Molecular Interactions in β-Lactoglobulin. VII. The "Hybridization" of β-Lactoglobulins A and B[2]

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The possibility of interchange of the low *p*H-dissociated subunits of β-lactoglobulins A and B has been studied. No electrophoretically distinguishable hybrids are formed when a mixture of the two proteins is acidified and reneutralized. Using radioactively labeled β-A it was shown that no hybrids of any kind are formed, indicating a specific structural difference in the area of subunit contact.

Introduction

β-Lactoglobulin, the major protein of milk whey, has been known for a number of years to be a mixture of two similar proteins.[4] In 1955, Aschaffenburg and Drewry[5] reported that the two proteins, β-lactoglobulin A (β-A) and β-lactoglobulin B (β-B),[6] were produced individually by some cows and in mixture by others. They showed by progeny tests that the type of protein produced is under the control of a single pair of genes. These results have since been confirmed by us on 192 cows of different breeds in the Beltsville, Maryland, herd.[7]

In previous communications we have shown[8,9] that the two genetic species of β-lactoglobulin undergo a reversible dissociation below *p*H 3.5. In the isoelectric region, *i.e.*, at *p*H's close to *p*H 5.2, the kinetic unit of both β-lactoglobulins has a mol. wt. of about 36,000. As the *p*H is lowered, however, the average molecular weight decreases as shown by a large decrease in sedimentation constant and turbidity. Archibald sedimentation experiments at low *p*H's have shown that the dissociated species have a molecular weight of about 18,000 or half of the isoelectric value.[10] Thus, at low *p*H's, β-lactoglobulin is split into subunits which are half of the isoelectric species. Upon neutralization, the original turbidity values, sedimentation constants and electrophoretic mobilities are regained and the proteins are recrystallizable.

The difference between β-A and β-B appears to be a small one. Tanford[11] has shown that β-A has two more titratable carboxyl groups that β-B, out of a total of close to 50. This difference is reflected in the electrophoretic properties of the two proteins, β-A having a higher mobility than β-B at *p*H's above the isoelectric point[12] and a lower isoelectric point.[13] This difference in mobilities permits electrophoretic separation of the two proteins in prolonged runs of 18 hr. or more. At *p*H's farther removed from the isoelectric, the percentage charge difference becomes increasingly smaller and a mixture of the two proteins migrates essentially as a single peak above *p*H 6. Other known differences between the two are: β-A migrates faster in paper electrophoresis at *p*H 8.4 than does β-B,[5] possibly reflecting a difference in denaturation rates; β-A associates to a tetramer (144,000 mol. wt.) at temperatures close to 0° between *p*H 3.7 and 5.2, whereas β-B does not.[14–16]

Detailed experiments on the dissociation of the two β-lactoglobulins below *p*H 3.5 have shown that ultracentrifugally the two are not distinguishable, while light scattering measurements have revealed only a small difference in the free energies of dissociation of the two[8]; thus, it is reasonable to conclude that the dissociation patterns of the two proteins must be quite similar. In the previous studies it has not been possible to conclude whether a mixed reassociation, which would form hybrid molecules made up of β-A and B subunits, can occur. It should be noted in this connection, that heterozygous cows produce no hybrid β-lactoglobulin, but only a mixture of the two discrete genetic types. Another important question is whether each species of β-lactoglobulin is composed of two identical half molecules or of two different subunits. A definite possibility is that the entire difference is concentrated in one chain, the other subunit being common to the two proteins. This is the case with hemoglobins A and S.[17–19] It is the purpose of this paper to present the results of experiments aimed at the elucidation of these problems.

Experimental

Materials and Methods.—Non-radioactively labeled β-lactoglobulins A and B: These were prepared from the milks of individual cows by the method of Aschaffenburg.[20]

Experimental Animal.—A Holstein–Sindhi crossbred cow, known to produce only β-A was made available. The animal weighed 1350 lbs. and was at the end of the lactation period.

