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Retinol and retinoic acid bind to a surface cleft in bovine β-lactoglobulin: a method of binding site determination using fluorescence resonance energy transfer

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Abstract

Two potential ligand binding sites in the lipocalin β-lactoglobulin have been postulated for small hydrophobic molecules such as retinol or retinoic acid. An agreement on one of the two alternatives, an interior cavity or a surface cleft, however, has not been achieved. In order to discriminate between these two possibilities, we measured the efficiency of fluorescence resonance energy transfer between the two intrinsic Trp-residues of β-lactoglobulin and the ligands retinol, retinoic acid and bis-ANS. Using the crystallographic coordinates of β-lactoglobulin, this efficiency could be accurately computed for both the interior cavity and the surface cleft as ligand binding sites. For the surface cleft, the theoretical value was found to be in excellent agreement with the measured value, whereas for the interior cavity any reasonable agreement would require a dramatic ligand-induced conformational change that can be ruled out due to the protein's known structural stability. Our conclusion that these ligands bind to the surface pocket rather than the interior cavity was further confirmed by competitive binding studies.

Keywords: β-Lactoglobulin; Retinol; Binding site; Fluorescence resonance energy transfer (FRET); Lipocalin

1. Introduction

β-Lactoglobulin, an 18-kDa polypeptide, has been identified as a member of the lipocalin superfamily of transporter molecules for small hydrophobic ligands [1–3]. Some examples of additional members of this protein superfamily include apolipoprotein D, retinol-binding protein, bilin-binding protein, insecticyanin, odorant binding protein, α-2μ-globulin, and α_1-microglobulin and tear lipocalins [4]. Although they show low sequence similarity (generally less than 20% identity), their remarkably similar topology, consisting of eight strands of antiparallel β-sheet twisted

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Abbreviations: bi-ANS, 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid dipotassium salt; Cr(III) pic, tris picolinato chromium(III); FRET, fluorescence resonance energy transfer

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into a cone-shaped barrel, indicate their evolution from a common ancestor [5,6]. β-Lactoglobulin is a milk-whey protein expressed in the glandular epithelium of the mammary gland in ruminants and other species. β-Lactoglobulin is known to bind several non-polar ligands such as retinol, fatty acids, protoporphyrin IX, cholesterol, estradiol and progesterone [7–10]. Several non-nutritional functions of β-lactoglobulin have been proposed [11], including the binding and transport of retinol through the stomach and its subsequent delivery to a specific receptor located in the intestine of the suckling neonate [5,12].

The molecular mechanisms of ligand binding by lipocalins are poorly understood. We have recently developed a fluorescence spectroscopic assay that, together with FRET analysis, allows the rapid and accurate determination of the kinetic and thermodynamic aspects of lipocalin-ligand interaction [10]. In the present studies we used this approach to discriminate between two alternative binding sites that have been proposed for the binding of retinol by β-lactoglobulin. Based on two separate crystallographic analyses, two alternative ligand binding sites, an evolutionarily conserved interior cavity or a cleft at the surface of the protein have been proposed (Fig. 1). By inference from the structural similarity of bovine β-lactoglobulin with human retinol-binding protein it has been suggested that retinol binds within the central calyx domain of β-lactoglobulin [3,12]. This view is supported by the strong topological similarity with other members of the superfamily, for which structural analyses have provided evidence that their non-polar ligands bind to the hydrophobic core where they are protected from the surrounding environment [13–15]. A further aspect of this structural similarity is a spatially conserved tryptophan residue at the base of the binding pocket of all the members of the lipocalin superfamily. In addition, absorption spectroscopy on retinylidenepropy-

