

Lactoglobulins A and B: The Environment of the Asp/Gly Difference Residue¹

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The environment of the aspartic acid-glycine substitution has been examined. A pair of peptides of differing mobility, showing the known asp-gly substitution of the native variants, has been isolated from tryptic digests of *S*-sulfonated β -lactoglobulins A and B. The amino acid composition of the peptides shows a large concentration of carboxylic residues in the neighborhood of the difference amino acid. Comparison with the physicochemical properties of the variants indicates that this area of the primary structure is probably implicated in the low-temperature aggregation, since differences between the two species in degrees of this reaction correlate with the density of carboxyl groups in the vicinity of the substitution.

In 1955 it was found that bovine β -lactoglobulins existed in two genetic forms that differed slightly in their electrophoretic behavior on paper at pH 8.6 (1). These forms are called β -lactoglobulin A (β -A) and β -lactoglobulin B (β -B) in order of decreasing mobility. (Recently, K. Bell (2) found a third genetic variant (β -C) that moved still more slowly on starch-gel electrophoresis than either of the other two.) At room temperatures these two proteins are otherwise essentially identical in their gross physical properties such as molecular weight (3, 4), optical absorptivity (5), rotatory dispersion properties (6), and association-dissociation reactions (3). Amino acid analysis has shown that the difference in electrophoretic properties arises from the fact that β -A has one more aspartyl residue and one less glycylic residue than β -B (7, 8) per polypeptide chain of 18,000 molecular weight. An additional amino acid substitution (β -A valine for β -B alanine) has also been found. That the proteins differ by

two carboxyl groups per 36,000 (the molecular weight of the isoelectric, two-chain units existing in aqueous solvents at room temperature) has been independently shown by titration experiments (5). The most striking physical difference between β -A and β -B is the extensive formation, in β -A, of a cyclic octamer (9, 10) of 144,000 molecular weight.³ As a result of this pronounced difference between the association properties of the two ($4\beta \rightleftharpoons \beta_4$). Equilibrium constants for the reaction at 2° are: β -A, 6×10^{11} ; β -B, 3.5×10^8 l³mole⁻³; S. N. Timasheff and T. Kumosinski, unpublished data), and the fact that the association is maximal near the p*K* of carboxyl groups, it has been proposed that the substituent β -A aspartyl residue is intimately involved in the octamerization site (11). It is also significant that upon titration of β -A in the cold, a large reversible uptake of protons is found

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³ Previously (3, 9, 10) the eight-chain unit has been designated a tetramer, the 36,000 mol. wt. two-chain unit a monomer, and the 18,000 mol. wt. single chain a subunit. It is suggested that the nomenclature be standardized as follows:

$2 \times 18,000 \rightleftharpoons 36,000$: pH values <3.5
(monomer \rightleftharpoons dimer)

$4 \times 36,000 \rightleftharpoons 144,000$: pH 3.8-5.1, cold
(dimer \rightleftharpoons octamer)

when passing through the region of octamer formation (pH 3.7–5.1), which is quantitatively ascribable to about 30 carboxyl side-chains being titrated (over and above the number predicted for that pH) for every octamer molecule formed (12).

An earlier publication from this laboratory (13) has reported that the β -A-aspartyl- β -B glyceryl difference occurs in a pair of single chymotryptic peptides, all other residues in the two peptides being equal in number, and all other peptides given by β -A and β -B having identical electrophoretic mobility in the pH 6.4 pyridine-acetic acid buffer used. (Peptides containing the alanine-valine difference have not, to the best of our knowledge, ever been isolated, so the belief that this, too, is a single amino acid substitution in otherwise identical structures remains unproven. It is highly probable, however, when one considers the overriding similarities of the two β -lactoglobulin types and their very few differences.) The β -A chymotryptic difference peptide contains seven carboxylic residues out of a total of nineteen, which is a fairly high concentration. It was desired to see if the suspected high density of carboxylic groups at the site of substitution could be also seen when smaller peptides were examined.

