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Nuclear Magnetic Resonance Spectroscopic Study of β-Lactoglobulin Interactions with Two Flavor Compounds, γ-Decalactone and β-Ionone

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Interactions between a well-characterized protein, β-lactoglobulin, and two flavor compounds, β-ionone and γ-decalactone, were studied by 2D NMR spectroscopy. NMR spectra were recorded in aqueous solution (pH 2.0, 12 mM NaCl, 10% D_2O) under conditions such that β-lactoglobulin is present in a monomeric state. TOCSY and NOESY spectra were recorded on the protein and the complexes between protein and ligands. The spectra of the NH−CH_α region showed the cross-signals due to the coupling between N- and C-bonded protons in the polypeptide backbone. The observed chemical shift variations in the presence of ligands can be assigned to changes in the protein conformation. It appears that the side chains of several amino acids are affected by binding of γ-decalactone point into the central cavity (Leu46, Ile56, Met107, and Gln120), whereas binding of β-ionone affects amino acids located in a groove near the outer surface of the protein (Leu104, Tyr120, and Asp129), as illustrated by molecular visualization. This NMR study provides precise information of the location of binding and confirms the existence of two different binding sites for aroma compounds on β-lactoglobulin, which was suggested in previous competition studies by fluorometry or affinity chromatography and by structural information obtained from infrared spectroscopy.

KEYWORDS: Aroma; binding site; protein; β-lactoglobulin; NMR spectroscopy; ionone; decalactone

INTRODUCTION

Interactions between proteins and flavor compounds have been the subject of numerous studies (*1*). As part of the main constituents of foods, or used as ingredients, proteins have the capacity to bind flavor molecules. These interactions of flavors with proteins significantly affect their impact on flavor perception (*2*, *3*). The studies conducted on these interactions are therefore motivated by the necessary knowledge development required for the control of food flavoring and for understanding flavor release in the mouth.

The structure and properties of bovine whey protein β-lactoglobulin (BLG) are well established (*4*−*6*). It is a small, water-soluble protein of 162 amino acid residues, belonging to the lipocalin family. It is built up of an eight-stranded, flattened β-barrel, or calyx, and a flanking three-turn α-helix. BLG is widely used as model in studies of interaction with flavor compounds (*7*).

Both reversible and irreversible binding may exist for aldehydes (*8*), whereas flavor compounds from other chemical classes such as esters, ketones, and alcohols bind to proteins by reversible interactions (*9*, *10*), characterized as being mostly hydrophobic in nature. Headspace analysis, equilibrium dialysis, or liquid chromatography have been used to quantify binding constants and numbers of binding sites of flavor retention by proteins (*7*). The effect of the medium, pH (*11*), ethanol, and salt concentration (*12*) on binding has been assessed and mainly explained by the changes in the conformation of the protein (*13*). A mathematical model has also been used to predict the equilibrium partitioning properties and the rate of release of methyl ketones (*14*). Competition studies have been made with different ligands (*15*, *16*), but interpretation of the results is difficult due to the lack of information concerning the localization of binding sites. The cocrystallizations of BLG with 12-bromododecanoic acid and with palmitic acid have independently been obtained, and X-ray diffraction studies have allowed binding of free fatty acids into the central cavity of the protein to be demonstrated (*17*, *18*). To our knowledge, no such information is available for aroma compounds. Concerning the binding site of retinol, the results of different authors are not in accordance. Considering the close structural resemblance of BLG and retinol binding protein, one might conclude that retinol binds in the central cavity (*19*), as confirmed by a recent X-ray study (*20*). However, other studies are in favor of an external binding site (*21*). Both sites seem to exist, with a preference of

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the fixation into the central cavity, the external binding site being occupied by retinol only in the presence of other ligands (*22*). Binding of retinol or β-carotene to this central cavity exerts a protective effect toward oxidation by preventing the fragmentation at the conjugated ene region (*23*).

The conformational and structural changes induced upon binding of various flavor compounds to BLG have been studied by Fourier transform infrared (FT-IR) spectroscopy (*24*, *25*). Similar spectral patterns have been obtained for retinol and tetradecanoic acid, leading to the conclusion that they might bind to the same site. Different patterns have been observed for the flavor compounds studied, but this method may allow only indirect determination of the localization of the binding sites.

