \quad (5)$$

J was found equal to 4.33×10^{-14} M^{-1} cm^3.

The Förster distance, R_0, (in Å) at which the efficiency of energy transfer is 50% was calculated with Eq. 2:[17,18]

$$R_0 = 9.78 \times 10^3 [\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (6)$$

where κ^2 is the orientation factor ($=2/3$), n the refractive index ($=1.33$) and Q_d the average quantum yield ($=0.064$).[21] R_0 is equal to 25.6 Å. This large distance suggests the presence of an efficient energy transfer between Trp residues and hemin that occurs specifically inside the formed complex.

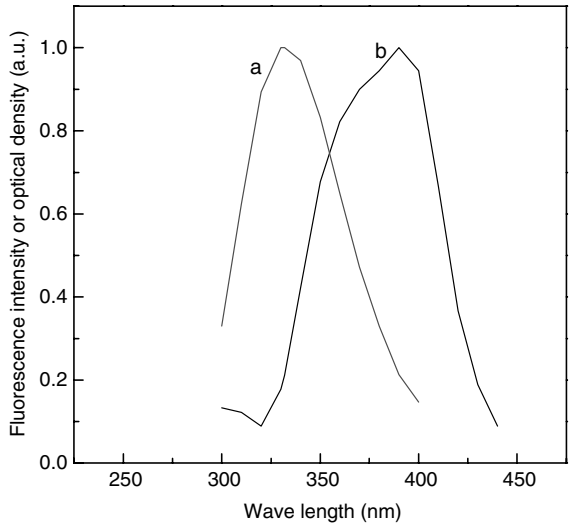

Figure 5. Spectral overlap between Trp residues fluorescence emission of α_1-acid glycoprotein (a) and absorption spectrum of hemin bound to the protein (b). Spectrum b was obtained by subtracting (protein) from (protein–hemin complex).

The efficiency of quenching (E) is equal to[22]

$$E = 1 - \frac{I}{I_0} \quad (7)$$

where I and I_0 are the fluorescence intensity in the presence and the absence of hemin.

The value of E calculated at infinite concentrations of hemin was obtained by plotting $1/E$ as a function of 1/[hemin] (Fig. 6). E was found equal to 0.80.

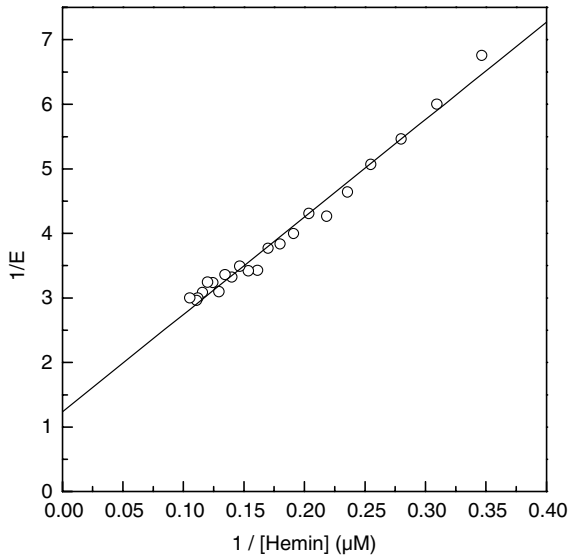

Figure 6. Determination of the efficiency of energy transfer from Trp residues of α_1-acid glycoprotein to hemin. The value of E determined at the extrapolation is equal to 0.80.

The distance that separates the donor from the acceptor was calculated with Eq. 8:[17,18,20]

$$R = R_0\left(\frac{1-E}{E}\right)^{1/6} \quad (8)$$

R is equal to 20.3 Å.

The constant rate of the energy transfer (k_t) can be calculated from Eq. 9:[17,23]

$$k_t = (1/\tau_0)(R_0/R)^6 \quad (9)$$

where τ_0 ($=2.285$ ns) is the fluorescence lifetime of Trp residues in α_1-acid glycoprotein, and k_t is equal to 2.8×10^9 s^{-1}, a value equal to 6.5 times that calculated for Trp → calcofluor energy transfer.[23]

4. Discussion

The stoichiometry of the α_1-acid glycoprotein–hemin complex (1:1) indicates that hemin binds to a specific site. Displacement of TNS by hemin gives the nature of this site: a hydrophobic one. Therefore, hemin does not bind to the surface of α_1-acid glycoprotein, a hydrophilic area. It binds to a hydrophobic domain of the pocket of the protein inducing a decrease of the fluorescence intensity of Trp residues of the protein via a Förster energy transfer mechanism.

Important energy transfer occurs between the Trp residues and hemin as it is revealed by the values of E (0.80) and R_0 (25.6 Å). It is interesting to compare these results with those obtained for the energy transfer between the Trp residues of α_1-acid glycoprotein and TNS and Calcofluor White. The two extrinsic fluorophores bind to the pocket of α_1-acid glycoprotein in two different domains, hydrophobic (case of TNS) and hydrophilic (case of Calcofluor White). Energy transfer studies between Trp residues and TNS and Calcofluor White gave values for R_0 equal to 28 Å[11] and 18 Å,[23] respectively. The very close values of R_0 for Trp → TNS and Trp → hemin energy transfer is also an indication of the fact that TNS and hemin bind to the same hydrophobic site within α_1-acid glycoptotein pocket.