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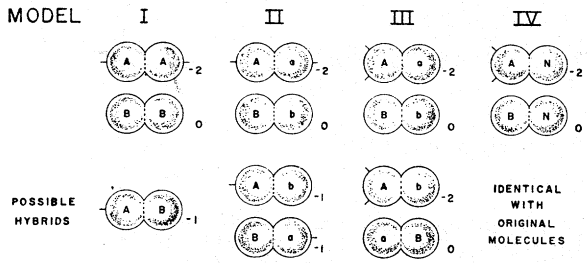

Fig. 1.—Models of β-lactoglobulins A and B and possible hybrids.

Source of Radioactivity.—500 microcuries of DL-valine-1-C^{14} hydrochloride was obtained from New England Nuclear Corporation[21] with specific activity of 46 μcuries/mg. This was dissolved in 10 ml. of sterile saline and injected into the jugular vein. Valine was selected for the experiment as it is one of the essential amino acids which occurs in reasonable amount in β-lactoglobulin and in not much greater proportion in casein.[22] Furthermore, it has been shown to be rapidly incorporated into β-lactoglobulin in the goat[23] and was available in high specific activity at the time the experimental animal became available.

Preparation of Labeled Protein.—The animal was milked (using a milking tube and milking as dry as possible) 5 times over a period of 46 hr. following administration of the radioactivity. After this time a laboratory monitor (1.9 mg./cm.^{2} window) was unable to detect any increase in radioactivity over normal background when the detector was held directly in the animal's expired breath. It was expected that the majority of the incorporated activity would be obtained in the first milkings[24] and that very little radioactivity would be present in milk produced after 36 hr.[23] A total volume of 4650 ml. of milk was obtained when the separate milkings were pooled. Crystalline β-lactoglobulin A was prepared from the milk following the procedure of Aschaffenburg.[20] The preparation gave 17 g. of crystalline slurry, about 35% of the theoretical yield,[25] with a measured activity of 18 c./min./mg. The slurry was stored in the refrigerator under distilled water saturated with toluene.

Preparation of Protein Samples for Counting.—Solutions of β-lactoglobulin, containing no more than 25 mg. of protein, were diluted to 5 ml. with water.

2.0 ml. of trichloroacetic acid (TCA) was added with stirring. The precipitated protein was allowed to stand 5 minutes and then filtered with suction onto a $^7/_8$ inch circle of Whatman No. 2 filter paper held in a special filter funnel (Tracerlab E-8B).[21] The paper discs had been stored in a desiccator over saturated $Mg(NO_3)_2$, (50.8% r.h. at 25°) and weighed to the nearest 10 micrograms in a 50% r.h. room. The precipitate was washed quantitatively onto the filter with a few ml. of 1 molar TCA, washed with 2 ml. of absolute ethanol, washed several times with ether and sucked dry. The discs were then removed from the filter, stored in the humid desiccator and subsequently weighed and counted.

In preliminary experiments, attempts had been made to precipitate the β-lactoglobulin by boiling, but the flocculent precipitate obtained would not pack onto the filter paper disc and when dried, would fall off. The discs were mounted for counting on thick aluminum planchets with retaining rings (Tracerlab E-7B planchets[21]). Counting was done on an ultrathin-window gas flow counter (Nuclear-Chicago Model C-110A[21]) for at least 1000 counts. Self-absorption was corrected for by precipitating a constant amount of tagged protein along with varying larger quantities of non-radioactive β-lactoglobulin and plotting the activity *versus* weight of precipitated protein in the usual manner.[26] From the graph so obtained, counting data were extrapolated to infinite thickness of protein layer.

Electrophoresis.—All electrophoretic experiments were carried out in a Spinco Model H apparatus[21] at 1.0°. Mobilities were determined in the descending limb with the aid of a microcomparator. Experiments involving the separation of β-A from β-B were done in the 11 ml. cell at *p*H 5.30, 0.1 ionic strength acetate buffer. An applied field strength of 7.3 v./cm. gave fairly good separation of the two components in 25–28 hr. without back-compensation. The β-A (more rapidly moving at *p*H 5.3) could be drawn off as the fast peak in the rising limb and the β-B as the slow component in the descending limb. In all cases the sampling needle was inserted almost to the minimum between the schlieren peaks and the suction applied until the entire peak had disappeared. In this way 1–2 ml. of protein solution could be obtained from each limb.