Besides FT-IR spectroscopy, which is a method well suited for studying conformational changes in proteins such as those occurring on binding with small ligands, nuclear magnetic resonance (NMR) spectroscopy gives access to structural information down to the atomic level of the mechanisms underlying the molecular processes. Thus, NMR has been used in studies on the conformational and aggregation state of proteins, influenced by factors such as high pressure and temperature (*26*, *27*). For BLG, besides its structural characterization (*6*, *28*, *29*), cation-mediated aggregation (*30*), thermal denaturation (*30*, *31*), and effect of high pressures (*32*) have been studied by ^{1}H NMR. Interactions of proteins with small ligands have also been studied by NMR (*33*, *34*). Recently, an NMR solution study of the complex BLG/palmitic acid has been published (*35*) after it was shown by X-ray crystallography that BLG binds palmitic acid within its central cavity (*18*).

The aim of the present study was to evaluate the benefits of two-dimensional (2D) NMR spectroscopy for the direct determination of the topology of binding of flavor compounds to BLG. For this purpose we selected β-ionone and γ-decalactone, two ligands having dissociation constants at pH 3, measured by affinity chromatography, of 50 and 300 μM, respectively (*15*), and which have been shown by infrared spectroscopy to induce nonidentical conformational changes in BLG on binding (*25*). It was furthermore attempted to relate the obtained information on binding site localization for these two ligands to those for the two most studied BLG ligand classes, namely, retinoids and fatty acids.

MATERIALS AND METHODS

Materials. The conditions used in this study in terms of pH, ionic strength, and protein concentration were chosen to ensure that BLG is present in a monomeric state (*28*, *36*). BLG variant A (purity ≥ 90%; Sigma, St. Louis, MO) was used as received and dissolved at a concentration of 1 mM in 12 mM NaCl solution, adjusted to pH 2.0 with 1 N HCl, and containing 10% D_2O. The ligand-containing solutions were prepared in an identical manner, with the addition of 2 mM γ-decalactone (SKW Biosystems, La Ferté sous Jouarre, France) (BLG concentration was 1 mM) or 500 μM β-ionone (Sigma) (BLG concentration was 500 μM), respectively, to the NaCl solution before dissolution of the protein. Protein and ligand concentrations were lower for the β-ionone solution in order to have an approximately equal ratio of complexed over free protein in both solutions [calculated from literature dissociation constants (*15*)], while taking into account the lower solubility of β-ionone. All solutions were allowed to equilibrate overnight before NMR analysis was performed. To obtain chemical shifts for the protons of the free ligands, solutions of the aroma compounds were prepared according to the same protocol but without addition of protein.

NMR Spectroscopy. NMR spectra were recorded at 35 °C on a Varian INOVA 600 MHz spectrometer (Varian Inc., Palo Alto, CA), equipped with a 5 mm z-gradient probe at the Centre de Biophysique Moléculaire du CNRS, Orléans, France. The spectra were referenced relative to the water signal. The 10% D_2O added to the solutions guaranteed a correct spin lock while precluding any significant H−D exchange. TOCSY and NOESY spectra were recorded in the States-TPPI mode with mixing times of 40 and 150 ms, respectively. The Watergate sequence was used to suppress the solvent line. For data acquisition and data processing, the standard Varian programs were used.

Molecular Visualization. The NMR structure of β-lactoglobulin (1CJ5) was obtained from the Brookhaven Protein Database (PDB), as deposited by Kuwata et al. (*6*). The PDB data were processed using WebLab ViewerPro software (Accelrys Inc., San Diego, CA).

RESULTS

TOCSY was used to generate maps of short- to long-range coupling correlations among protons. One region of these spectra is of particular interest here: the NH−CH_α region, which shows the cross-signals due to coupling between *N*- and C_α-bonded protons in the polypeptide backbone. The former show signals in the δ 6.5−10 range, whereas the latter can be observed in the δ 4−6.5 range. The thus-defined region can be considered to be a fingerprint region, and the chemical shifts of protons within this region are highly conformation sensitive. A change in the protein conformation, such as the one accompanying a ligand-binding event, can cause local twisting of the polypeptide chain and a corresponding variation of the chemical shift of the coupling signals. The aim of the TOCSY experiments was thus to monitor such potential variations and to identify those amino acids that are involved in conformational changes.