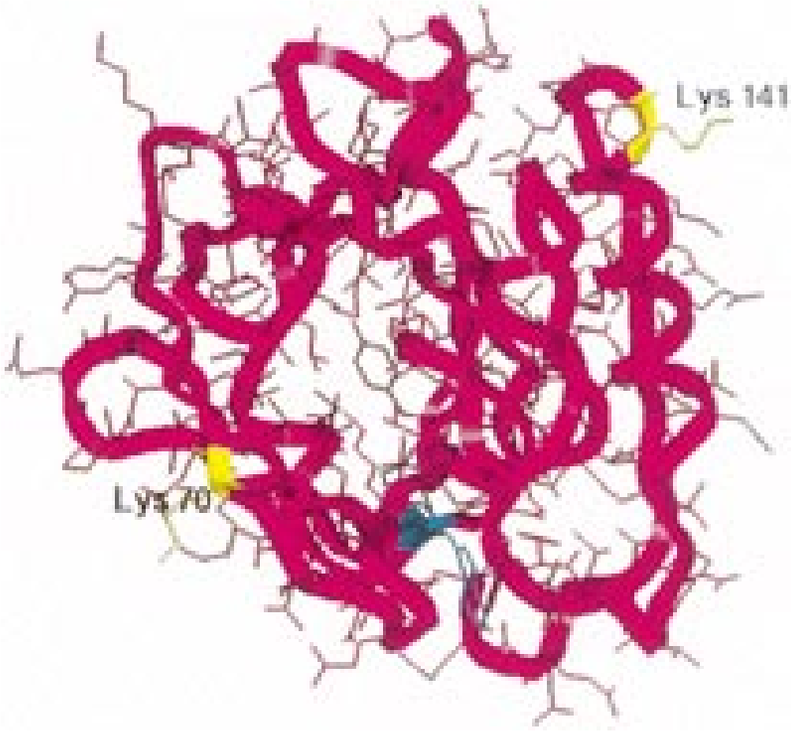

Fig. 1. Crystallographic model for bovine β-lactoglobulin. Lys^{141} and Lys^{70} (yellow) are taken as reference points for the surface pocket and the interior cavity, respectively. The two Trp-residues are shown in blue.

lamine, a retinyl Schiff base analog of retinol, that had been complexed with site-directed mutations of β-lactoglobulin supports the notion that the retinol-binding site of β-lactoglobulin does indeed lie within the central calyx [16]. In this model, the Trp^{19} residue is located close to the β-ionone ring and the retinol hydroxyl group can then interact with the ε-amine of Lys^{70} [17]. However, this model is inconsistent with the result of a later crystallographic analysis of a β-lactoglobulin-retinol complex, in which the retinol appears to be bound to the surface pocket [18]. In this case, Phe^{136} and Lys^{141} replace the roles of Trp^{19} and Lys^{70}, respectively.

In order to discriminate between these two models, we made use of the spectroscopic properties of all-*trans*-retinol, all-*trans*-retinoic acid and bis-ANS and investigated their binding to β-lactoglobulin followed by determination of the efficiency of fluorescence resonance energy transfer between the two intrinsic Trp-residues (donors) and each of the ligands (acceptors). The experimental data was then compared to the values obtained by calculations based on the crystallographic structure of β-lactoglobulin, assuming both the interior cavity and then the surface pocket as two possible alternative binding sites. Taking all possible errors into account, the outcome is remarkably clear, giving strong support to the proposal that the binding of retinol and retinoic acid takes place in the surface cleft rather than the interior cavity. Competitive binding studies with all-*trans*-retinol/-retinoic acid and bis-ANS further support this conclusion.

2. Materials and methods

A 1.2 μM solution of β-lactoglobulin in 50 mM TRIS buffer (pH 7.1) was prepared. The tryptophan fluorescence, following excitation at 290 nm, was measured at room temperature using a Perkin Elmer LS50B luminescence spectrometer. The quenching of tryptophan fluorescence upon consecutive addition of small amounts of a 100 μM all-*trans*-retinol [all-*trans*-retinoic acid] solution in 100% ethanol was monitored. The minimum fluorescence intensity was reached at saturation of the protein with an ~3-fold molar excess of ligand. The absorption spectra of all-*trans*-retinol, all-*trans*-retinoic acid and bis-ANS in TRIS (pH 7.1) were obtained and normalized to their respective extinction. Care has to be taken as these solutions are very light sensitive. Based on the known values in ethanol, the extinction coefficients in TRIS buffer were calculated to be 28 000 M^{-1} cm^{-1} for retinol at 325 nm and 10 040 M^{-1} cm^{-1} for retinoic acid at 350 nm.