The electrophoretic runs used to obtain mobilities for identification were carried out in the 2 ml. cell, *p*H 5.30, 0.1 ionic strength acetate, 12 hr. at 12.3 v./cm. in the case of β-A and 15 hr. at 10.5 v./cm. for β-B.

C^{14} Transfer Experiments.—A 2.2 g. sample of the radioactive β-A crystal slurry (containing approximately $^2/_3$ water) was dissolved in 18 ml. of *p*H 5.30 acetate buffer and dialyzed overnight *vs.* 2 l. solvent. A like solution of nonlabeled β-B was prepared and dialyzed. The final concentrations were adjusted to the same value by addition of dialysate, *i.e.*, 3.7 g./100 ml. 6 ml. aliquots of the two solutions were taken, mixed, dialyzed overnight and used as the control run.

The remaining 12 ml. of each of the two solutions were mixed and adjusted to *p*H 1.99 by slow addition of concentrated HCl at 25°. Under these conditions the equilibrium can be calculated[7,8] to lie at approximately 25% dissociation. The protein mixture was allowed to stand 90 minutes at 25°, readjusted to *p*H 5.3 with NH_4OH and dialyzed overnight *vs.* 4 l. of *p*H 5.30 acetate buffer. Electrophoretic runs were done in duplicate so that enough of each protein could be sampled to check its electrophoretic mobility before precipitation.

Another acidification was done (*p*H 2.0, 60 minutes) on an equimolar mixture of tagged β-A and untagged β-B, at a total concentration of 1 g./100 ml., where the equilibrium lies at *ca.* 40% dissociation. The solution, after neutralization, was concentrated by pervaporation and separated electrophoretically.

Other Measurements.—All *p*H's were measured with a Beckman Model G[21] *p*H meter at 25°. Protein concentrations were determined by ultraviolet absorption at 278 mμ, using the value of 0.96 l. cm.$^{-1}$ g.$^{-1}$ for the absorptivity of both species.[15]

Results

The possible models of the two isoelectric β-lactoglobulin species are shown in Fig. 1. The two sphere diagrams used are in accord with the configuration found by Green and Aschaffenburg in X-ray studies.[27] Since β-A has two more titratable carboxyls than β-B, the relative charges of the various species have been included next to each model. In model I each species is composed of two identical halves with the two extra carboxyls of β-A being located one on each half-molecule. Hybridization here would produce a mixed molecule with an intermediate carboxyl content. In models II and III, each lactoglobulin is composed of two different sub-molecules. In II the two extra carboxyls of β-A are located one on each subunit and hybridization would produce two hybrids, each with an intermediate number of carboxyls. In III the two extra carboxyls of β-A are located on a single subunit and mixed reassociation would result in two hybrids having charges identical with the original molecules. In IV, the entire difference between β-A and β-B is present in one subunit, the other (designated by N) being identical in the two. Hybridization here would result in species indis-

(21) Mention of the above does not imply endorsement by the U. S. Department of Agriculture over others not mentioned.

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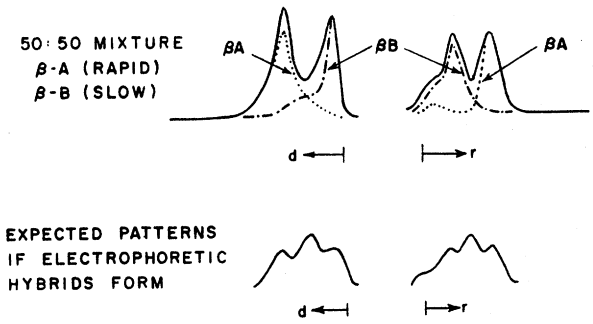

Fig. 2.—Electrophoretic diagrams of the β-lactoglobulins: upper, patterns given by β-A and β-B at *p*H 5.30, 0.1 ionic strength, are shown by the broken lines, the solid line is the sum of the two; lower, expected pattern if electrophoretic hybrids form.

tinguishable from the starting materials. In models I, II and III, hybrids may or may not form, depending on whether the difference between the species is close to the surface of bonding or not; in IV hybridization must occur, since the N subunits are identical and must be exchangeable.

