Interaction of β-Lactoglobulin with Small Hydrophobic Ligands As Monitored by Fluorometry and Equilibrium Dialysis: Nonlinear Quenching Effects Related to Protein−Protein Association

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Although a thorough characterization of binding parameters is essential for application of β-lactoglobulin as a carrier for a variety of small hydrophobic ligands, the binding parameters derived in various studies using various techniques are inconsistent. The bindings of several small ligands as detected by fluorometry and equilibrium dialysis were compared. Fluorescence spectroscopy showed that β-ionone, retinol, and fatty acid lactones all bound in the vicinity of a tryptophan residue. Retinol and fatty acid lactone competed for the same binding site. Exclusively for ligands that quench the β-lactoglobulin fluorescence through a resonance energy transfer mechanism, fluorometry yielded a systematically higher binding affinity than equilibrium dialysis. The binding overestimation in fluorometric measurements can be explained by oligomer formation of protein, together with an underestimation of the limiting quenching level at saturating ligand concentrations due to the use of a limited set of data points.

Keywords: *β-Lactoglobulin; flavor compounds; fluorescence spectroscopy; equilibrium dialysis; binding parameters; hydrophobic and steric effects*

INTRODUCTION

Proteins are basic ingredients of food systems. Not only do they have important nutritional value but also, like other macroingredients present in foodstuff, they strongly influence the overall flavor perception due to the interaction with other food constituents (*1*−*3*). The ability of proteins to interact selectively and reversibly with flavor components and, consequently, to influence their flavor release can be used to develop efficient controlled-release systems. These systems can provide efficient protection against degradation or oxidation while providing the active ingredient(s) at a specific rate by applying an appropriate release stimulus. Lipid-binding proteins, partly responsible for the transport of hydrophobic molecules within a cell system, are especially interesting for their potential use in the development of ligand-specific controlled-release systems (*4*).

By far the most studied of the hydrophobic transporter molecules is the 18 kDa globular milk whey protein, β-lactoglobulin (*5*, *6*). β-Lactoglobulin is a useful model protein for studying ligand−protein interactions because its conformation and physical properties are well-defined. A variety of compounds have been reported in the literature to be bound by β-lactoglobulin (*3*, *4*, *7*−*12*), although different numerical binding parameters were derived in different studies, probably as a result of different experimental conditions and methodologies.

The correct understanding of the nature of the factors contributing to the β-lactoglobulin binding properties is a prerequisite to the use of this protein as a specific carrier in the development of protein-based controlled-release systems. Apparently, β-lactoglobulin tightly binds one retinol molecule per monomer in vitro (*13*). On the basis of structural analogies with retinol-binding protein and bilin-binding protein, it has been postulated that β-lactoglobulin binds retinol inside the main hydrophobic binding pocket formed by the β-barrel (*14*). Although some authors originally suggested additional binding sites on the protein (*6*), there are many experimental data that support the role of the central calyx in the binding of retinol and other compounds such as fatty acids (*4*, *15*, *16*).

Fluorescence spectroscopy is a valuable tool in the investigation of the structure, function, and reactivity of proteins and other biological molecules. It is a fast and relatively simple technique and has the advantage of 100−1000-fold higher sensitivity than light absorption techniques. Wavelengths shifts (from 350 nm in highly polar media to 315 nm in nonpolar media) and changes in intensity of the fluorescence emission peak of tryptophan residues can be used to monitor the environment of these residues in proteins, providing information on local interactions. However, the extreme sensitivity to local environmental effects (e.g., changes of solvent relaxation, local dielectric constant, and pH) and the interference of optical effects, such as inner filtering and light scattering, easily disturb a correct interpretation of fluorometric results. Probably due to unnoticed fluorometric pitfalls, large differences between binding parameters estimated from fluorometric and other measurements have been reported (*15*, *17*).

In this paper, the interaction of β-lactoglobulin with several small ligands as monitored by fluorescence spectroscopy as well as equilibrium dialysis is compared at pH 7. From a careful analysis of the data and from model calculations, it is concluded that oligomer forma-

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tion of protein, together with underestimation of the limiting quenching level at saturating ligand concentrations, can explain the overestimation of the binding affinity in fluorometric experiments. Competition of different ligands and the influence of steric and hydrophobic effects upon binding were also investigated. Some preliminary data were presented at several COST Action '96 confidential meetings and have been mentioned in corresponding review papers (*2*, *3*, *10*).

MATERIALS AND METHODS

Materials, Standard Conditions, and Procedures. All chemicals were of analytical grade. They were used without further purification. β-Lactoglobulin from bovine milk was purchased from Sigma (product L6879, 1× crystallized and lyophilized).

All experiments were performed in 50 mM phosphate buffer, pH 7. Concentrations of β-lactoglobulin were determined spectrophotometrically at 278 nm, using a molar absorption coefficient (ϵ_{278}) of 17600 M^{-1} cm^{-1} per β-lactoglobulin monomer (*15*), or calculated from the dissolved protein mass and the total solution volume, using a monomer molecular mass of 18 kDs.

Fluorescence Spectroscopy. Excitation as well as emission spectra of β-lactoglobulin were recorded in the absence as well as in the presence of the added compounds (ligands) on a Perkin-Elmer luminescence spectrometer LS50B equipped with a thermostated multiple-cell holder. All experiments were performed at 20.0 ± 0.1 °C, in the ratio mode, using 10 mm fluorescence cuvettes and 5 nm excitation and emission bandwidths. In ligand titrations, fixed excitation and emission wavelengths of 278 and 332 nm, respectively, were routinely used. At these wavelengths, the fluorescence of β-lactoglobulin is dominated by the contribution of the tryptophanyl residues. Three milliliters of β-lactoglobulin solutions of ~9 μM were titrated with 3−5 μL increments of the ligand solution in ethanol (1 mM for retinol and β-ionone, 20 mM for undecanoic and dodecanoic acid lactone, and 150 mM for nonanoic acid and decanoic acid lactones), injected with a microsyringe. After each addition and before each measurement, the cuvette was well shaken and ~5 min was allowed. This yielded stable fluorescence signals. At the end of the various titrations, the ethanol concentration did not exceed 2% v/v. All fluorescence data were corrected for the inner filtering, that is, for light absorption, with a factor of $10^{(A_{ex}+A_{em})/2}$, where A_{ex} and A_{em} are the absorbance at the emission and excitation wavelengths. In all cases, the initial tryptophan (β-lactoglobulin) fluorescence in the absence of ligand and solvent was normalized at $F_{332nm} = 100.0$, after correction for inner filtering and before correction for the blanks. Thus, all fluorometric data were expressed as relative fluorescence intensities (RFI). Inner filtering appeared to be negligible in experiments with fatty acid lactones. For these lactones, a blank containing β-lactoglobulin solution titrated with ethanol was used as control. The RFI changes induced by the blank were subtracted from the RFI changes induced by the ligand at every titration point. In parallel titrations of L-tryptophan solutions with an absorbance at 287 nm equal to that of the protein, the RFI changes of the free tryptophan, induced by undecanoic acid γ-lactone, were negligible (not shown). In titrations with retinol or β-ionone, non-negligible corrections were made for inner filtering at each ligand concentration. In parallel titrations of l-tryptophan solutions with an absorbance at 287 nm equal to that of the protein, the RFI changes of the free tryptophan induced by retinol were approximately ~5 times smaller than the RFI changes of β-lactoglobulin induced by retinol. This showed that the changes of the RFI of β-lactoglobulin induced by the ligand largely reflected the complexation of the ligand by the protein.

