

# $\beta$ -Lactoglobulin binds retinol and protoporphyrin IX at two different binding sites

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Measurement of tryptophan fluorescence quenching and the excitation energy transfer from tryptophanyl residues to the bound ligand indicates that  $\beta$ -lactoglobulin binds tightly to heme and protoporphyrin IX in a ligand-to-protein stoichiometric ratio. The apparent dissociation constants of heme- $\beta$ -lactoglobulin and protoporphyrin IX- $\beta$ -lactoglobulin complexes are  $2.5 \times 10^{-7}$  M and  $4 \times 10^{-7}$  M, respectively. The addition of  $\beta$ -lactoglobulin (final concentration = 10  $\mu$ M, phosphate buffer 50 mM, pH 7.1) to the solution containing retinol and protoporphyrin IX triggers an energy transfer between  $\beta$ -lactoglobulin tryptophan and protoporphyrin IX as well as between retinol and protoporphyrin IX. The efficiency of energy transfer depends on the distance between the donor (retinol) and the acceptor (protoporphyrin IX). Using the Förster theory, a retinol-protoporphyrin IX distance of 25 Å was calculated. These results indicate that retinol and protoporphyrin IX are bound to the  $\beta$ -lactoglobulin monomer at two different sites.

$\beta$ -Lactoglobulin; Protoporphyrin IX; Retinol; Binding site

## 1. INTRODUCTION

Living organisms make widespread use of small hydrophobic molecules such as lipids, steroids, bilins, porphyrins and retinoids. The insolubility of such molecules in water leads to specific problems of their recognition, transport and regulation in living organisms. Recent developments in structural studies of small proteins interacting with hydrophobic ligands suggest that these proteins belong to the same 'super-family' of hydrophobic molecule transporters [1]. Retinol-binding protein [2], bilin-binding protein [3], insecticyanin [4] and  $\beta$ -lactoglobulin [5,6] are the best known proteins of this class. All these proteins share a common three-dimensional structural pattern: an 8 stranded anti-parallel  $\beta$ -barrel flanked on one side by an  $\alpha$ -helix constituting a hydrophobic pocket.

It is known that, in vitro,  $\beta$ -lactoglobulin binds tightly to one retinol molecule per monomer [7]. In the case of the  $\beta$ -lactoglobulin-retinol complex, the binding site of retinol is not well characterized. It has been postulated on the basis of the structural analogies between retinol binding protein [2] and bilin binding protein [3], where ligands are clearly situated inside the main hydrophobic binding pocket formed by the  $\beta$ -barrel, that  $\beta$ -lactoglobulin binds retinol there too [5]. This assumption

has been questioned by Monaco et al. [6], who have suggested that the retinol is localized in a hydrophobic pocket situated on the external surface of the  $\beta$ -lactoglobulin molecule.

The binding of heme to some globins yields a holo-protein with remarkable properties including, in the case of hemoglobin, the capacity of the reversible and cooperative binding of a number of gaseous ligands [8]. Globins, however, are not the only proteins binding heme. Serum albumin, an abundant constituent of plasma, has one high affinity heme binding site with a dissociation constant of approximately  $2 \times 10^{-8}$  M as well as additional sites of much lower affinity [9–11].

The results of fluorescence experiments which are aimed at the elucidation of interactive properties of  $\beta$ -lactoglobulin with heme, fluorescent protoporphyrin IX and retinol are presented and discussed in this study.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Heme (Sigma) was dissolved in 0.1 M NaOH to give a stock solution of 10 mM. A stock solution (10 mM) of protoporphyrin IX (PPIX) di-sodium salt (Sigma) in methanol was prepared. In order to prevent oxidation and isomerization, retinol (Sigma) was dissolved in ethanol, under anaerobic conditions and in the dark, to give a 0.5 mM stock solution. All chemicals used were reagent grade.  $\beta$ -Lactoglobulin (BLG) variant B was obtained from homozygote cow's milk following the method of Maillart and Ribadeau-Dumas [12] and, as judged from high performance liquid chromatograms on a C<sub>18</sub> column and polyacrylamide gel electrophoresis, it was more than 95% pure. The absorbance spectra were recorded on a Cary-1 spectrophotometer (Varian). Concentrations of BLG were determined

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Abbreviations: BLG,  $\beta$ -lactoglobulin; PPIX, protoporphyrin IX

spectrophotometrically using, for calculation purposes, a molecular absorption coefficient  $\epsilon_{278} = 17\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$ .