The electric charges of the hybrids shown in models I and II are intermediate between those of β-A and β-B, and therefore their electrophoretic mobilities would also be intermediate. In Fig. 2 are shown electrophoretic patterns that should be obtained in these cases if hybridization did and did not occur. The upper diagram is the summation of the electrophoretic patterns of β-A and β-B at *p*H 5.3 in acetate buffer of 0.1 ionic strength. The dashed lines are tracings of the patterns obtained with the two individual proteins. The solid line is obtained by their addition and represents the situation that exists when an equimolar quantity of β-A and β-B is present and there are no hybrids.[11] Since reassociation with hybrid formation would yield a species distribution AA:AB:BB = 1:2:1, the area under the hybrid molecule peak should be half of the total area under the electrophoretic diagram. Such a hypothetical pattern for β-A and β-B has been constructed and is shown in the lower half of Fig. 2.

Electrophoretic runs were carried out at *p*H 5.3 on equimolar mixtures of β-A and β-B; a solution was prepared at *p*H 5.3, one aliquot was adjusted to *p*H 2 for 1 hr. (25°) and then brought back to *p*H 5.3; the other aliquot was subjected to electrophoresis without the acid treatment. As can be seen in Fig. 3 the patterns in the two cases are indistinguishable; they are essentially identical with the upper summation pattern of Fig. 2 and no traces of a species of intermediate mobility is detectable in the mixture that had been acidified. Thus, if the structure is that represented by models I or II no hybridization takes place, while III and IV would yield hybrids undetectable electrophoretically.

In elegant experiments using radioactively tagged proteins, Singer and Itano[18] were able to show that the system hemoglobins A and S, corresponds to Model IV.

In order to determine if the same situation exists in β-lactoglobulins, mixed dissociation experiments were carried out using β-A which had been tagged

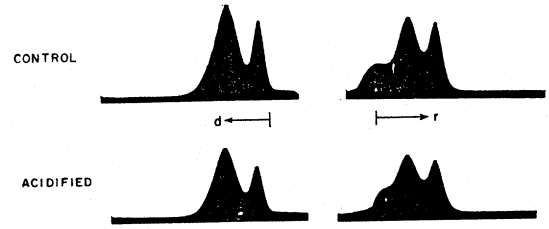

Fig. 3.—Mixture (50:50) of β-A and β-B: total concn. 1 g./100 ml., *p*H 5.30, 0.1 ionic strength acetate buffer; 22 hr. at 4.8 v/cm. Upper, non-acidified control; lower: subjected to *p*H 2 for 1 hr., then readjusted to *p*H 5.3.

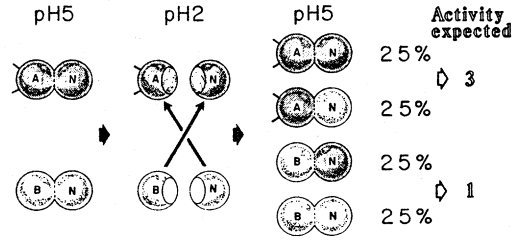

Fig. 4.—Model showing expected transfer of radioactivity to slow electrophoretic species if hybridization can occur.

with C^{14}. It is assumed throughout that the labeled amino acid is incorporated equally into both subunits of the protein. Equimolar quantities of tagged β-A and untagged β-B in solution were mixed, brought to *p*H 2 for 1–1.5 hr. and then readjusted to *p*H 5.3. The individual proteins were than separated electrophoretically and the radioactivity of each was measured. Under the conditions of this experiment the four sub-molecules of model IV become distinguishable and, on recombination, four molecular species should be found in equal amounts, as shown schematically in Fig. 4. The two upper species would migrate electrophoretically as β-A, the two lower ones as β-B. In the exchange of the N subunits, the activity found under the β-A peak should be reduced to 75% of the original, while β-B should contain 25% of the total radioactivity. The results of these acidification and separation experiments are presented in Table I. Two sets of values are control experiments. In the first, the proteins were mixed and then separated without passing through the acid treatment. In the second control the two proteins

