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Homology of β-Lactoglobulin, Serum Retinol-Binding Protein, and Protein HC

Abstract. *The milk protein β-lactoglobulin has been extensively studied but its function has not been identified. A clue regarding the function of a protein can be obtained by discovering a genetic relationship with a protein of known function through comparisons of amino acid sequence. Such comparisons revealed that β-lactoglobulin is similar to human serum retinol-binding protein and to another human protein of unknown function known as complex-forming glycoprotein heterogeneous in charge (protein HC). β-Lactoglobulins from several species have been found to bind retinol, while the absorption and fluorescence properties reported for the unidentified heterogeneous prosthetic group of protein HC are retinoid-like. The role of serum retinol-binding protein in vitamin A transport in the circulation suggests that the other two homologous proteins may function in the binding and transport of retinoids; β-lactoglobulin may facilitate the absorption of vitamin A from milk and protein HC may mediate the excretion of retinol-derived metabolites.*

β-Lactoglobulin (BLG) is the major globular protein component of bovine milk whey. It exists as an equilibrium mixture of monomer (molecular weight of 18,500) and dimer under physiological conditions (*1*). BLG has only been found in the ruminants (as the dimeric form) and in the pig (as the monomer). Milks from rodents, primates, and lagomorphs are devoid of BLG (*2*). The milk of a member of each of two species of aquatic mammals, the dolphin (*Tursiops truncatus*) and the manatee (*Trichechus manatus*), contains high concentrations (up to 2 percent weight to volume) of monomeric BLG's. A partial sequence has been obtained for one dolphin BLG (Fig. 1). While comparing the sequences of this protein and bovine BLG (BBLG) to those of other proteins, we observed the similarity of both BLG's to the published sequence (*3*) of human serum retinol-binding protein (RBP). The similarity became apparent when residue 1 of the BLG's was aligned with residue 6 of RBP (Fig. 1) and three gaps were placed in each sequence. RBP is also longer and extends six residues beyond the end of the alignment. Dolphin BLG (in regions where the sequence is known) was identical to RBP at six sites that are not among the 33 residues that were identified in BBLG and RBP.

The strength of the case for homology of a pair of proteins can be enhanced by the addition of a third divergent homolog to the alignment; the probability of the similarity between all three proteins being coincidental is very low (*4*). The addition of a protein known as the complex-forming glycoprotein heterogeneous in charge (protein HC) to the alignment (Fig. 1) provided further links between the sequences of RBP and BLG's. Protein HC is an α_1-glycoprotein that was originally isolated from the urine of patients with chronic cadmium poisoning (*5*). It is also found in normal urine, plasma, and cerebrospinal fluid in relatively low amounts but is present in high concentrations in the urine of patients with tubular proteinuria and in both blood and urine of patients on renal dialysis (*6*). The sequence of HC (*7*) was added to the alignment (Fig. 1) so that residue 1 of RBP corresponded with residue 2 of HC, and residue 1 of BBLG with residue 7 of HC, with gaps inserted to optimize the homology. Although the number of identical residues between HC and RBP and between HC and BBLG were less than between RBP and BBLG, the pattern of identities provided evidence of a familial relationship. In comparing all three proteins, 64 positions were identical between one or another pair of proteins, while 15 positions had identical residues in all three complete sequences. A large proportion of these residues were amino acids that occur infrequently in proteins (*4*), which decreases the possibility of a coincidental similarity. Comparison of the codons for the amino acids in corresponding positions shows that the percentage of amino acids that are identical or differ by one base in their codons was 62 percent for BBLG and RBP, 62 percent for HC and BBLG, and 58 percent for HC and RBP. The percentages of identical residues and the normalized alignment scores (NAS) of computer aligned sequences (Table 1), in comparison with the corresponding scores for randomized sequences of the same composition, indicate a relationship between the three proteins (*4*).