## 2.2. Fluorescence spectroscopy

Fluorescence spectra were recorded at 20°C on an Aminco SLM 4800C spectrofluorimeter in the ratio mode. The excitation and emission slits were set at 4 nm. We have investigated tryptophan, retinol and protoporphyrin fluorescence emission spectra.

The binding of the small hydrophobic molecules was measured by following the fluorescence quenching of protein tryptophans. The following procedure was used for titration of BLG or its derivatives with various ligands: 2 ml of protein solution, approximately 10  $\mu\text{M}$ , were placed in a cuvette and small increments of 4  $\mu\text{l}$  of the ligand solution were injected with a micropipet. As the titration proceeded towards its end, larger amounts of ligand were added. The experiments were performed in 50 mM phosphate, pH 7.1.

In order to exclude the possibility of unspecific interactions of the studied ligands with tryptophan indoles in BLG, the titrations of *N*-acetyl-L-tryptophanamide solutions with an absorbancy at 287 nm equal to that of the protein were used as controls. Apparently, this compound displays a fluorescence typical to the tryptophans in proteins but, according to the obtained results, it is unable to interact with the studied compounds.

## 2.3. Determination of the apparent dissociation constants

Differences in fluorescence intensity at 332 nm between the complex and free protein (excitation at 287 nm) were monitored in order to measure the apparent dissociation constants of BLG with various ligands, i.e. hemin and protoporphyrin IX. It was assumed that the change in fluorescence depends on the amount of protein-ligand complex, and the apparent dissociation constants were determined according to Cogan et al. [13]. By plotting  $(P_0 \cdot a)$  vs  $B[a/(1-a)]$ , a straight line is obtained with an intercept of  $K'_d/n$  and a slope of  $1/n$ ; where  $K'_d$  is the apparent dissociation constant;  $n$  is the apparent molar ratio of ligand/BLG at saturation;  $P_0$  is the total protein concentration and  $B$  is the total ligand concentration.  $(a)$  is defined as the fraction of unoccupied binding sites on the protein molecules. The value of  $(a)$  was calculated for every desired point on the titration curve of fluorescence quenching intensity using the relationship:

$$a = (F - F_{\min}) / (F_0 - F_{\min})$$

where  $F$  represents the fluorescence intensity at a certain  $B$ ,  $F_{\min}$  represents the fluorescence intensity upon saturation of BLG molecules and  $F_0$  is the initial fluorescence intensity.

# 3. RESULTS AND DISCUSSION

## 3.1. Binding of hemin and protoporphyrin IX to $\beta$ -lactoglobulin

The fluorescence emission spectra of BLG were studied as a function of added compounds. The observed tryptophan fluorescence quenching may be due to the polarity changes induced in the neighbourhood of indoles [14] and also due to energy transfer to the heme. The decrease of tryptophan fluorescence was taken as evidence for a complex formation. The addition of hemin or protoporphyrin IX induces a significant quenching of BLG tryptophan fluorescence. After correction for the blank, the maximum fluorescence quenching is obtained for hemin-BLG and PPIX-BLG ratios of 1:1 (Fig. 1). In addition, the static spectrum of BLG-heme-CO exhibits a peak Soret absorbance at 416 nm (data not shown). The Soret peak of BLG-heme-CO complex is quite similar to that of serum albu-

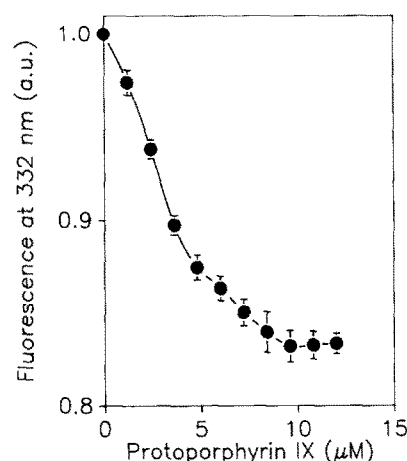


Fig. 1. Corrected fluorescence titration curve of  $\beta$ -lactoglobulin with protoporphyrin IX. The experiments were performed in 50 mM phosphate, pH 7.0 at 20°C. The  $\beta$ -lactoglobulin concentration was 10.2  $\mu\text{M}$ .

min-heme-CO (415 nm) and it is situated between hemoglobin (419 nm) and free heme-CO (407 nm) [15].