2.1. Determination of distances

According to Förster's classical model of fluorescence resonance energy transfer, the spectroscopic properties of two chromophores, i.e. the donor (intrinsic tryptophan) and the acceptor (ligand) are related to their spatial separation as follows:

$$R_0^6 = 8.785 * 10^{-28} \text{mol} * \frac{\Phi_D * \kappa^2}{n^4} * (\text{OI}) \qquad (1)$$

Φ_D, quantum yield of donor; κ^2, orientation factor (taken to be 2/3 as for the case of dynamic averaging over two randomly oriented dipoles can be assumed and later taken into error consideration); n, index of refraction in aqueous solution, generally taken to be 1.4. OI is the 'overlap integral':

$$\text{OI} = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)^4 \, d\lambda$$

F_D, integral-normalized fluorescence spectrum of the donor; ε_A, molar absorption coefficient of the acceptor. The efficiency of fluorescence resonance energy transfer is given by

$$E = 1 - \frac{F_{DA}}{F_D} = \frac{1}{1 - \left(\frac{R}{R_0}\right)^6} \qquad (2)$$

where F_D is the fluorescence intensity in the absence, and F_{DA} in the presence of A with equal concentration of donor, and R is the donor-acceptor separation [19–22].

R_0 was calculated for each Trp-ligand-pair ac-

cording to (1). Owing to the sixth-order dependence of R_0, assuming an average value for the tryptophan quantum yield results in a negligible error, although their spectroscopic properties vary due to different local environments. The average quantum yield was taken to be 0.12 as obtained by comparison with 0.14 for free tryptophan. The distances between each Trp-residue and Lys^{141} (taken as reference point for the surface cleft) and Lys^{70} (reference point for the interior cavity) were calculated using the crystallographic coordinates of β-lactoglobulin. As a function of these distances the total (theoretical) FRET efficiency, i.e. the mean of the efficiencies for the two individual Trp-ligand pairs, was computed according to (2), making the following simplifying assumptions: (i) the protein concentration is small enough for the energy transfer between different proteins being negligible; and (ii) due to the relatively large Stoke's shift of Trp, self-quenching, i.e. energy transfer between Trp-residues, can be neglected. This theoretical value for the efficiency was compared to the experimental value that was determined by measuring the quenching of fluorescence intensity according to (2).

2.2. Error considerations

As this method involves a number of simplifying assumptions, a reliable interpretation of the data requires great care in the evaluation of the uncertainty ranges for the above quantities. The major uncertainties emerge from the following simplifications: (i) given the fixed locations of at least the acceptor moieties, the assumption of dynamic averaging over two randomly oriented dipoles does not necessarily hold. However it has been emphasized [23,24] that $\kappa^2 = 2/3$ is usually a reasonable approximation even if the donor and acceptor molecules do not have total orientational randomization. (ii) Although the spectroscopic properties of the two Trp-residues might vary due to different local environments, we have taken an average value for the quantum yield. Owing to the sixth-order dependence of R_0 on these parameters, even the largest possible errors that we assumed for these and the other quantities result in an error in R_0 of only 6% (see Table 1) with all errors being treated as independent. (iii) Other factors to be considered are the resolution of the crystallographic coordinates (2.8 Å) and the fact that we took the Lys^{141} and Lys^{70} as reference points for the binding sites of the in reality extended acceptor molecules. The error in the respective distances contributes to the uncertainties in the theoretical FRET efficiency as shown in Table 1.

The major advantage of this method is that even considerable uncertainties in the mentioned quantities result in error ranges for the FRET efficiencies that are comparatively small and, in this case, can clearly be separated from each other, i.e. they do not overlap. This allowed us to set spatial limits on the protein's dimensions by optimizing the distance parameters in order to match the theoretical and experimental FRET efficiency values.

3. Results and discussion

A typical spectrofluorimetric titration of β-lactoglobulin with all-*trans*-retinol with excitation held at 290 nm is shown in Fig. 2. Maximal quenching, i.e. saturation of the protein, was reached at an ~3-fold molar excess of ligand. The values for FRET efficiency, E^{exp}, as obtained from this fluorescence quenching according to (2), are given in Table 1 for all three ligands, retinol, retinoic acid and bis-ANS. The distances from the Trp-residues to the reference points of the two potential binding pockets as determined from a crystallographic model of β-lactoglobulin (with resolution of 2.8 Å) are: 23 Å (Trp^{19}–Lys^{141}); 31 Å (Trp^{61}–Lys^{141}) for the surface cleft and 16 Å (Trp^{19}–Lys^{70}); 18 Å (Trp^{61}–Lys^{70}) for the interior cavity. Assuming either the surface cleft or the interior cavity as alternative binding sites, we calculated the FRET efficiencies (Table 1), E^{theor}, according to (2) as a function of these distances and of the critical distances R_0 for each Trp-ligand pair. A comparison of these results with the experimentally determined values shows excellent agreement for the surface cleft as the binding site: all values of E^{exp} lie very well within the value range of E^{theor}. In contrast, only dramatic conformational changes of the β-lactog-