Depending on the situation, different methods were used to derive the binding parameters from the changes of RFI as a function of the total ligand concentration, on the basis of the assumptions that (1) the ligand-binding sites on the protein are discrete, (2) each site is saturable and can bind a single ligand molecule, (3) all sites have the same intrinsic binding affinity, (4) each protein monomer can harbor one ($n = 1$) or more than one ($n > 1$) sites, and (5) a linear relationship exists between the ligand-induced change of RFI and the fraction of binding sites occupied by a ligand (i.e., every binding event results in the same change of RFI, irrespective of previous changes).

Normally, the method of Cogan et al. (*18*) was used, which is based on a linear regression of plots of $P\alpha$ versus $L_T[\alpha/(1-\alpha)]$, where P is the total molar protein monomer concentration, α is the fraction of binding sites unoccupied, and L_T is the total molar ligand concentration. Under the assumptions above, it yields an intercept of $-K_d$/N and a slope of $1/N$, where K_d is the apparent dissociation constant and N is the limiting number of ligand-binding sites available per protein monomer, whereas α can be calculated for various L_T using the relationship

$$\alpha = (RFI_{corr} - RFI_{sat})/(100 - RFI_{sat}) \quad (1)$$

RFI_{corr} being the corrected RFI at a certain L_T and RFI_{sat} the (apparent) limiting corrected RFI at L_T values that are judged to elicit (approximate) binding saturation.

When the limiting RFI under saturating conditions was apparently difficult to reach, for example, due to the limited solubility of ligands, the iterative method of Yoshida (*19*) was used to derive the apparent K_d. This method requires the additional assumption that the limiting stoichiometry N be one ligand per protein monomer and thus allows estimation of the fraction α for various L_T, starting from a preliminary estimate (hypothetical value) of K_d, according to

$$\alpha_{est} = \{(1 + T + \kappa_d) - [(1 + T + \kappa_d)^2 - 4T]^{0.5}\}/2 \quad (2)$$

T being the molar ratio of total ligand to protein monomer (L_T/P) and κ_d being the preliminary estimated K_d/P ratio. Plotting the experimentally determined, ligand-induced changes of corrected RFI ($\Delta F_{corr} = 100 - RFI_{corr}$) versus α_{est}, for various preliminary estimates of K_d, will give a family of curves, each curve corresponding to one K_d value. The closest approximation of the correct K_d will yield the best approximation of a straight line and the highest correlation coefficient in linear regression. The correct apparent K_d value was thus approximated by computer-mediated iteration.

Equilibrium Dialysis. The dialysis tube (Visking size 2, $^{18}/_{32}$in., 12000−14000 MWCO, from Medicell U.K.), boiled for 30 min in 0.1 M EDTA and thoroughly rinsed with deionized water (Milli-Q water purification system, Millipore, Bedford, MA) and buffer, was filled with 2.5 mL of β-lactoglobulin solution of ~5 mg/mL (i.e., ~0.694 μmol of β-lactoglobulin monomers), brought into a stirred 75 mL bath of the same buffer containing ligand (10−60 μM β-ionone or 45−260 μM undecanoic acid γ-lactone), and incubated for 48 h at 4 °C under continuous stirring. β-Ionone was added from a 3 mM undecanoic acid γ-lactone from a 20 mM stock solution in ethanol, the final ethanol concentration being maximally 2% v/v. From previous analysis of various time points, it was found that the equilibrium was reached in <36 h. No detectable amount of protein was present in the external (bath) compartment. When equilibrium was reached, the tube was separated again from the bath, the bath and the interior of the tube were individually sampled, and the ligand was quantitatively extracted with an equal volume of hexane. The free (bath) and total (free plus bound, tube) ligand concentrations (L_F and L_T, respectively) were determined by gas chromatography using an internal standard. It was verified by gas chromatography that in single-compartment systems containing a mixture of known amounts of ligand and proteins, all ligand was recovered by extraction with hexane. The concentration of bound ligand L_B was calculated according to $L_B = L_T - L_F$. The binding stoichiometry N and the apparent K_d were estimated by linear regression of a plot of B/L_F versus B, in which B is the molar L_B/P ratio (bound ligand/protein monomer). Under

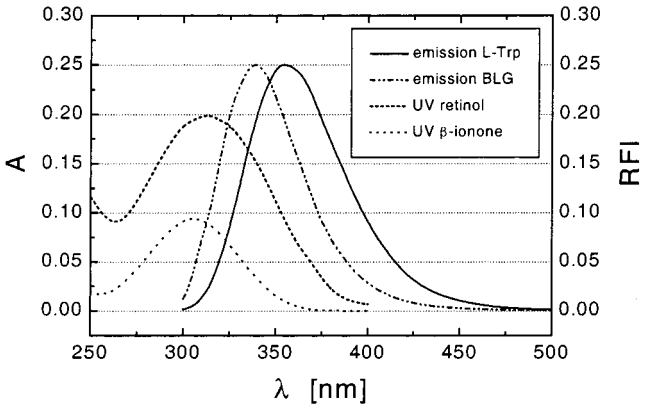

Figure 1. Fluorescence emission spectra of β-lactoglobulin (BLG) and L-tryptophan (L-Trp) and UV light absorption spectra of retinol and β-ionone. Fluorescence excitation was at 278 nm; arbitrary units are used on the *Y*-axis.

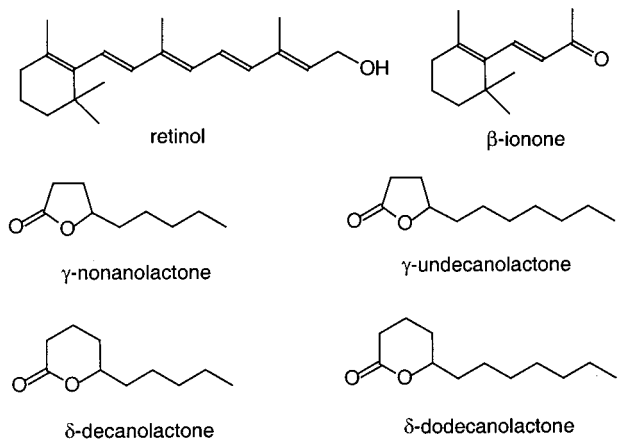

Figure 2. Various compounds used in the present binding study. (Abbreviations: γ-nonanolactone, nonanoic acid γ-lactone; δ-decanolactone, decanoic acid δ-lactone; γ-undecanolactone, undecanoic acid γ-lactone; δ-dodecanolactone, dodecanoic acid δ-lactone.

the assumptions 1−4 used for the fluorometric analyses above, this yields an intercept N/K_d and a slope $-1/K_d$ according to Scatchard (*20*).