The fluorescence quenching data are shown in Fig. 1. The apparent binding constants of ligand-BLG complexes and the apparent molar ratio of ligand-BLG at saturation were calculated from the intercept and the slope of the traced lines (Fig. 2), respectively. The numerical values for hemin-BLG and PPIX-BLG complexes were  $2.5 \times 10^{-7}\text{ M}$  ( $n=0.85$ ) and  $4 \times 10^{-7}\text{ M}$  ( $n=0.79$ ), respectively. The results were interpreted in terms of apparent dissociation constants since the exact physical state of unbound ligand in the aqueous environment is unknown; unbound hemin and PPIX exist in different forms which are in equilibrium. Cogan et al. [13] used the law of mass action in similar studies of retinol binding by the retinol binding protein in order to derive the apparent binding constant of the ligand.

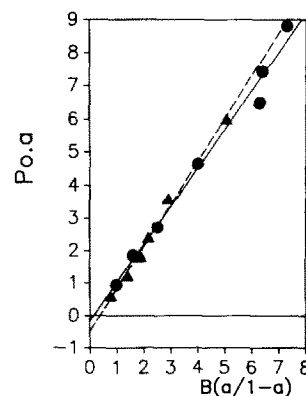


Fig. 2. Graphic representation (Cogan et al, 1976) of  $(P_0 \cdot a)$  versus  $B[a/(1-a)]$  for the titration of  $\beta$ -lactoglobulin with hemin (●) and protoporphyrin IX (▲).  $B$  is the ligand concentration,  $P_0$  is the BLG concentration and  $(a)$  is the fraction of unoccupied sites.

### 3.2. Binding of protoporphyrin IX to retinol-BLG complex

The characterization, at a molecular level, of the BLG binding site(s) seems particularly interesting. Fluorescence spectroscopy is an ideal technique for the study of such problems, providing that the system has fluorophores. Protoporphyrin IX is a fluorescent porphyrin which after excitation at 400 nm displays an emission spectrum with two maxima located at 622 nm (maximum emission) and 685 nm. Retinol fluorescence is weak in aqueous solution alone, but it is greatly enhanced when bound to BLG ( $K_d = 2 \times 10^{-8}$  M). Its complex with BLG exhibits a typical fluorescence spectrum (excitation at 342 nm) with a maximum emission at 480 nm [16].

The analysis of the excitation spectrum of the sample including retinol, PPIX and BLG, measured at 622 nm (maximum of PPIX emission) can give information on the binding to BLG of retinol and PPIX, and on the energy transfer between BLG, retinol and PPIX. If there is no energy transfer between the fluorescent ligands and protein, then the PPIX excitation spectra with or without BLG and retinol should be identical. In contrast, if energy is absorbed by BLG or retinol and then transferred to PPIX, the observed excitation spectrum of PPIX (considering emission at 622 nm) should resemble the total of BLG, retinol and PPIX absorbance spectra. As shown in Fig. 3, when PPIX is added to a solution of BLG in a molar ratio of 1:1, the excitation spectrum of PPIX displays an additional peak at 280 nm, i.e. the energy absorbed by the tryptophans of BLG is transferred in part to PPIX. The excitation spectrum of the sample containing PPIX, retinol and BLG in a molar ratio of 1:1:1 (or 1:10:1) displays three peaks at 280 nm, 345 nm and 400 nm (Fig. 3) showing that, even in 10-fold excess of retinol, energy transfer occurs between BLG and PPIX as well as between retinol and PPIX. Fig. 4 shows the absorption

spectra of PPIX, PPIX-BLG complex (1:1 molar ratio) and PPIX-retinol-BLG complex (1:1:1 molar ratio). The peak absorbance of PPIX, located at 377 nm in phosphate buffer 50 mM pH 7.1, is observed at 390 nm when PPIX is bound to BLG.

The excitation spectrum, for PPIX emission, of a solution of PPIX and retinol in a molar ratio of 1:1 was measured, as a control and it was found to match the PPIX excitation spectrum (see Fig. 3). This shows that the observed energy transfer between retinol and PPIX is mediated by BLG. Moreover, when the predominantly  $\beta$ -sheet secondary structure of BLG rearranges into mostly  $\alpha$ -helical structure under the influence of alcohol [17], BLG binds neither PPIX nor heme-CO and the energy transfer between BLG, retinol and PPIX is no longer observed in these conditions.

BLG displays various oligomeric states as a function of pH, concentration and temperature [18]. In applied experimental conditions (BLG solution = 10  $\mu$ M, phosphate buffer 50 mM, pH 7.1), BLG is monomeric [18]. The obtained fluorescence and retinol and PPIX binding data are consistent with the model of binding of these two ligands at two different binding sites on or within monomeric BLG.