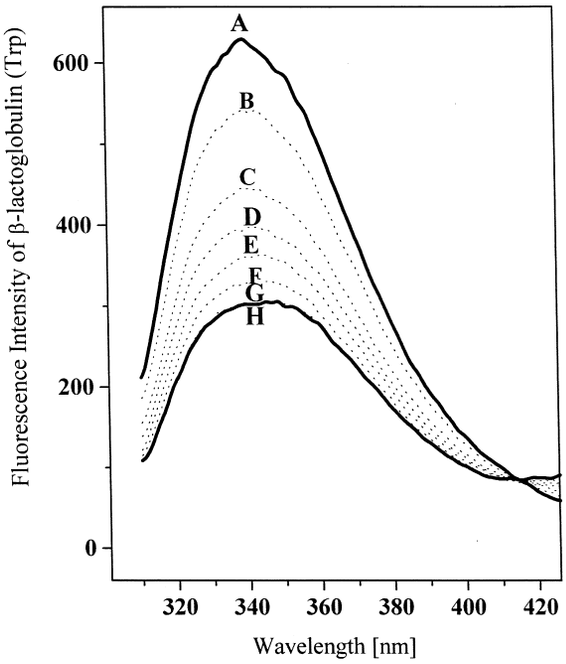

Fig. 2. Spectrofluorimetric titration of β-lactoglobulin with all-*trans*-retinol. (A) Fluorescence intensity of 1.2 μM β-lactoglobulin in 50 mM TRIS-buffer. (B–H) After addition of 0.2, 0.6, 1.0, 1.6, 2.0, 2.7, 3.2 μM all-*trans*-retinol. Bold: quenching of 46% due to FRET. Virtually identical graphs were obtained for all-*trans*-retinoic acid and bis-ANS. Excitation was held at 290 nm.

lobulin-ligand complexes could explain the values for the interior cavity. Quantitatively speaking, such a conformational change would have to involve a simultaneous increase of at least 10 Å in both Trp-ligand distances in order for the error range of E^{theor} to just overlap with that of E^{exp}. This method of comparing the efficiency value obtained from fluorescence quenching to that predicted by means of crystallographic models proved fairly accurate also in previous studies [10] on two other lipocalins, apolipoprotein D and insecticyanin, for which the locations of the binding sites had been previously established. Despite the fact that ligand binding is accompanied by dimerization, our previous results suggest a remarkably rigid and stable conformation of apolipoprotein D and insecticyanin, which are homologs of β-lactoglobulin. Therefore, it appears unlikely that a conformational change of β-lactoglobulin, as dramatic as required for fitting the theoretical data for the interior cavity as the ligand binding site, is taking place.

This conclusion was further strengthened by our findings that the ligands, bis-ANS and all-*trans*-retinol, as well as bis-ANS and all-*trans*-retinoic acid, compete for the same binding pocket in β-lactoglobulin. As we have previously shown, such competitive binding analysis can be readily conducted owing to the suitable spectroscopic properties of bis-ANS [10]. For instance, binding of bis-ANS to apoD results in more than 100-fold enhancement in its fluorescence emission and a 44-nm blue shift of the peak maximum and can therefore be discerned unambiguously. Fig. 3 shows the fluorescence emission at 480 nm of bis-ANS bound to β-lactoglobulin with excitation held at 394 nm. β-Lactoglobulin was first saturated with bis-ANS ($\sim$ 7.5 μM). Subsequent addition of all-*trans*-retinol in steps of 0.7 μM resulted in a marked decrease in bis-ANS-fluorescence intensity, owing to the displacement of bis-ANS from its binding pocket by retinol. This decrease cannot be explained on the basis of energy transfer occurring between bis-ANS

Table 1
Comparison between experimental and theoretical FRET efficiencies for the two possible binding pockets

Ligand	Experimental value for FRET efficiency E^{exp}	Theoretical value E^{theor} assuming surface cleft as binding site	Theoretical value E^{theor} assuming interior cavity as binding site
Retinoic acid ($R_0 = 26 \pm 2$ Å)	$43 \pm 1\%$	$44 \pm 14\%$	$92 \pm 7\%$
Retinol ($R_0 = 27 \pm 2$ Å)	$46 \pm 1\%$	$50 \pm 14\%$	$94 \pm 5\%$
Bis-ANS ($R_0 = 28 \pm 2$ Å)	$54 \pm 1\%$	$52 \pm 13\%$	$95 \pm 5\%$