RESULTS AND DISCUSSION

Binding Parameters Derived from Fluorometric Data. Figure 1 shows the emission spectra of β-lactoglobulin and free tryptophan and the UV absorption spectra of retinol and β-ionone (see Figure 2 for the ligands used in this work). The emission maximum of β-lactoglobulin (332 nm) is blue-shifted by ~20 nm with respect to that of free tryptophan (354 nm), possibly because the tryptophanyl residues are in a less polar environment inside the protein (*21*).

The fluorescence emission spectra of β-lactoglobulin were studied as a function of the concentration of added retinol and other compounds. The changes of the fluorescence of β-lactoglobulin, induced by these ligands, were due to resonant energy transfer (RET) between the excited indole (tryptophan) rings and the ligand, changes of polarity in the neighborhood of the tryptophanyl residues, or both effects. The emission maximum of β-lactoglobulin was not shifted any further upon addition of retinol or β-ionone (not shown). The addition of retinol induced a significant quenching of β-lactoglobulin fluorescence, as shown in the top panel of Figure 3. In comparison, the addition of retinol to free tryptophan

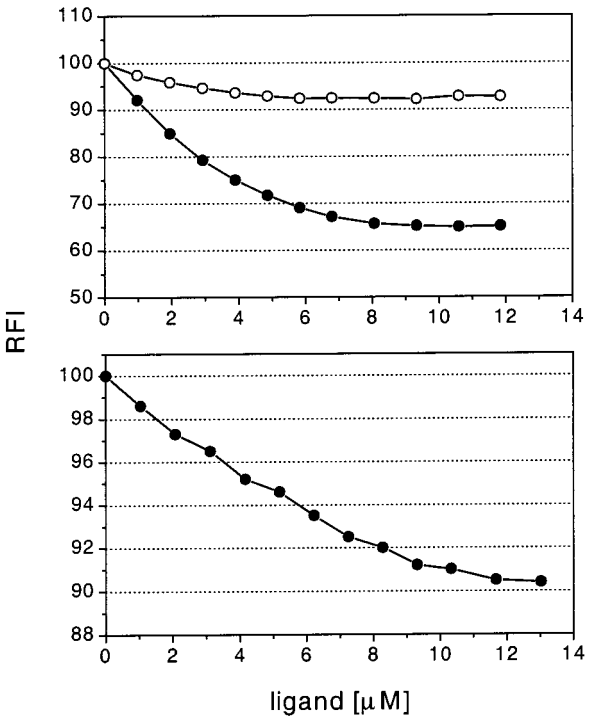

Figure 3. Fluorescence changes of β-lactoglobulin or free tryptophan, elicited by retinol (top) or β-ionone (bottom): (●) β-lactoglobulin (at 8.8 μM in the top panel and at 10.1 μM in the bottom panel); (○) free tryptophan in the absence of β-lactoglobulin, at the same 278 nm extinction as 8.8 μM of the protein (for comparison). Excitation was at 278 nm and emission at 332 nm. Fluorescence was normalized to 100 in the absence of retinol.

induced comparatively little fluorescence quenching of free tryptophan, at a tryptophan concentration corresponding to the same extinction at 278 nm as the protein (top panel of Figure 3). This indicates that the quenching of β-lactoglobulin fluorescence by retinol is largely due to binding of retinol to the protein. When the quenching of β-lactoglobulin was analyzed using the Lehrer equation (*22*), $F_o/\Delta F = 1/(fK_Q[Q]) + 1/f$, a linear plot was obtained that gave an apparent quenching constant $K_Q = 0.13 \times 10^6$ M^{-1}. Using this value, and the fluorescence lifetime of β-lactoglobulin, $\tau_o = 1.4$ ns (*13*), the apparent bimolecular rate constant $k_q = K_Q/\tau_o$ was calculated to be 0.93×10^{14} M^{-1} s^{-1}. This exceeds any possible diffusion-controlled process, the maximum rate constant of diffusion being ~1.4×10^{11} M^{-1} s^{-1}. Again, this shows that complex formation (or nonspecific adsorption) occurs and that the β-lactoglobulin fluorescence is mainly quenched statically by the bound or adsorbed retinol. Thus, the quenching can be used to monitor ligand binding.

The extinction coefficient of the retinol analogue β-ionone is 2.5-fold lower than that of retinol, and the integrated overlap of the β-lactoglobulin emission and β-ionone absorption spectra is much smaller than the overlap of the lactoglobulin and retinol spectra (Figure 1). In accordance, β-ionone caused smaller RET-related quenching of the β-lactoglobulin fluorescence than that seen with retinol (Figure 3, lower panel). As the spectral overlap of β-ionone and free tryptophan is much lower than that of retinol and free tryptophan (Figure 1), the effect of β-ionone on the fluorescence of free tryptophan was also much lower than the effect of retinol. Therefore, also the quenching of the β-lactoglobulin fluores-

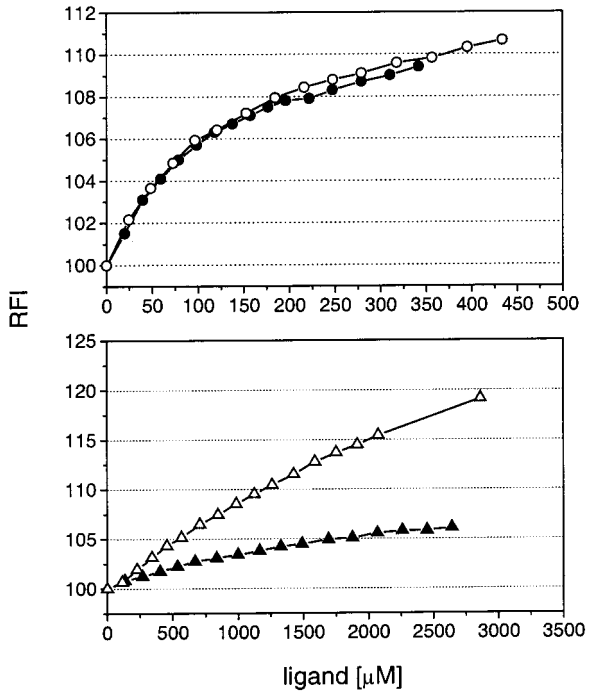

Figure 4. Binding of various fatty acid lactones to β-lactoglobulin as detected by protein fluorescence (excitation at 278 nm; emission at 332 nm): (▲) nonanoic acid γ-lactone; (△) decanoic acid δ-lactone; (●) undecanoic acid γ-lactone; (○) dodecanoic acid δ-lactone (see Figure 2 for the molecular structures of these compounds). β-Lactoglobulin concentration was 7.4 μM.

cence by β-ionone (Figure 3, lower panel) must have been largely due to β-ionone binding to the protein.