Displacement of TNS by hemin (Fig. 1) is direct proof that the two chromophores bind to the same site. Also, the structural nature of hemin prohibits it from binding specifically to the surface of α_1-acid glycoprotein. In general, hemin interacts with the hydrophobic domain of the hemoprotein pocket.[13] Also, it is important to indicate that the fluorescence intensity decrease of Trp residues in presence of saturated concentrations of hemin is not equal to 100%, as is the case for TNS fluorescence decrease. Trp residues are part of the protein, and they are not displaced by the binding of hemin. Therefore, the intensity decrease of Trp residues is the result mainly of their energy transfer to the hemin. However, TNS is displaced by hemin. TNS does not fluoresce when it is free in solution, and each time a defined concentration of hemin binds to the pocket, it displaces a defined concentration of TNS. The fluorescence that results occurs from TNS still bound to the protein. Upon reaching saturation (1 hemin for 1 α_1-acid glycoprotein), all the TNS will be free in solution, and the fluorescence intensity recorded will be equal to zero. We plotted $1/E$ versus 1/[hemin] from the data of Figure 1 and found a value for E equal to 1.44 (not shown). This simply means that energy transfer between TNS and hemin does not occur since efficiency of quenching cannot be higher than 100%. TNS is simply displaced by hemin as the result of hemin binding to the same site. Therefore, the dissociation constant of the hemin–α_1-acid glycoprotein complex calculated from the data of Figure 1 is the correct one, since it was obtained from binding experiments only and not by a combination of binding and high-energy transfer phenomena.

Eq. 4 yields a value for the dissociation constant equal to 0.67 ± 0.67 μM. If this value is correct, this means that around this concentration, we should have a decrease by 50% of the fluorescence intensity of Trp residues. Looking closely to the data of Figure 4, one can notice that at 0.7 μM hemin, the intensity decrease is only 4% of the initial fluorescence intensity. Thus, this value cannot be considered as the dissociation constant for the α_1-acid glycoprotein–hemin complex.

Let us consider now the lowest fluorescence intensity value calculated by the computer with Eq. 4. This value was found equal to 57. For simplicity, let us consider it equal to 60. In other terms, the fluorescence intensity of Trp residues begins at 100 in absence of hemin and reaches 60 at saturation concentrations of hemin. Thus the decrease is equal to 40. In this case, the K_d calculated in Figure 4 corresponding to a 50% intensity decrease, and thus to an intensity equal to 80, will be equal to 4 μM. This value is twice that found when displacement of TNS was performed (Fig. 1). This difference is due simply because the intensity at saturation is not equal to 60 as generated by the computer.

Then how can we analyze the data of Figure 4? If we consider that binding of hemin to α_1-acid glycoprotein induces energy transfer from Trp residues to the heme and thus fluorescence intensity quenching of Trp residues, the decrease in the observed fluorescence intensity should stop when the stoichiometry reaches 1 mol of hemin for 1 mol of protein. The same analysis applies for TNS fluorescence intensity quenching. When we reach 1 mol of hemin for 1 mol of protein, the fluorescence intensity of TNS is equal to zero (Fig. 1). Quenching experiments in Figure 4 were performed on 7.5 μM of α_1-acid glycoprotein. Thus, saturation should be reached at 7.5 μM of hemin. The corresponding intensity is around 70. This means that the dissociation

constant of the complex will be calculated for a fluorescence intensity of 85. For this value, the plot in Figure 4 yields a K_d equal to 2.8 μM, a value close to that (2.3 μM) obtained from Figures 1 and 2 or from Eq. 2.

Thus, the decrease of the fluorescence intensity of Trp residues beyond the stoichiometry concentration is the result of a nonspecific quenching mechanism by aggregated hemin binding nonspecifically to the protein.[24]

In hemoproteins such as myoglobin and hemoglobin, the iron of the heme interacts with two histidine residues of the pocket so that stability is maintained.[13] Studies concerning hydrophobic domain of α_1-acid glycoprotein pocket do not show any histidine residue,[2,12] and thus binding of hemin to the pocket does not allow a real docking on the wall of the pocket. For this reason also, interaction between hemin and pocket of α_1-acid glycoprotein could be of a nature different from that we know in hemoproteins. This problem could be investigated in another work dealing with the properties of the amino acids of the wall of the pocket of α_1-acid glycoprotein.

The present work shows that hemin can bind to a non-hemin protein on a specific site. The presence of a pocket within α_1-acid glycoprotein facilitates this binding. The diameter of the hemin is estimated to be 12 Å.[13] Thus, the volume that hemin occupies within the α_1-acid glycoprotein pocket is approximately equal to 904 Å.[3] Considering that α_1-acid glycoprotein is approximately spherical, its radius and volume will be equal to 26 and 72,453 Å,[3] respectively. Therefore, hemin occupies a very small volume compared to that of the protein. Since the pocket of α_1-acid glycoprotein is formed by two domains, one hydrophobic and the other hydrophilic, its volume should have a size higher than the volume occupied by the hemin.

The carbohydrate residues possess a well-defined structure when bound to the protein,[25–27] and their presence in the pocket confers to them their specific structure. However, we do not know the volume occupied by the carbohydrate residues within the pocket, although some fluorescence data tend to indicate that the carbohydrate residues cover most of the interior surface of the pocket.[8] Fluorescence, X-ray and electron microscopic analysis on crystals of α_1-acid glycoprotein would reveal the structure of the protein and the carbohydrate residues.

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