TABLE I

	Protein	Acidified conc., g./100 ml.	Protein recovered, mg.	C./min./mg.[a]
βA	Not acidified	..	6.1	15.0
βB		..	5.7	0.73
βA	Acidified together	3.7	3.0	15.8
βB			1.4	0.97
βA	Acidified together	3.7	4.1	18.3
βB			1.7	1.74
βA	Acidified together	1	10.5	11.94
βB			3.5	0.58
βA	Acidified together	3.7	15.3	12.6
βB			18.1	0.64
βA	Acidified separately	3.2	6.76	16.92
βB		3.2	1.60	1.18

[a] Corrected for background and self-absorption.

were acidified separately, neutralized, then mixed and separated electrophoretically. In neither of these can transfer of radioactivity take place. It is found that in both a small amount of radioactivity is found in β-B after separation. This is probably due to contamination with β-A, the electrophoretic pattern of which skews backward in the descending limb, as shown in Fig. 2. In one of the sampling experiments, the proteins withdrawn from the electrophoresis cell were re-dialyzed against a *p*H 5.3 buffer and their mobilities determined. These are compared in Table II with the

TABLE II

Protein	Mobility cm. sec.$^{-1}$ volt^{-1}, *p*H 5.30 acetate buffer Γ/2 = 0.1
Leading peak of descending limb (β-A)	0.79×10^{-5}
Known β-A	$.76 \times 10^{-5}$
Trailing peak of rising limb (β-B)	$.22 \times 10^{-5}$
Known β-B	$.11 \times 10^{-5}$

known mobilities of β-A and β-B at this *p*H. In this range, the difference between $u = 0.11$ and 0.22×10^{-5} would correspond to an error of *ca.* 0.02 *p*H in the buffer. Thus, the two proteins removed from the electrophoresis cell and counted can be considered as essentially β-A and β-B lactoglobulins. The results shown in the other four sets of values represent four individual separation runs after acidification of the mixture; three were carried out on the same mixture of proteins at various total protein concentrations, and the last was a completely separate experiment.

The variation in specific activity of the recovered β-A is somewhat puzzling but is probably due to irreproducibilities in counting geometry. It was noticed that occasionally the precipitated protein, when stored in the humid chamber, would crack and curl up from the surface of the filter paper disc. It should be noted, however, that the β-B activity did not increase in the cases where the β-A activity is lower and in all experiments the activity ratio of β-A to β-B is very far from the 3:1 ratio required if subunit transfer were possible. Furthermore, the small amount of activity found in β-B after separation is never significantly greater than that present in the two controls. These results show that no subunit interchange between the two β-lactoglobulins takes place and that no hybrid molecules of any type can be found. They also eliminate completely model IV as a possible structure.

Discussion

The experiments described above permit immediately certain conclusions: the subunit pattern of the β-lactoglobulins is different from that of hemoglobins A and S (model IV) and must be model I or II (the difference between II and III is really trivial and represents the two possible distributions of the extra carboxyls of β-A). Since no hybrids are formed, the structure of the β-A and β-B submolecules must differ in the region of contact between the two (had that area of the molecule been identical for A and B, cross-reassociation would have been possible).

The structural difference, which reflects the genetic difference, may be of three types: (1) a difference in amino acid composition; (2) a difference in amino acid sequence; (3) a difference in chain folding. A difference in amino acid composition is anticipated from the difference in carboxyl content. If this is the only difference between the two proteins, model III would reduce to IV and, therefore, would be eliminated by the C^{14} experiments. In models I and II the carboxyls would have to be present in the area of contact between the two sub-molecules and face each other, since if they faced another group, this group would have to be different for β-A and β-B in order to eliminate the possibility of cross-combination.