(donor) and retinol (acceptor) that might possibly bind to the second binding pocket other than that occupied by bis-ANS for the following reasons: (1) with the distance between the two binding sites, i.e. from Lys^{70} to Lys^{141}, being 30 Å, the overlap of the absorption spectra of retinol and the fluorescence spectra of bis-ANS is far too small to allow for such large values of fluorescence quenching by FRET. (2) In earlier studies we could establish that another molecule, Cr(III) pic, does bind to β-lactoglobulin but does not displace bis-ANS, and therefore most probably binds to the interior cavity. (It has been previously proposed that there are other ligands whose binding site differs from that of retinol, as for example the porphyrin, protoporphyrin IX [9]). Cr(III) pic does not absorb in the emission range of bis-ANS and can therefore not play the role of an acceptor in FRET. Therefore, in the case of bis-ANS residing in the surface cleft and retinol in the interior cavity of the same protein, the displacement of retinol by Cr(III) pic should severely weaken the deactivation mechanism of FRET, resulting in an increase of bis-ANS fluorescence intensity. In the opposite case, i.e. if retinol binds to the surface cleft and bis-ANS to the interior cavity, addition of Cr(III) pic should eventually release bis-ANS entirely from the protein, leading to a decrease in bis-ANS-fluorescence intensity due to the molecule's almost vanishing quantum yield in the unbound state. However, not the slightest change in intensity was observed upon addition of Cr(III) pic, not even for concentrations as high as 100 μM.

It is still conceivable that retinol (or retinoic acid) and bis-ANS bind to different sites in β-lactoglobulin, and that through allosteric effects the binding of retinol at one site would decreases the protein's affinity for bis-ANS at a separate site, much like the Bohr-effect in hemoglobin. However, this change in affinity would need to be very large (Fig. 3), which would be somewhat surprising considering that the binding of Cr(III) pic has no effect on any of the ligand's affinity, in accordance with the earlier proposed rigidity of the protein. Therefore, we consider it most likely that retinol and bis-ANS compete for the same

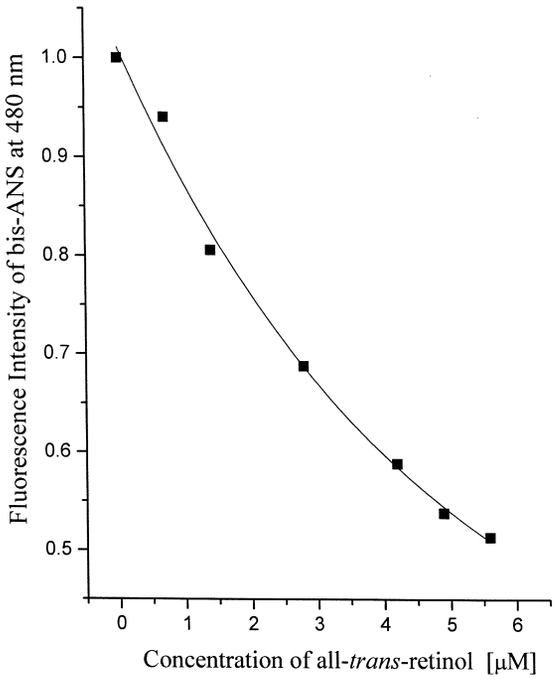

Fig. 3. Competitive binding of all-*trans*-retinol and bis-ANS in β-lactoglobulin. The fluorescence intensity of bound bis-ANS (7.5 μM) at 480 nm decreases exponentially with increasing concentration of all-*trans*-retinol. Virtually identical behavior was observed for all-*trans*-retinoic acid. Excitation was held at 394 nm.

binding pocket. Analogous results were obtained from competitive binding studies with bis-ANS and retinoic acid.

In summary, all the evidence obtained from analyzing energy transfer between intrinsic tryptophan and various suitable ligands as well as from competitive binding studies leads us to strongly favor the surface cleft of β-lactoglobulin as binding site for retinol, retinoic acid and bis-ANS over its interior cavity. This is consistent with the crystallographic analysis of a β-lactoglobulin-retinol complex by Monaco et al. [18], but in contradiction to various other, more recent studies. Considering the general role of lipocalins in the transport of small hydrophobic and labile molecules, which could be used for therapeutic drug delivery, the current findings may prove useful in the design and functional characterization of recombinantly engineered β-lactoglobulins.

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