Although there are differences between the numbers of disulfide bonds in RBP, BBLG, and HC, the arrangements of these bonds based on reported data (*1, 3, 7*) are consistent with the view that the

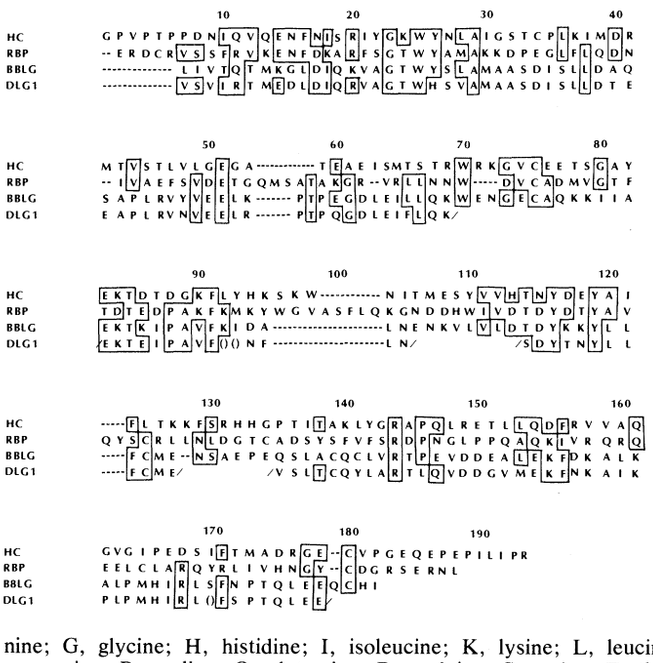

Fig. 1. A comparison of the amino acid sequences of protein HC (HC), human serum retinol-binding protein (RBP), bovine β-lactoglobulin (BBLG), and dolphin β-lactoglobulin-1 (DLG-1). The sources of the sequences are given in the text, apart from DLG-1 (*17*). The alignments are slightly adjusted from that given by the pairwise computer alignments of sequences, so as to give an optimal alignment of the four sequences. Boxed residues are identical in pairs of nonisologous sequences. D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

Table 1. A quantitative comparison of the sequences of protein HC (HC), bovine β-lactoglobulin (BBLG), and human serum retinol-binding protein (RBP). Percent identities, normalized alignment scores (NAS), and statistical significances (n) in standard deviations for each aligned pair of sequences were obtained by computer analysis as described (*4*). Numbers in parentheses indicate the sequence length.

Protein 1	Protein 2	Identity (%)	Gaps	Gaps/100	NAS	(n)
HC(181)	BBLG(162)	18.5	3	1.8	151	3.0
HC(181)	RBP(182)	18.2	2	1.1	160	5.0
RBP(182)	BBLG(162)	24.1	6	3.5	168	3.6

proteins may have similar three-dimensional structures. The three proteins have one bond between corresponding cysteinyl residues based on the alignment in Fig. 1, and RBP and BBLG have a second bond which produces a disulfide loop (Fig. 2). One cysteinyl residue in this loop is at a homologous site (position 125), but the residue with which it is paired does not correspond in the two proteins (position 134 in RBP; position 139 in BBLG). In about 50 percent of the molecules in bovine BLG preparations (*1*), the disulfide bond links cysteines 125 and 141 (Fig. 1) with residue 139 rather than 141 having a free thiol group. This would suggest that the loop region may be flexible, so that the abbreviated loop in RBP would be compatible with a similar conformation to BBLG. However, dolphin (as well as porcine) BLG does not have a cysteine at position 141. If disulfide bonds are more frequently acquired rather than lost during the course of evolution (*8*), the disulfide bond arrangements, as well as quantitative sequence differences, suggest that HC diverged at an earlier time from the line that led to BBLG and RBP.

Recently, the three-dimensional structure of the complex of RBP with retinol has been determined at a resolution of 3.1 Å by x-ray crystallography (*9*). The general features of BLG at low resolution (*4*), when considered in relation to secondary structure predictions (*10*), suggest that BLG and RBP may have closely similar overall conformations. The major features of RBP are an eight-stranded β-barrel core that encloses the retinol molecule, and a rod of α-helix encompassing residues 147 to 160 (positions 151 to 165 in Fig. 1). Like RBP, the central region of BLG is thought to consist of β-sheet (*1, 10*). A prominent rod with an electron density equivalent to about 20-Å length is present on one edge of the molecule. This appears to correspond to a section of polypeptide chain in a rod of α-helix that corresponds almost exactly in location and length to the α-helical region in RBP (BBLG residues 129 to 143; positions 149 to 163 in Fig. 1). The main deletions in both the BBLG and HC sequences in the alignment (Fig. 1) around positions 55 and 100 correspond in location to a β-turn and a loop, respectively, in RBP and therefore could be accommodated without disrupting the conformation of RBP. Furthermore, the additional six residues at the COOH-terminus of RBP that are not present in BBLG (positions 183 to 188) do not appear to play a significant role in the structure, since they were not located on the electron density map, and are probably disordered (*9*).