If differences other than the content of free carboxyls exist between β-A and B, models I, II and III remain as possibilities. The indications are strong that such differences do exist. A difference in the denaturation rates of the two proteins would point in the direction of differences in configuration which probably involve the tertiary structures of the two species. Furthermore, there is indication that the extra carboxyls of β-A participate in the tetramerization of that protein between *p*H 3.7 and 5.2.[16] In order to be compatible with the geometry of that aggregation, these carboxyl groups must be located in a region outside of the area of contact between the subunits. This necessitates another structural difference in that area. Thus, it would seem that at least two types of difference exist between β-A and β-B, *i.e.*, one in the number of titratable carboxyls, the other in the chain configuration in the area of subunit contact. It should be recalled that the differences existing between the two proteins in that area cannot be major since the patterns of dissociation are almost identical.[8] If the recombination requires a steric fit at specific sites, then a small alteration in tertiary structure in the area of contact would be sufficient to render cross-combination impossible. It is quite possible that the substitution of one amino acid for another in the polypeptide sequence could lead to a difference in folding over a fairly large section of the chain.

The problem of the identity or non-identity of the molecular subunits in each protein cannot be answered with certainty at the present time. Certain arguments can be advanced, however, in favor of model I (identical halves). First, practically all known groups are present in even numbers per 36,000 mol. wt.; this includes all titratable groups,[28] two identical N[29] and C[30] terminal residues, and two abnormal carboxyl groups.[31,32] In studies on ion binding with the formation of stoichiometric complexes it has been found that, in each case, two ions of each type are bound.[27,33,34] Sulfhydryl content, using various reagents, appears to be close to 2

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moles-SH per 36,000 even though pooled β-lactoglobulin, containing both genetic species in unknown ratio, was used.[35–37] Secondly, Green and Aschaffenburg[27] have demonstrated a dyad axis of symmetry in both molecules, indicating that the two halves have very nearly, if not identical configurations. All these data suggest that each protein is made up of two identical chains of *ca.* 18,000 mol. wt., and strengthen the assumption that a tagged amino acid would be distributed equally between both subunits.

Model I would be more elegant than II or III with regard to the genetic control of the synthesis of the β-lactoglobulins. As pointed out earlier, it is known that the difference between β-A and β-B is controlled by a single pair of genes. In I, each half of the molecule could be synthesized on the same RNA template, controlled by one specific DNA locus. The two halves would then combine spontaneously to the AA and BB molecules (either on the template or in solution) at the intracellular *p*H. Although not excluded by this argument, the synthesis of models II and III is less straightforward than the first one.

On the basis of the above, it seems more likely that the correct structure is represented by I. This viewpoint is strongly supported by experiments involving tryptic digestion of β-A and β-B, followed by 1-dimensional ionophoresis, which will be reported elsewhere.[38] The acid dissociation phenomenon can be best depicted then by the mechanism of Fig. 5. Here, each species is composed of identical half molecules, there being two differences

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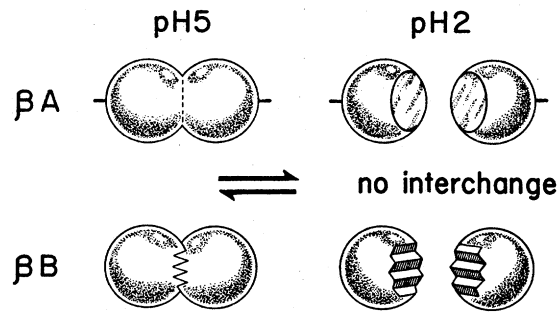

Fig 5.—Schematic representation of the low *p*H dissociation of β-A and β-B.

between β-A and β-B, namely a difference in chain folding at the surface of subunit contact and the presence of one extra titratable carboxyl on each subunit of β-A.

Due to the difference in tertiary structure no cross combination can take place. Although the model advanced in Fig. 5 seems to represent the simplest explanations of the experimental facts, the possibility of models II and III has not been completely eliminated by these experiments. To settle this question, more detailed structural information on the two lactoglobulins is necessary. Such a study has been presently undertaken in our Laboratory.

Acknowledgments.—We would like to thank Professor S. J. Singer for suggesting to us the radioactive tagging technique for the solution of the hybridization problem as well as for making available to us prior to publication his manuscripts with Dr. H. A. Itano. We would also like to thank Drs. Murray Strassmann and Bernice Friedman of Cancer Research Institute, Philadelphia, who made their counting equipment available to us.