In contrast to β-ionone and retinol, various fatty acid lactones (Figure 2) induced an *increase* of β-lactoglobulin fluorescence upon binding, as shown in Figure 4. The fluorescence of tryptophan or tryptophanyl residues normally increases upon transfer from a polar to a nonpolar environment (see, for example, ref *23*). Therefore, the fluorescence increase induced by the fatty acid lactones most probably reflects a decrease of polarity in the tryptophan microenvironment, caused by the displacement of water molecules from the binding cavity by the nonpolar ligand. (The proposed binding site on β-lactoglobulin is lined by hydrophobic amino acid side chains, among which is the side chain of the Trp19 residue.) A similar change of polarity must also have occurred upon binding of retinol and β-ionone, but the corresponding fluorescence increase was apparently masked (i.e., overcompensated) by RET, the resulting net effect being a fluorescence decrease.

The binding parameters could be calculated from the data such as in Figures 3 and 4 under the assumption that (i) the change of fluorescence induced by the added compounds is linearly related to the amount of compound bound by the protein and that (ii) the limiting binding level, that is, the limiting fluorescence change, can be derived from the data (see Materials and Methods). Note that for practical reasons, such data are always restricted to a limited range of ligand concentrations. The resulting binding parameters of retinol, β-ionone, and undecanoic acid γ-lactone are shown in Table 1, together with the data from other research groups.

Competition between Retinol and Undecanoic Acid γ-Lactone for the Same Binding Sites. Figure 5 shows the results of a titration of the β-lactoglobulin−retinol complex (at equimolar concentrations of retinol and β-lactoglobulin) with undecanoic acid γ-lactone, as compared to a titration of the free protein in the absence of retinol. The larger increase in fluorescence of the β-lactoglobulin−retinol complex as compared to that of the free protein is due to a loss of RET between β-lactoglobulin and retinol. It shows that the retinol molecules are displaced from the binding site by the lactone. At concentrations of ~300−400 μM undecanoic acid γ-lactone, the fluorescence of β-lactoglobulin did not reach the level initially observed in the absence of retinol, indicating incomplete displacement of retinol. This is in general accordance with the range of dissociation constants of the two ligands compiled in Table 1, taking into account the total amount of the respective ligands present and assuming a single common binding site for the two ligands per protein monomer, as illustrated in Table 2 (columns I−III). A K_d of retinol between 0.02 and 67 μM would imply that between 91 and 3%, respectively, of all binding sites on β-lactoglobulin are still occupied by a retinol molecule at 350 μM γ-lactone (Table 2, column I). If bound retinol can quench maximally ~35−45% of the original fluorescence of ligand-free β-lactoglobulin (Figure 3, top panel, and

Table 1. Apparent Dissociation Constant (K_d) and Apparent Number (N) of Ligand Molecules That Can Be Bound by β-Lactoglobulin, Estimated with Different Techniques

	fluorescence spectroscopy		equilibrium dialysis	
ligand	K_d (μM)	N (mol/mol)	K_d (μM)	N (mol/mol)
β-ionone	0.52[a,b]	0.8[a,b]	66.6[a]	0.85[a]
	0.60[d,b]	1.1[d,b]	52.2[c]	
undecanoic acid γ-lactone	111.6[a,e]	1.0[f]	111.3[a]	0.99[a]
			100.8[c]	
retinol	<0.20[a]	1.0[a]		
	0.02[g,b]	1.0[g,b]		
	0.047[h,b]	0.9[h,b]	0.38[h]	
	0.045[i,b]	0.8[i,b]	66.7[j]	1.0[j]

[a] From our present work. The Cogan plot (*18*) for retinol in our present study was curved, leading to a negative K_d; when the lowest retinol concentration was left out, a K_d of 0.2 μM was obtained. The Yoshida analysis (*19*) did not yield a straight line even for extremely low estimated K_d values (down to 0) and could thus not be used. [b] Binding parameters derived essentially according to the method in ref *18*. [c] From ref *9*. [d] From ref *32*. [e] Parameters derived according to the method in ref *19*. [f] Assumed value. [g] From ref *13*. [h] From ref *15*. [i] From ref *33*. [j] From ref *17*.

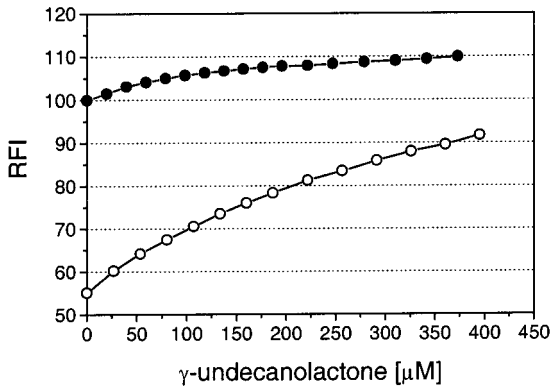

Figure 5. Displacement of retinol from β-lactoglobulin by increasing concentrations of undecanoic acid γ-lactone (γ-undecanolactone), as detected by protein fluorescence (excitation at 278 nm; emission at 332 nm): (○) β-lactoglobulin (8.7 μM) in the presence of 8.7 μM retinol; (●) control titration (β-lactoglobulin in the absence of retinol).

Table 2. Competition between Undecanoic Acid γ-Lactone and Retinol for Common Binding Sites on β-Lactoglobulin: Theoretical Dependence on the Dissociation Constant of Retinol[a]

	under conditions of Figure 5[b]				under conditions of Figure 6[c]			
$K_{d(R)}$[d] (μM)	I $f_{B(R)}$[d]	II $f_{B(\mu UL)B}$[d]	III RFI[e]	IV RFI[f]	V $f_{B(R)}$[d]	VI $f_{B(\mu UL)B}$[d]	VII RFI[e]	VIII RFI[f]
0.02	0.907	0.071	64.8	55.6	0.959	0.032	62.1	55.3
0.05	0.857	0.108	67.3	55.9	0.915	0.068	64.4	55.6
0.2	0.736	0.201	73.6	56.9	0.788	0.169	71.0	56.5
0.5	0.617	0.290	79.7	58.5	0.657	0.273	77.8	58.0
1	0.509	0.372	85.2	61.0	0.536	0.370	84.1	60.4
2	0.391	0.461	91.3	65.7	0.406	0.473	90.8	65.2
5	0.243	0.573	98.9	76.2	0.246	0.601	99.2	76.2
10	0.152	0.641	103.5	86.2	0.152	0.676	104.1	86.7
20	0.088	0.689	106.8	95.3	0.087	0.727	107.4	96.1
50	0.039	0.726	109.3	103.7	0.038	0.766	110.0	104.5
67	0.030	0.733	109.8	105.4	0.029	0.773	110.4	106.2