The homology of the three proteins suggests the possibility of common functional properties. This is partially supported by previous reports that BBLG forms stable stoichiometric complexes with retinol (*11*). Using similar methodology we have found that both dolphin BLG's, together with the manatee and porcine proteins, bind retinol to form complexes that are stable to isolation by gel filtration. Although the binding of retinol by BLG could reflect a general affinity for small hydrophobic molecules, the relationship between BLG and RBP suggests that binding is a significant and specific phenomenon that probably reflects BLG function. High concentrations of esters of vitamin A are found associated with fat in milk. Since vitamin A is important in development, it is reasonable to speculate that BLG might function in binding, protecting, and facilitating the uptake of retinol in the intestine of suckling young animals as it is released during the dispersion and digestion of milk fat. BLG is unique among the major milk proteins in its solubility at low *p*H values (*12*), so that it would pass rapidly through the stomach and reach the intestine in an intact form. The observed presence of BLG in the urine of young suckling ruminants attests to its survival in intact form and specific absorption by the intestine (*13*). The very high levels of BLG's in dolphin and manatee milk would then be related to the extremely high fat content of their milk (20 to 30 percent weight to volume) (*14*). Since retinol and its derivatives are both insoluble and labile, a specific transport system of this type could be nutritionally beneficial to the young animal.

Proteins present only in milk are of particular evolutionary interest since they might be expected to be coeval with the mammals. Another specific milk protein, α-lactalbumin, is homologous with the type C lysozymes; distribution and comparative sequence studies indicate that the milk protein arose after duplication of a lysozyme structural gene at a time close to the origin of the mammals (*15*). In the case of BLG and RBP (or HC), the level of similarity is far less than that between α-lactalbumin and lysozyme, suggesting that their most recent common ancestor appeared before the mammals.

The function of HC is unknown, as is the nature of the prosthetic group responsible for its yellowish-brown color (*5*). The color is associated with one or more fluorescent chromophores which have a broad absorption band between 320 and 550 nm and a shoulder at 345 mm (*5*). Although the chromophore remains associated with the protein after denaturation, suggesting possible covalent attachment (*5*), the chromophore was not located at a specific site in amino acid

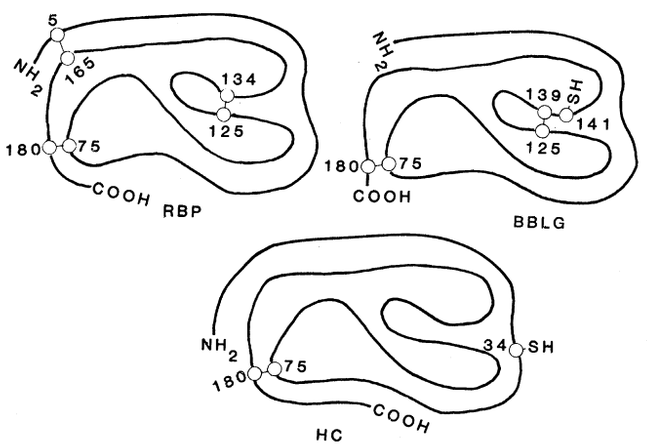

Fig. 2. Arrangements of disulfide bonds and cysteinyl residues in HC, RBP, and BBLG. Residue numbering corresponds to that in Fig. 1.

sequence studies (*7*). Since all amino acids in the sequence were identified, this would appear to suggest noncovalent binding. Because of the homology of HC with RBP and BLG, we suggest that the chromophoric group in HC may be a retinoid, which would only be removed by extraction with organic solvents. Previous studies have suggested that HC might play a role in excretion, since large amounts are found in the urine of patients with tubular proteinuria, presumably as a result of a failure of the kidney to catabolize the protein, as well as in a high concentration in the plasma of patients maintained on hemodialysis (*5*). HC may be a vehicle for the controlled excretion of retinol metabolites.

Other workers have suggested that two intracellular retinoid-binding proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRAB), and two lipid-binding proteins, P2 myelin protein and Z protein, show sequence similarity (*16*). In the region where the intracellular proteins are most similar (near the NH_2-terminus) they have a small amount of similarity with RBP, BLG, and HC. Their similarity in other regions is not sufficient to justify the conclusion that they are all related, but it appears possible that the intracellular and extracellular retinol-binding proteins might also have a common, though distant, relationship.