Figure 1a is a partial view of the TOCSY spectrum of a 1 mM BLG solution, showing cross-peaks belonging to the NH−CH_α region. The F2 domain corresponds to the NH signals, whereas the F1 domain corresponds to the CH_α signals. Amino acid assignments are based on the complete assignments by Uhrínová et al. (*29*). Resonances belonging to the α-helix show F1 chemical shifts in the 3.9−4.4 ppm range, whereas those of other structures such as β-sheet and loops are spread out from 3.6 to 6.6 ppm. Most of the signals in **Figure 1a** could clearly be assigned, except those in the crowded region of δ_{F2}/δ_{F1} 7.8−8.4/4.1−4.5.

Figure 1b shows a spectrum recorded under identical conditions from a solution containing 1 mM BLG and 2 mM γ-decalactone. Overall, the pattern obtained is very similar. This confirms earlier findings that the BLG structure at this pH is globally unaffected by ligand binding (*17*, *18*). However, a number of shift variations can clearly be identified; these are listed in **Table 1**. All affected amino acids are located on the β-barrel, which forms the wall of the central cavity. In **Figure 2a**, these amino acids are identified on a cartoon of the BLG structure. It appears that two outer surface sites are formed by the side chains of Lys47, Leu57, Lys70, and Ile72, on the one hand, and Val81 and Glu89, on the other hand, whereas Leu46 and Ile56 are in close vicinity to Lys 47 and Leu 57, but on the inner face of the β-barrel. The residues Met107 and Gln120 are located at the opposite wall of the β-barrel, on strands G and H, respectively, and point toward the inside.

Similarly, the complex between BLG and β-ionone was studied by TOCSY. **Figure 1c** shows the spectrum of the NH−CH_α region obtained from a solution containing 500 μM BLG and 500 μM β-ionone. Again, the overall pattern remains unchanged, and again some shift variations can be observed. Compared to BLG in the presence of γ-decalactone, these are, however, fewer in number and concern entirely different amino acids and different regions on the protein. **Table 2** lists these shifts, and **Figure 2b** identifies the affected residues on the BLG