[a] The competition was calculated by solving the following five simultaneous equations for R_F, γUL_F, R_B, γUL_B, pS_F: $K_{d(R)} = R_F \cdot pS_F/R_B$, $K_{d(\gamma UL)} = \gamma UL_F \cdot pS_F/\gamma UL_B$, $R_T = R_F + R_B$, $\gamma UL_T = \gamma UL_F + \gamma UL_B$, $pS_T = pS_F + R_B + \gamma UL_B$. Here, R, γUL, and pS are the concentrations of retinol, undecanoic acid γ-lactone, and binding sites on the protein, respectively, and K_d is the apparent dissociation constant of the corresponding ligand. The subscripts F, B, and T refer to free (nonbound), protein-bound, and total (free plus bound), respectively. Rather than combining this set of equations into a single-third power equation in one variable, the set was solved as follows by iterative calculation of pS_F: $L_B = R_B + \gamma UL_B = R_T \cdot pS_F/(pS_F + K_{d(R)}) + \gamma UL_T \cdot pS_F/(pS_F + K_{d(\gamma UL)})$, where L_B is the total amount of ligand bound to the protein. If a preliminary test value of L_B was smaller than a preliminary test value of $pS_B = pS_T - pS_F$, it followed that the preliminary pS_B was yet too large and, thus, the preliminary pS_F was yet too small. A new preliminary pS_F was therefore calculated as $pS_{F(new)} = pS_{F(old)} + (pS_{B(old)} - L_{B(old)})/A$, where A is a factor attenuating the speed with which the correct value of pS_F is iteratively approached. To prevent an overshoot during calculation, A was gradually increased as the deviation of pS_F became smaller. $K_{d(\gamma UL)}$ was assumed to be 111 μM, and the number of binding sites per protein monomer to be 1, as derived from Table 1. [b] 350 μM undecanoic acid γ-lactone, 8.7 μM retinol, 8.7 μM β-lactoglobulin. [c] 440 μM undecanoic acid γ-lactone, 10 μM retinol, 8 μM β-lactoglobulin. [d] K_d(R), apparent dissociation constant of retinol; $f_{B(R)} = R_B/pS_T$, $f_{B(\gamma UL)B} = \gamma UL_B/pS_T$: fractions of protein sites occupied by retinol and undecanoic acid γ-lactone, respectively. [e] Calculated tentative RFI of β-lactoglobulin, based on the preliminary assumption that upon binding, a retinol molecule quenches 40% of the original fluorescence of the protein molecule to which it is bound, and a undecanoic acid γ-lactone molecule increases the original fluorescence by 15%. As discussed in the text after the presentation of the dialysis results, this assumption may not be correct. [f] Calculated tentative RFI of β-lactoglobulin, based on the nonlinear model used for Figures 8 and 9.

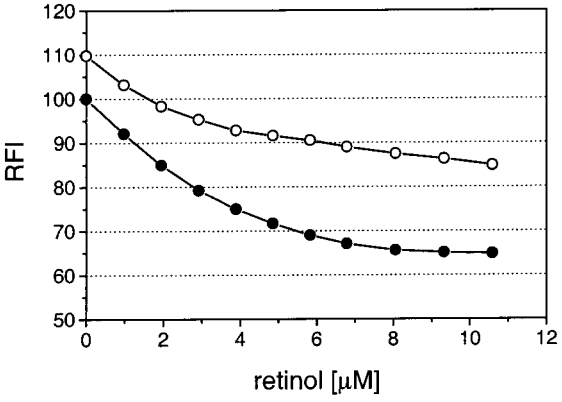

Figure 6. Displacement of undecanoic acid γ-lactone from β-lactoglobulin by increasing concentrations of retinol, as detected by protein fluorescence (excitation at 278 nm; emission at 332 nm): (○) β-lactoglobulin (8.0 μM) in the presence of 440 μM undecanoic acid γ-lactone; (●) control titration (β-lactoglobulin in the absence of undecanoic acid γ-lactone).

Figure 5 at 0 μM lactone) and if bound undecanoic acid γ-lactone can increase the fluorescence by ~10−15% (Figure 4), the RFI should be in the range of 65−110 (Table 2, column III). The RFI actually observed was ~89 around 350 μM undecanoic acid γ-lactone (Figure 5). Assuming that the fluorescence is linearly related to the binding levels of the two ligands, this points to a K_d of retinol of 1−2 μM (Table 2, column III), much higher than the K_d (Table 1) derived from the top panel of Figure 3. As will be discussed after the description of the equilibrium dialysis data below, this assumption, which influences the K_d derived from Figure 3 and other fluorometric data (Table 1) as well as the value of 1−2 μM derived from Table 2, may be wrong.

Figure 6 shows the titration of the β-lactoglobulin−undecanoic acid γ-lactone complex with retinol (γ-undecanolactone/β-lactoglobulin concentration ratio 55), as compared to the lactone-free protein. The fluorescence of the β-lactoglobulin−lactone complex was quenched to a much lower extent by retinol than the lactone-free protein. This result is consistent with the data shown in Figure 5 and again in general accordance with the range of dissociation constants of the two ligands compiled in Table 1 (see Table 2, columns V−VII). Apparently, these two compounds compete for the same binding site. The RFI actually observed was ~86 around 10 μM retinol (Figure 6). Like the comparison of Figure 5 and Table 2, a comparison of this RFI (Figure 6) with Table 2 (column VII) points to a K_d of retinol of 1−2 μM, assuming a linear relationship between the fluorescence and the binding level. However, as indicated above, this assumption may be wrong.

Binding Parameters Derived from Equilibrium Dialysis Data: Comparison to Fluorometric Data. In the equilibrium dialysis experiments, the difference in the concentration of ligand in the tube and in the bath directly corresponds to the concentration of ligand bound by the protein in the tube. The difference was shown to be zero in the absence of protein, and the ligand mass balance in single-compartment experiments showed that all protein-bound ligand was recovered by extraction with hexane (see Materials and Methods). Thus, the equilibrium dialysis experiments allowed determination of the amount of bound drug in an unequivocal manner, in contrast to the fluorescence experiments. Figure 7 presents the Scatchard plot for the interaction of β-lactoglobulin with β-ionone and undecanoic acid γ-lactone, as assessed by equilibrium dialysis.

The binding parameters derived from the present fluorescence spectroscopy and equilibrium dialysis experiments, as well as data from other studies, could thus be compared (see Table 1). Fluorescence experiments

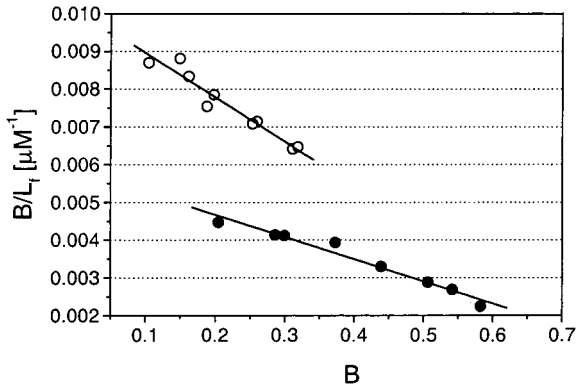

Figure 7. Scatchard plot of ligand binding to β-lactoglobulin in equilibrium dialysis experiments: (○) β-ionone; (●) undecanoic acid γ-lactone (β-lactoglobulin concentration = 9.6 μM). B is the molar ratio of bound ligand to protein monomer (L_B/P); L_F is the micromolar concentration of free (nonbound) ligand.

and equilibrium dialysis gave similar K_d values for undecanoic acid γ-lactone. In contrast, for β-ionone the fluorometric data yielded a lower K_d than the dialysis data. Others have reported lower dissociation constants derived from fluorometry than from dialysis also for retinol (Table 1). An apparent limiting binding stoichiometry not higher than 1 was consistently found, that is, not more than one molecule ligand was bound per protein monomer.