Note added in proof: Feigelson (*18*) has noted a significant, but limited, sequence relationship of the three proteins discussed here with α_2-globulin, and the structure of BBLG at 2.8-Å resolution shows similarities to RBP (*19*).

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Cyclosporin A Binding to Calmodulin: A Possible Site of Action on T Lymphocytes

Abstract. *Cyclosporin A, a potent immunosuppressive agent, has been widely used to treat patients with solid organ transplants. Although its precise mechanism of action is unknown, it appears to inhibit subsets of T lymphocytes at an early stage in cell activation. Fluorescent, fully active derivatives of cyclosporin A and calmodulin, a protein that binds calcium and is therefore essential to normal cell function, were utilized to demonstrate that cyclosporin A binds to calmodulin. Flow cytometry showed that the calmodulin inhibitors R24571 and W-7 competitively inhibited binding of cyclosporin A to cloned T lymphocytes. Cyclosporin A inhibited the calmodulin-dependent activation of phosphodiesterase in a dose-dependent manner. Binding of cyclosporin A to calmodulin may prevent the latter's role in the activation of the second messengers and enzymes required for effective cell proliferation and function in the immune response.*

Cyclosporin A (CsA) has become a principal immunosuppressive agent in solid organ transplantation and offers the possibility of an effective treatment for graft-versus-host disease, autoimmune disorders, malaria, and schistosomiasis, disorders that may affect over 1 billion people worldwide (*1*). There has been

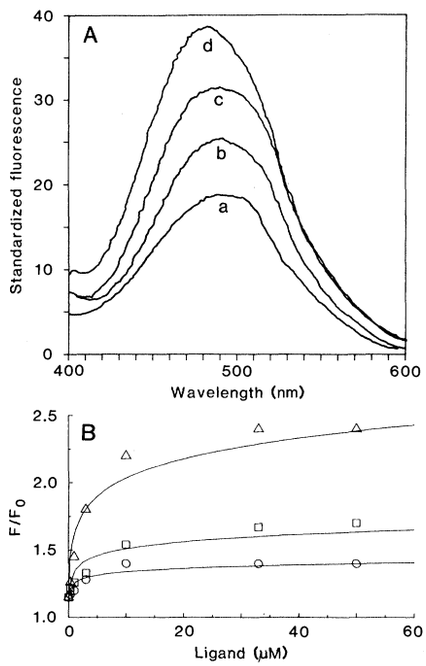

Fig. 1. (A) Fluorescence emission spectra of $1 \times 10^{-6}M$ dansylated calmodulin alone (a) and in the presence of $3 \times 10^{-5}M$ W-7 (b), $3 \times 10^{-5}M$ CsA (c), and $3 \times 10^{-5}M$ R24571 (d). Spectra obtained with insulin (0.1 mg/ml) and an ethanol control were identical to the spectrum of dansylated calmodulin alone. Solutions contained 0.2*N* KCl, 1.0 m*M* $CaCl_2$, and 50 m*M* 4-morpholinepropanesulfonic acid (MOPS) in glass-distilled water buffered to *p*H 7.3 at 25°C. Insulin was initially solubilized with HCl and buffered to *p*H 7.3. All studies were performed on a Mark I spectrophotometer (Farrand Optical). Maximum excitation wavelength was 345 nm for all ligands tested. Maximum increase in fluorescence occurred within minutes. Maximum emission wavelength was 495 nm, although a small blue shift in wavelength was noted with R24571. Control solutions of MOPS buffer, CsA, R24571, W-7, and ethanol without dansylated calmodulin yielded no intrinsic fluorescence. (B) Increase in fluorescence versus concentration of ligand. Ligands R24571 (△), W-7 (○), and CsA (□) were initially solubilized in 99 percent ethanol to a concentration of $10^{-2}M$. Serial dilutions of each ligand were made from 5×10^{-5} to $1 \times 10^{-8}M$ in MOPS buffer and incubated with 1 μ*M* dansylated calmodulin. The increase in fluorescence for dansylated calmodulin (F/F_0) was calculated at each concentration of ligand; where F is observed fluorescence of dansylated calmodulin bound to ligand minus fluorescence of ligand alone and F_0 is fluorescence of unbound dansylated calmodulin.