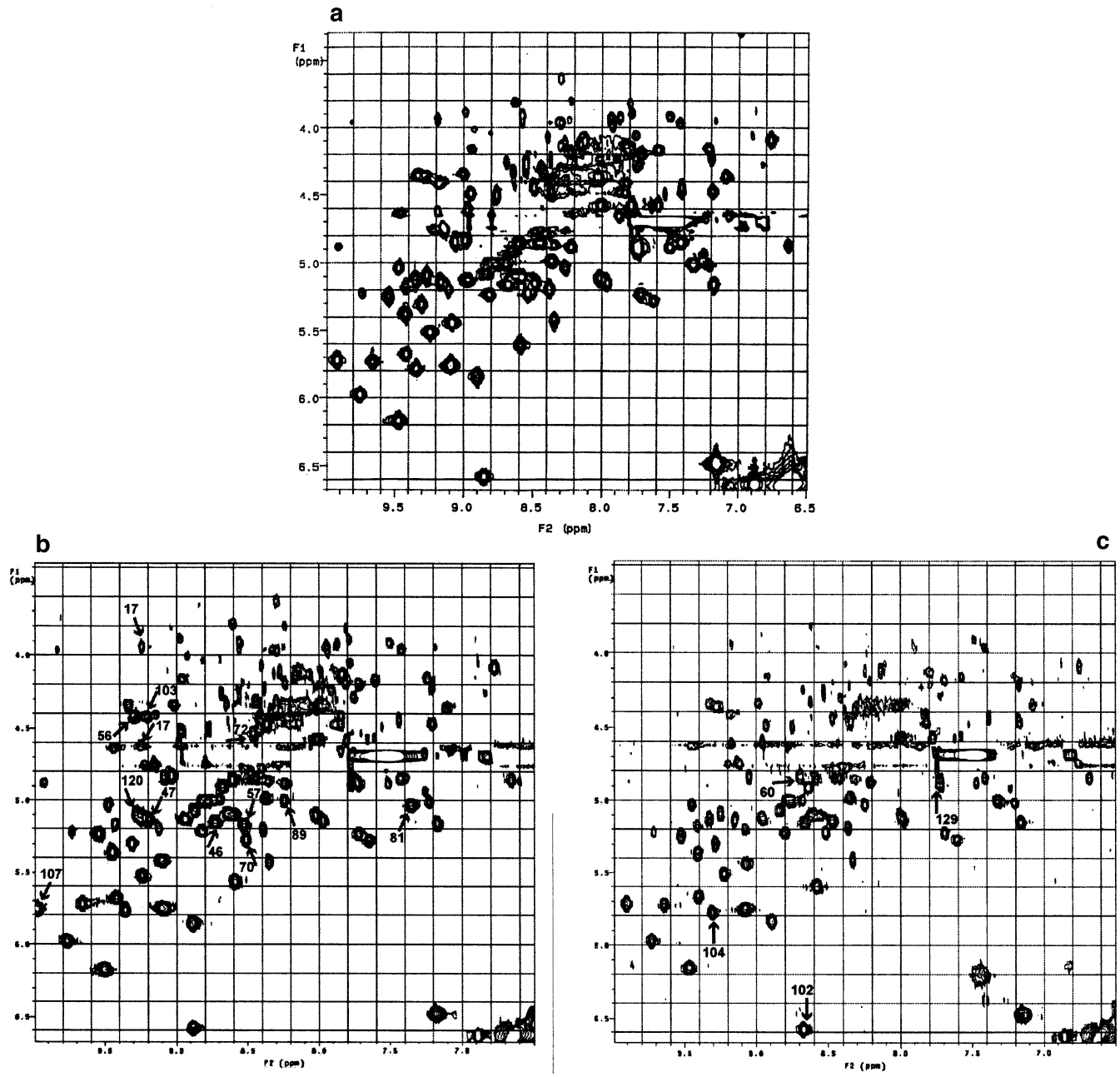

Figure 1. TOCSY spectra of BLG at pH 2.0 and 35 °C: (a) BLG alone (1 mM solution); (b) BLG (1 mM) and γ-decalactone (2 mM); (c) BLG (500 μM) and β-ionone (500 μM). Mixing time was 40 ms. Only the NH–CH_α regions are shown.

cartoon. The upfield shift in the F2 domain of Lys60 and Tyr102, amino acids with hydrogen-bonding side chains, is particularly pronounced. Lys60 is located on the β-barrel, at the outer surface of the protein. Leu104 and Tyr102 are also located on the β-barrel, opposite to Lys60 on strand G, whereas Asp129 is on the loop at the C-end of the α-helix. However, their three side chains are close together, in a groove between the outer side of the β-barrel and the α-helix (**Figure 2b**).

In an attempt to characterize these complexes even further, NOESY was used. This technique provides information about spatial relationships between protons. In contrast to TOCSY, there is no need for connectivity between protons to observe a correlation; ligand protons and protein protons can yield cross-peaks in the spectrum, provided they are close enough in space. Hence, the aim was to identify NOE effects that could help to define the relative positions of the complexing partners.

The bands of γ-decalactone are exclusively in the aliphatic region of the 1D spectrum, one signal at δ 4.59 (>CH−O−)

Table 1. NH-CH_α Shifts in the BLG–γ-Decalactone Complex, As Observed by TOCSY

residue	amino acid	secondary structure	$\Delta\delta_{F2}$[a] (ppm)	$\Delta\delta_{F1}$[a] (ppm)
17	Gly	β-sheet (strand A)	+0.06	±0.00
46	Leu	β-sheet (strand B)	+0.06	–0.02
47	Lys	β-sheet (strand B)	+0.04	±0.00
56	Ile	β-sheet (strand C)	+0.01	+0.06
57	Leu	β-sheet (strand C)	+0.03	+0.02
70	lys	β-sheet (strand D)	+0.03	+0.04
72	Ile	β-sheet (strand D)	–0.03	+0.09
81	Val	β-sheet (strand E)	+0.02	+0.02
89	Glu	β-sheet (strand F)	–0.03	–0.04
107	Met	β-sheet (strand G)	+0.07	+0.03
120	Gln	β-sheet (strand H)	+0.07	–0.03

[a] $\Delta\delta_{F2}$ and $\Delta\delta_{F1}$ are the observed variations upon complexation of the chemical shifts in the F2 (NH signals) and F1 (CH_α signals) domains, respectively.