Possible Explanations for the Discrepancy between Fluorometry and Equilibrium Dialysis. From Table 1, it appears that the discrepancy between fluorometric and equilibrium dialysis data is specifically observed for ligands that quench the β-lactoglobulin fluorescence and not for ligands that enhance the fluorescence.

Therefore, it could be that RET from β-lactoglobulin to free (nonbound) ligand plays some role. The rate of RET can be quantified by use of the Förster theory for energy transfer. Energy transfer from tryptophan (donor) to retinol (acceptor) can occur only if the distance between the chromophore groups (i.e., the distance between the centers of mass of the two transitions) is $<\sim$3.4 nm (*13*). This distance is larger than the sum of van der Waals molecular radii of the RET pair, suggesting that static quenching can occur even if the retinol is not in physical contact with the tryptophan fluorophore at the exact moment that it becomes excited. Although quenching studies using acrylamide and iodide as external quenchers indicated that the β-lactoglobulin tryptophan residue(s) are buried [quenching constants: K_Q (acrylamide) = 1.9 M^{-1}, K_Q (iodide) < 0.1 M^{-1}], the "buried" tryptophan(s) must be very close to the protein surface. As β-lactoglobulin has an overall radius of $\sim$2 nm, nearly 60% the mass of such a particle would be within 0.5 nm of the surface and almost 90% within 1 nm. Moreover, the free retinol molecule can diffuse an additional 2.0−2.5 nm, that is, move within the sphere of action of β-lactoglobulin, during the lifetime of excited tryptophan(s). Thus, quenching can be elicited if the donor and acceptor are at an initial distance of $\leq$3.4−5.9 nm. When the concentrations of free retinol and β-lactoglobulin are both $\sim$10 μM, that is, when the average distance between the β-lactoglobulin and retinol molecules is $\sim$39 nm, the fraction of β-lactoglobulin fluorescence quenched by free (non bound) ligand could thus *maximally* be 6−15%, assuming a completely random spatial distribution of both free ligand and protein and 100% quenching efficiency. In general accordance with this, 6−12 μM retinol induced only 7−8% quenching of the fluorescence of free tryptophan (Figure 3). According to the relative overlap of the retinol absorption spectrum with the tryptophan and β-lactoglobulin spectra (Figure 1), RET to free (nonbound) retinol is not expected to be significantly larger in the case of β-lactoglobulin than in the case of free tryptophan. However, the quenching level of β-lactoglobulin (35−45%, Figure 3, top panel) was much larger than either the quenching of free tryptophan (Figure 3) or the theoretical maximum of quenching derived above (6−15%). This shows that RET from β-lactoglobulin to free (nonbound) ligand cannot have been the cause of the discrepancy between fluorometric and dialysis data. Moreover, RET to free retinol could not have been reversed upon addition of undecanoic acid γ-lactone and would thus be in conflict with the observations shown in Figures 5 and 6.

RET to nonspecifically adsorbed ligand is also not a likely explanation because nonspecific adsorption to the protein should also have been detected after equilibrium dialysis and because nonspecific adsorption is not likely to be more important than binding to specific sites (normally associated with high affinity), at low ligand concentration.

Whereas equilibrium dialysis directly provides the amounts of free and bound ligand, fluorometry does not. The amount of bound ligand can only be inferred from the fluorescence changes relative to the "limiting" fluorescence at high ligand concentration. The latter is difficult to estimate, even if the titration curves seem to approach saturation. The analysis according to Cogan et al. (*18*) appears to be heavily dependent on the estimation of the limiting fluorescence. Small errors in the estimate of the fluorescence limit will result in serious underestimation of K_d (overestimation of affinity). To check this, a theoretical binding curve was calculated and computer-translated into an RFI curve, assuming (a) a single specific binding site per protein monomer, (b) independent binding and quenching behavior of all protein monomers, (c) a theoretical K_d of 5−15 μM and (d) a linear relationship between the protein fluorescence and the fraction of binding sites occupied by a ligand molecule (model explained in detail in Table 2). Using the RFI at 15 μM ligand as the final ("limiting") RFI, graphic analysis (not shown) of the calculated fluorescence data between 0 and 15 μM ligand according to the method of ref *18* indeed yielded incorrectly low (apparent) values for the apparent K_d (Table 3, top rows). This is probably a partial explanation of the discrepancy between the fluorescence and equilibrium dialysis data.

Although errors in the estimation of the limiting fluorescence level can thus result in serious overestimation of the apparent binding affinity, such errors do still not explain the *shape* of the fluorometric binding curves such as shown in Figure 3. This shape seems indeed to point to a lower apparent affinity than observed in equilibrium dialysis. Half-saturation apparently occurs at 5 μM for β-ionone (Figure 3, lower panel), whereas, in dialysis, half-saturation occurred at a free β-ionone concentration of 66.7 μM (Table 1). We discovered that, using only the assumptions mentioned above, it was not possible to make the shape of the calculated binding curves equal to the shape of the real binding curves in

Table 3. Comparison of Apparent Binding Parameters Derived by Graphical Analysis of the Data (Using the Wrong Limiting Fluorescence Values) and True (Theoretical) Binding Parameters

model parameters used to generate theoretical data		apparent values[a]		true values	
		K_d[b] (μM)	N[c] (mol/mol)	K_d[b] (μM)	N[c] (mol/mol)
fluorescence independent of protein oligomerization		0.23	1.08	5	1
and linearly related to ligand binding level		0.22	1.19	15	1
tetramer model[d]	retinol data	0.10	0.67	5	1
	(limiting RFI = 55)	0.18	0.76	10	1
	β-ionone data (limiting RFI = 82)	0.24	1.16	66.6	1
octamer model[d]	retinol data (limiting RFI = 65)	0.047	0.60	10	1

[a] Apparent binding parameters derived from graphic analysis of the data according to the method of ref *18*, using the fluorescence corresponding to the largest ligand concentration in the data set (15 μM) as the "limiting" fluorescence. [b] Apparent dissociation constant defined as the product of the free ligand and the unoccupied binding site concentrations, divided by the concentration of occupied binding sites. [c] Limiting number of specific binding sites per protein monomer. [d] Model assuming protein tetramerization (or octamerization) and nonlinear dependence of the fluorescence on the ligand binding level. The was used for the simulation of the data in Figures 8 and 9 and columns IV and VIII of Table 2. It was assumed that static quenching by nonbound or nonspecifically adsorbed ligand did not occur, that all protein molecules were present as oligomers, that a single bound ligand could quench all protein molecules in such an oligomer, and that binding of additional ligand molecules to oligomers containing already one or more bound ligand molecules would not result in additional quenching of β-lactoglobulin fluorescence.

Figure 3 (not shown). Then we analyzed the curves in Figure 3 in more detail. Thus, it became clear that at least the extent of quenching in the top panel of Figure 3 cannot possibly be linearly related to the level of occupation of individual binding sites by retinol. (This was one of the assumptions upon which the calculations were based.) According to a linear relationship, the shape of the curve in the top panel would indicate half-saturation to occur at a total retinol concentration of ~2.1 μM. However, the β-lactoglobulin concentration was 8.8 μM and, thus, a total concentration of >4.4 μM retinol (free plus bound) would be required to occupy 50% of the available binding sites.