### 3.3. Estimation of retinol-PPIX distance

The efficiency of energy transfer depends on the distance between the donor (retinol in this case) and the acceptor (protoporphyrin IX). Using the Förster theory for the energy transfer [19], the retinol-protoporphyrin distance can be calculated from the relation  $F_0/F = 1 + (R_0/R)^6$ , as previously done for the tryptophan-retinol distance in BLG [16] and the change in tryptophan-heme distance in myoglobin [20]. Since the orientation factor is not known; the standard value of 2/3 for a rotationally free system was used. The critical distance  $R_0$  for 50% quenching was calculated to be 24 Å. The observed quenching of retinol-BLG fluore-

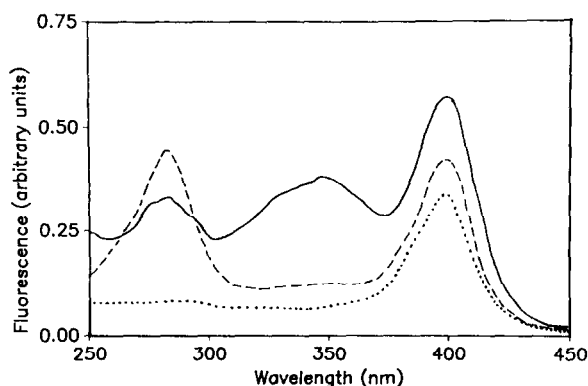


Fig. 3. Excitation spectra for protoporphyrin IX emission at 622 nm for BLG-protoporphyrin IX-retinol (—), BLG-protoporphyrin IX (---) and protoporphyrin IX with or without retinol (.....) at protein to ligand ratio of 1:1 (or 1:1:1). The spectra were recorded in 50 mM phosphate, pH 7.0 at 20°C. The  $\beta$ -lactoglobulin concentration was 10.2  $\mu$ M.

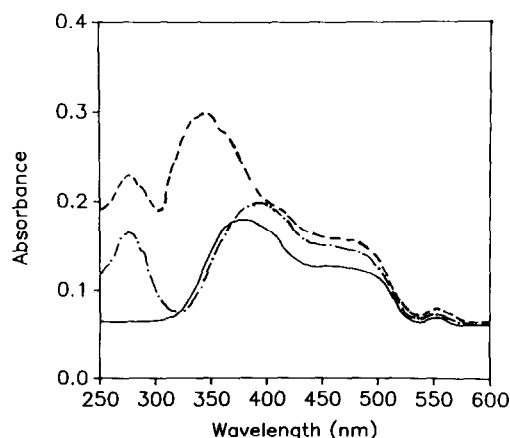


Fig. 4. Absorption spectra of protoporphyrin IX (—), protoporphyrin IX-BLG complex (---) and protoporphyrin IX-retinol-BLG complex (.....) at protein to ligand ratio of 1:1 (or 1:1:1). The spectra were recorded in 50 mM phosphate, pH 7.0 at 20°C. The  $\beta$ -lactoglobulin concentration was 6.5  $\mu$ M.

scence (emission at 480 nm) by protoporphyrin IX (or hemin) is about 45%; a value of  $F_{\min}/F_0 = 0.55$  is obtained with protoporphyrin IX (data not shown). This yields a retinol-porphyrin distance of 25 Å. The retinol-PPIX distance may be of importance in distinguishing between models for locating the retinol molecule on BLG monomer. As the entire end to end length of BLG monomer is less than 40 Å, the large distance of 25 Å between the retinol and PPIX bound by BLG does not support a model with retinol bound in the ideal center of the globulin. Since retinol and PPIX are probably bound in different hydrophobic pockets on or within the molecule, the maximum distance between these two ligands should not exceed 30–32 Å. Also, it is known that alcohol dehydrogenase can rapidly oxidize the retinol molecule bound to BLG [7]. This fact supports the present results suggesting the binding of retinol near one extremity of BLG monomer, rather than in a more protected central cavity. One possible error in the distance assessment can result from a perpendicular positioning of the retinol fluorescence dipole in relation to the heme group. The orientational factor would then be close to zero and this could explain the weakness of the transfer. However, both molecules would have to be rigidly constrained to stabilise such an orientation.

Prevailing evidence indicates that BLG may bind retinol at the highly conserved area of this protein super-family, way inside [5,21] the main hydrophobic pocket. PPIX could be bound elsewhere, may be at the surface hydrophobic site described by Monaco et al. [6]. Obtained results do not definitively exclude the existence of an external retinol binding site on the BLG molecule. They may support, however, its slightly eccentric placement inside the BLG molecule.

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