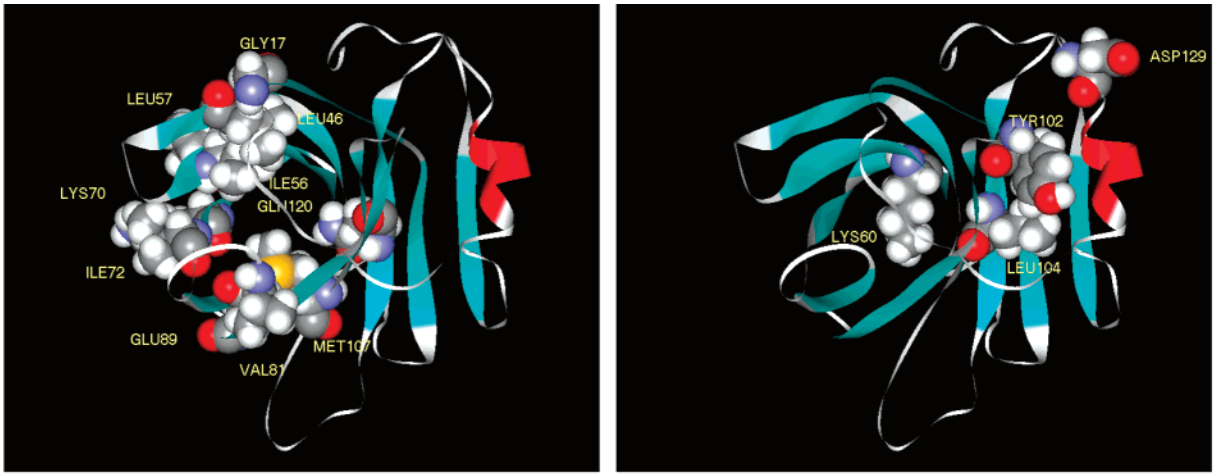

Figure 2. CPK display of amino acid side chains affected by complex formation with (a) γ-decalactone and (b) β-ionone, superimposed on a strands display of the BLG structure.

Table 2. NH-CH_α Shifts in the BLG–β-Ionone Complex, As Observed by TOCSY

residue	amino acid	secondary structure	$\Delta\delta_{F2}$[a] (ppm)	$\Delta\delta_{F1}$[a] (ppm)
60	Lys	β-sheet (strand C)	−0.29	−0.02
102	Tyr	β-sheet (strand G)	−0.18	±0.00
104	Leu	β-sheet (strand G)	−0.04	−0.02
129	Asp	loop H-α	+0.05	−0.03

[a] $\Delta\delta_{F2}$ and $\Delta\delta_{F1}$ are the observed variations upon complexation of the chemical shifts in the F2 (NH signals) and F1 (CH_α signals) domains, respectively.

and the other signals between δ 1 and 2. Due to the large number of resonances in the latter region of the spectrum, no NOEs involving those ligand protons could be identified. However, two new cross-peaks are clearly visible in the presence of γ-decalactone, at δ_{F2}/δ_{F1} 9.20/4.40 and 8.52/4.40 (spectra not shown). The observed shift in the F1 domain does not exactly correspond to the >CH−O− shift in the 1D reference spectrum; this may be a result of ligand complexation. Although a precise assignment of the shifts in the F2 domain is difficult due to the fact that a few NH groups have shifts close to these new cross-peaks, it is interesting to note that they exactly match NH signals that have already shown shift variations on complexation in the TOCSY experiment. These are Lys70, shifted from 8.49 to 8.52 ppm, and Lys47, shifted from 9.16 to 9.20 ppm.

The second ligand studied, β-ionone, contains two olefinic protons, with chemical shifts of 6.23 and 7.49 ppm. The other signals lie again within the aliphatic region of the spectrum. In the NOESY spectrum, one new cross-peak appears at δ_{F2}/δ_{F1} 8.23/7.50 (spectra not shown). Again, this signal is likely related to a backbone NH; its assignment requires further work.