As none of the above possibilities provided a totally satisfactory explanation, we finally considered the possibility that the shape derived from the fluorometric binding experiments could have been influenced by multimer formation of β-lactoglobulin. β-Lactoglobulin is known to form dimers and oligomers at moderate pH (see refs *24*−*26*). To check this possibility, the fluorometric binding curve was again calculated with the help of a computer, but now with the additional assumptions that β-lactoglobulin forms oligomers and that a single bound ligand can contribute to the quenching of all monomer units present in the β-lactoglobulin multimer. The observed fluorometric behavior (Figure 3) could be easily simulated in this way, using a K_d of ~10 μM for retinol and using the same K_d of β-ionone as was found in equilibrium dialysis (66.6 μM), as shown in Figure 8.

Graphic analysis of the calculated data in Figure 8 according to ref *18* again yielded incorrect low K_d values, as expected (Table 3). With the method of Yoshida (*19*), it was not even possible to obtain a linear relationship for retinol, whereas an estimated K_d of ~15 μM (instead of 66.6 μM) yielded a straight line for β-ionone.

Although the simulation in Figure 8 corresponds to a situation in which β-lactoglobulin tetramers are formed, a significantly (although not sufficiently) improved shape of the binding curve was already observed in simulations where dimers occurred (not shown). It was verified that the trend calculated for dimer and tetramer formation persisted when going to higher oligomerization states, even though formation of octamers or higher oligomers is not very likely under our conditions. An acceptable shape of binding curve was still observed when octamers were assumed (not shown).

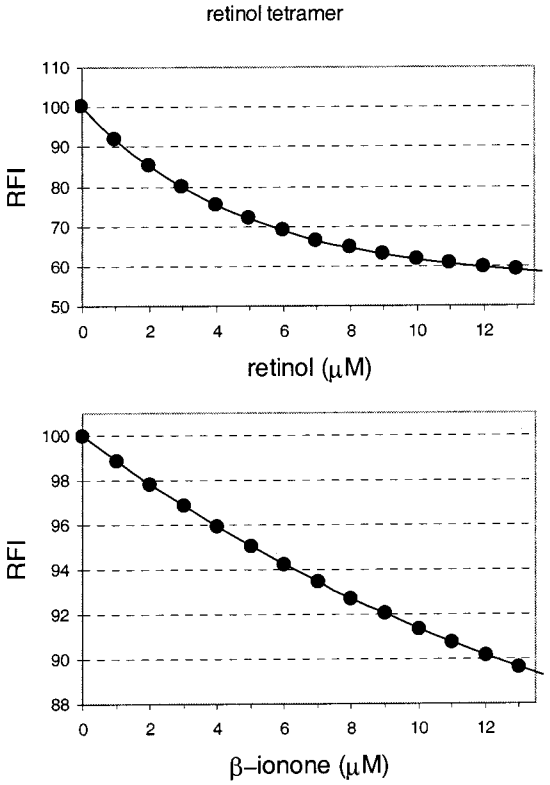

Figure 8. Simulated β-lactoglobulin fluorescence changes induced by ligand binding to β-lactoglobulin calculated for a β-lactoglobulin concentration of 10 μM: (top) simulation of retinol data with an apparent K_d of 10 μM for each individual binding site and a limiting RFI of 55; (bottom) simulation of β-ionone data with an apparent K_d of 66.6 μM for each individual binding site and a limiting RFI of 78. It was assumed that the binding stoichiometry was one ligand molecule per protein monomer, that static quenching by nonbound or nonspecifically adsorbed ligand did not occur, that all protein molecules were present as tetramers, that a single bound ligand could quench all four protein molecules in such an tetramer, and that binding of additional ligand molecules to oligomers containing already one or more bound ligand molecules would not result in additional quenching of β-lactoglobulin fluorescence.

Graphic analysis according to ref *18* of the simulated data again resulted in an incorrectly low apparent K_d of retinol: 0.047 μM (N = 0.60, Table 3). The best simulation results (i.e., the best shapes of the simulated

Table 4. Binding Parameters and Structural Descriptors for Fatty Acid Lactones

compound	K_d[a] (mM)	ΔG[b] (kJ/mol)	log P[c]	volume[d] (nm^3)	surface[d] (nm^2)
nonanoic acid γ-lactone	2.3230	−14.77	2.431	0.165	2.1
decanoic acid δ-lactone	4.706	−13.05	2.967	0.181	2.35
undecanoic acid γ-lactone	0.112	−22.18	3.503	0.199	2.63
dodecanoic acid δ-lactone	0.128	−21.84	4.039	0.214	2.80

[a] Estimated from fluorometric data according to the method of Yoshida (*19*), assuming $N = 1$, in agreement with equilibrium dialysis data. [b] Free energy of binding, $\Delta G = RT \ln K_d$. [c] Logarithm of 1-octanol/water partition coefficient (*34*). [d] Estimated using a Monte Carlo integration technique (*35*).

fluorescence binding curves) were obtained by assuming tetramer formation. The optimal K_d value for retinol, appearing from the tetramer simulations, was ~10 μM. With the same K_d value of 10 μM and the same set of model parameters used in Figure 8, we could also surprisingly well simulate the experimental data shown in Figures 5 and 6 (reflecting the competition with undecanoic acid γ-lactone, see Figure 9 and Table 2, columns IV and VIII). Without suggesting a particular number of monomers in the protein, this exercise indicates that β-lactoglobulin oligomer formation, in addition to graphical underestimation of limiting fluorescence levels [which distorts the Cogan- and Yoshida-type analysis (*18*, *19*) of binding parameters], is a likely explanation for the overestimation of the binding affinity by simple fluorometric analysis.

Various Methods for the Determination of Binding Constants. In conclusion, the data obtained from equilibrium dialysis appear to be more reliable than those from fluorometry. When fluorometry is preferred for its ease and time efficiency, the data should always be verified ("calibrated") using another method. As the binding parameters of fatty acid lactones derived from fluorometry appeared to be comparable to those derived from equilibrium dialysis (see Table 1 for undecanoic acid γ-lactone), the fatty acid lactone data obtained with either method appear to be reliable.

In other studies, dynamic methods involving column chromatography have been used to determine equilibrium ligand binding constants of β-lactoglobulin. Either ligand solutions were passed through a lactoglobulin column (*9*) or β-lactoglobulin solutions were passed through columns with adsorbed ligand (*12*). The advantage of those methods is convenience, speed of experimentation, and, thereby, minimization of possible oxidative ligand degradation. On the other hand, they do not allow the determination of binding stoichiometries, and it is not certain if binding equilibrium is always reached during passage through the column. In any case, the dissociation constants of β-ionone (52 μM) and undecanoic acid γ-lactone (101 μM) derived using a β-lactoglobulin-containing column (*9*) were closely in agreement with those found by us (Table 1).

Re-estimation of the Apparent Dissociation Constant of Retinol. If the K_d would have been 20−200 nM, as often stated (*13*, *27*, *28*), 86−91% of all bound retinol would have stayed in place, that is, bound to β-lactoglobulin, at 350 μM undecanoic acid γ-lactone and 8.7 μM retinol (Table 2, column I). According to Table 2 (column III), the corresponding RFI of retinol in Figure 5 could then never have been more than 65−74 at those ligand concentrations, assuming a linear dependence of fluorescence on binding. According to the nonlinear tetramer model described above, the RFI could not even have been more than 56−57 (Table 2, column IV). The RFI actually observed in Figure 5, at 350 μM undecanoic acid γ-lactone and 8.7 μM retinol, was clearly higher, namely 89. This indicates additional displacement of retinol by undecanoic acid γ-lactone and, thus, a higher K_d (lower affinity). Taking into account all of our findings, including the dialysis data shown in Table 1, the displacement of retinol by undecanoic acid γ-lactone shown in Figures 5 and 6 and analyzed in Table 2, and finally the simulations shown in Figures 8 and 9, we

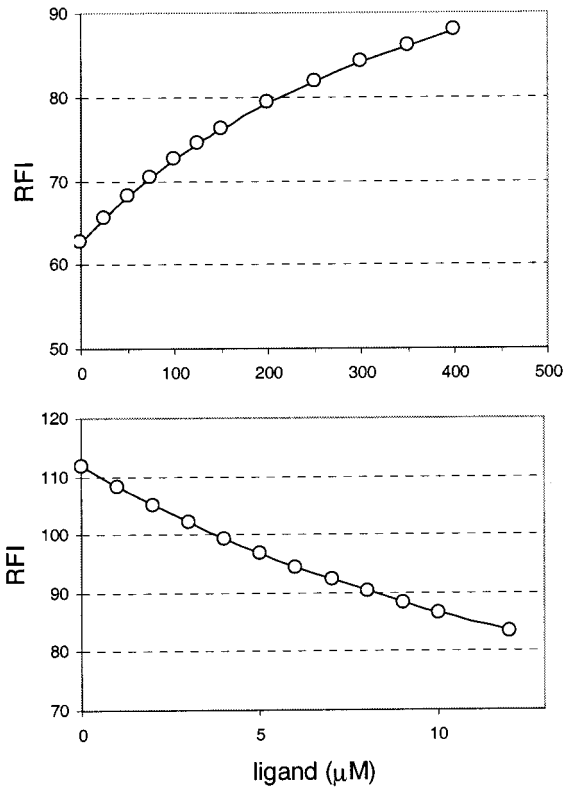

Figure 9. Simulated β-lactoglobulin fluorescence changes induced by competition between retinol and undecanoic acid γ-lactone for binding to β-lactoglobulin: (top) titration of β-lactoglobulin (8.7 μM) with undecanoic acid γ-lactone, in the presence of 8.7 μM retinol; (bottom) titration of β-lactoglobulin (8.0 μM) with retinol, in the presence of 440 μM undecanoic acid γ-lactone. It was assumed that the binding stoichiometry was one ligand molecule per protein monomer, that the apparent model K_d values of retinol and undecanoic acid γ-lactone were 10 and 111 μM, respectively, that the RFI of protein molecules having one bound undecanoic acid γ-lactone per monomer was 115, and that static quenching by nonbound or nonspecifically adsorbed ligand did not occur. Furthermore, it was assumed that all protein molecules were present as tetramers, that a single bound ligand could quench all four protein molecules in such an tetramer, and that binding of additional ligand molecules to oligomers containing already one or more bound ligand molecules would not result in additional quenching of β-lactoglobulin fluorescence. Finally, the quenching of the fluorescence of all monomers belonging to the same protein tetramer, induced by binding of the first retinol to that oligomer, was assumed to be 45%. This would result in a limiting RFI of $\{(m/4)\cdot 115 + [1 - (m/4)]\cdot 100\}\cdot 0.55$ of protein tetramers, which are carrying m bound undecanoic acid γ-lactone molecules ($m = 0, 1, 2, 3, 4$) and at least one retinol molecule.

estimate that the real K_d for retinol is ~10 μM, rather than 20−200 nM. In view of the price of retinol, we did not check this in dialysis experiments.

Comparison of the Binding of Various Fatty Acid Lactones. Influence of Hydrophobic and Steric Effects. In nature, fatty acid lactones serve, among others, as pheromones and flavor compounds. A set of lactones with different ring sizes and side chains was selected to evaluate the influence of hydrophobic and steric characteristics on β-lactoglobulin binding properties. The observed binding constants are presented in Table 4, together with structural parameters of these compounds.

The free energy of β-lactoglobulin−ligand association, ΔG, can be computed from the value of K_d by $\Delta G = RT \ln K_d$. The differences in K_d between γ- and δ-lactones correspond to an average $\Delta(\Delta G)$ of −3.703 and −4.393 kJ/CH_2 group, respectively. These results are in agreement with those reported in the literature for alkanones, −3.451 kJ/CH_2 group (*29*), and alkanes, −4.476 kJ/CH_2 group (*30*). The effect of chain length on the free energy of interaction indicates that the interaction between β-lactoglobulin and lactones is mainly hydrophobic. This is in agreement with the linear relationship between log K_b and log *P* observed for other compounds (*9*, *10*). No significant difference between the binding of undecanoic acid γ-lactone and dodecanoic acid δ-lactone was found, suggesting that in this case the protrusion of the alkyl chain in the binding pocket is deep enough to overcome the difference between the ring sizes. The affinity of β-lactoglobulin for the two lactones with a smaller alkyl chain is lower, and the influence of the different ring sizes, potentially reflecting steric hindrance, becomes larger. For the same lactone ring the dissociation constants have the same trends as log *P* and van der Waals surface and volume, these parameters reflecting the hydrophobic and steric interactions, respectively. For the same alkyl chain but different ring sizes, the apparent correspondence between K_d and log *P* disappears. In this case, K_d seems to reflect the van der Waals volume and surface, as a measure of the shape and size of the ligands. Thus, the interaction between β-lactoglobulin and these lactones is mainly hydrophobic (see also ref *31*), although steric interactions seem to play an important role as well.

This can be considered as indirect information about the location of the binding site. If the binding site were located at the surface of the protein, the interaction between β-lactoglobulin and ligands would be expected to be rather nonspecific and insensitive to (small) steric changes. However, the β-lactoglobulin binding site appears to have a narrow specificity, reflecting the steric requirements of a deep high-affinity binding cavity (*32*).

Clearly, β-lactoglobulin can well be used as a specific carrier of small hydrophobic flavor compounds and pheromones, such as fatty acid lactones, in addition to vitamins, polyunsaturated fatty acids, etc. By adapting the size of the cavity in recombinant β-lactoglobulin, using genetic engineering techniques, we expect that the protein can well be further optimized for its use as a high-affinity and high-specificity carrier protein in controlled-release application (see, for example, ref *4*). Having a reliable method for the analysis of the binding properties of such engineered proteins and taking into account the observations and pitfalls described above will certainly contribute to the development of such applications.

ABBREVIATIONS USED

RFI, relative fluorescence intensity with respect to β-lactoglobulin or tryptophan fluorescence in the absence of ligands; RET, resonance energy transfer.

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