DISCUSSION

A certain number of NH and/or CH_α chemical shift variations were clearly observed in the presence of the ligands. A closer examination of these shift variations in the context of the localization of the respective amino acid residue within the protein molecule allows conclusions on the likely position of the bound ligand relative to the protein to be drawn. TOCSY of BLG in the presence of excess amounts of γ-decalactone reveals that most of the conformational changes take place on those amino acid residues that form the wall of the protein's central cavity (Leu46, Ile56, Met107, and Gln120), although binding also exists on the outer surface, especially on the β-sheet area formed by the strands B, C, and D (Lys47, Leu57, Lys70, and Ile72). The existence of the central cavity as a ligand-binding site has been well established by various methods, but so far it is still not entirely clear if this is the preferred binding site for retinol or if binding to a surface pocket (*21*) is predominant. However, a recent X-ray study (*20*) of the BLG−retinol complex indicates that under the conditions used for growing the crystals retinol is bound to the central cavity. Fatty acids have conclusively been shown to bind to this central cavity (*17*, *18*, *35*), although the latter authors have found ligand affinity to decrease markedly with decreasing pH. At pH 2, the amount of bound palmitic acid has been found to be reduced by 80% relative to pH 7.3 (*35*). On the other hand, the fatty acid analogue 5-doxylstearic acid has been reported to bind to BLG at pH 2 (*37*). The strengths of retinol binding (*38*) and of various aroma compounds (*11*) have also been shown to be pH dependent in the pH range 3−9. The observation that γ-decalactone also binds to the central cavity of BLG at pH 2 where the protein exists in its monomeric native state confirms our previously published results obtained by infrared spectroscopy (*25*) showing that retinol, tetradecanoic acid, and γ-decalactone induce similar conformational changes on binding to BLG and are thus likely to bind to the same site. A recent fluorometric study on retinol and γ-undecalactone binding to BLG (*16*) has shown that there is competition between both ligands. The effect of the chain length on the free energy of interaction between BLG and different lactones indicated that the interaction is mainly hydrophobic, which led the authors (*16*) to conclude that the preferential binding site for the lactones is likely to be the central cavity. Taken together, the present work and the latter two studies (*16*, *25*) not only confirm the preferred binding site for aliphatic lactones but also provide further evidence in favor of retinol binding to the central cavity. This had already been proposed previously by several authors (*19*, *39*−*41*) but is in contradiction to the work of other researchers (*21*, *42*).

Second, TOCSY of BLG in the presence of β-ionone shows that binding of this ligand affects entirely different amino acid residues compared to γ-decalactone. These amino acid residues (with one exception, Lys60) are located near the outer surface of the protein, in a groove close to a region that has been described by Monaco et al. (*21*) as a hydrophobic surface pocket. In particular, the side chain of Tyr102 lies in the same region

as the side chains of three amino acids (Phe136, Ala139, and Leu140), identified by these authors as being part of this site. This again fits well with previous results (*25*) obtained by infrared spectroscopy, which indicate a different binding site for this ligand as opposed to retinol, tetradecanoic acid, and γ-decalactone. Furthermore, this seems to be in agreement with the findings of Sostmann et al. (*15*), who observed, by affinity chromatography, the absence of competition or at least reduced competition between β-ionone and γ-octalactone, and with the results of Jouenne et al. (*43*), who hypothesized that the high variations of binding constants for β-ionone as a function of pH are also in favor of an external binding site, resulting from the surface exposition of residues normally hidden in the structure at low pH values. The observed large shift of Lys60 NH may arise from hydrophobic interaction. This residue is at the connection between strand C and loop C−D, the aliphatic chain pointing toward the outer surface and being anchored by a hydrogen bond between the terminal NH_2 and Glu62.

It is very interesting to note that a number of residues of the proposed preferential binding sites for both ligands, namely, Met107, Gln120, Tyr102, and Asp129, include hydrogen bond donor and/or acceptor functions on their side chains. In addition to hydrophobic interactions, these functional groups may contribute to the formation of ligand−BLG complexes by hydrogen bonding (*44*).

In conclusion, it was shown that 2D NMR techniques are a valuable tool in the study of binding of flavor compounds to BLG. The detailed view of the ligand-binding event provided by this technique and the fact that the experiments can be performed in solution and with native nonmodified protein clearly make it superior to other techniques, such as ligand competition studies by various methods, fluorimetry, electron spin resonance spectroscopy, and chemical modification studies. The resolving power of NMR can be matched only by X-ray studies on crystalline protein complexes, which are notoriously difficult to obtain and potentially not always representative of the conditions in solution.

The results obtained for both ligands, β-ionone and γ-decalactone, show that they preferentially bind to different sites on the BLG molecule. Binding of γ-decalactone seems to take place in the central cavity, whereas β-ionone appears to bind to a region in what has previously been described as a hydrophobic surface pocket. This is in good agreement with published ligand competition studies and confirms structural information gained by infrared spectroscopy while providing for the first time precise information on the location of binding. The potential role played by hydrogen bonding in BLG−ligand interaction possibly deserves more attention and is currently addressed by work in our laboratory.

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