EXPERIMENTAL AND RESULTS

β -Lactoglobulins A and B were prepared from the milk of homozygous animals by the method of Aseffenburg and Drewry (14) and recrystallized three times from sodium acetate buffers. Performic acid-oxidized and *S*-sulfonated derivatives were prepared as described previously (13). The trypsin used was a TCA-purified preparation from Worthington Biochemical Corporation (Lot 519). This preparation was shown to be essentially free of chymotryptic activity by complete lack of change in the 256 m μ absorbance of a mixture of enzyme and benzoyltyrosine ethyl ester (1:150) over a period of 75 minutes at 25°C.

Trypsin, like chymotrypsin (13), was incapable of extensive digestion of native β -lactoglobulin in short times, but readily attacked the derivatives in which the disulfide bonds had been cleaved. Protein derivative digestion was carried out at 37°C and pH 8.0 in unbuffered solutions in a pH-stat with a trypsin-substrate ratio of 1:200 and digestion times of 60–90 minutes, following the methods of Ingram (15). Digestion was essentially complete after 1 hour, as judged by

the uptake of 0.1 *M* NaOH. In a few instances additional trypsin was added at this time, but no increase in base uptake was observed. At the end of this period dilute HCl or HCOOH was added to bring the pH to 6.4, a small amount of gel-like precipitate was removed by centrifugation, and the supernatants were stored frozen. Analytical high-voltage paper electrophoresis (ionophoresis) were done on a cooled plate, at a loading representing about 4 mg of protein, with a pH 6.4 pyridine-acetic acid buffer and a voltage drop of 45 V per centimeter. After 2 hours ionophoresis, the strip was air-dried and the bands were visualized by dipping in a 0.2% solution of ninhydrin in acetone and heating to 80°C for a few minutes.

IONOPHORETIC RESULTS

Figure 1 shows the ionophoretic pattern obtained with performic acid-oxidized β -A and β -B. No differences are visible in the cationic bands, but one clear difference is shown in the peptides possessing a negative charge at pH 6.4. (The fastest moving cationic band, on the extreme left of the figure, is identical in mobility with free lysine under this and other pH conditions.) By numbering the bands arbitrarily from the right, the suspected difference peptide is the B₄-A₅ pair. The presence of a visible band at B₅ is due to the fact that band A₅ is not a single peptide, since upon elution of the latter with 1 *M* acetic acid, concentration, and re-ionophoresis at either pH 3.5 or 1.9, two bands of differing mobility are observed.

Since performic acid oxidation destroys the tryptophan residues of the β -lactoglobulins, the *S*-sulfonated derivatives were also examined. These derivatives are white flocculent powders that are insoluble in dilute salt from pH 4 to 6 but are readily soluble at pH 7 and above. With the exception of a small amount (<5%) of a heavy component ($S_{20,w} \cong 17$), they were essentially homogeneous ultracentrifugally and gave an $S_{20,w}$ at 1% concentration of 2.6–2.8, compared with the native β -lactoglobulin value of 2.7 ± 0.02 at the same concentration and conditions (25°, pH 7 phosphate buffer). (Upon Tiselius electrophoresis, however, the *S*-sulfo preparations were far from homogeneous and gave four peaks of greatly differing mobilities, which indicates that either the oxidation of the

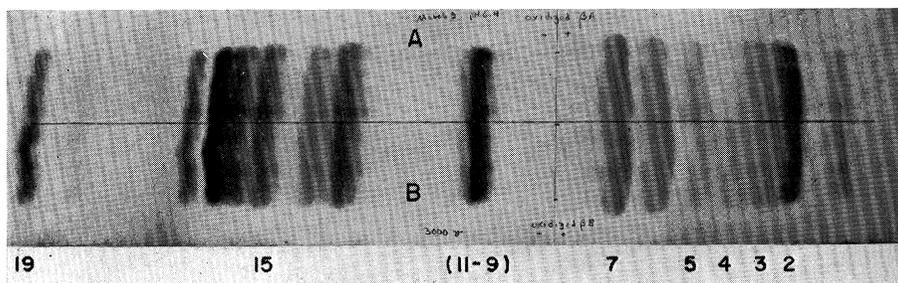


FIG. 1. Performic acid-oxidized β -A and β -B tryptic peptides. Vertical line is point of application. Whatman 3 MM paper; pH 6.4 pyridine-acetic acid buffer; 2 hours at 45 V/cm; positive pole at right.

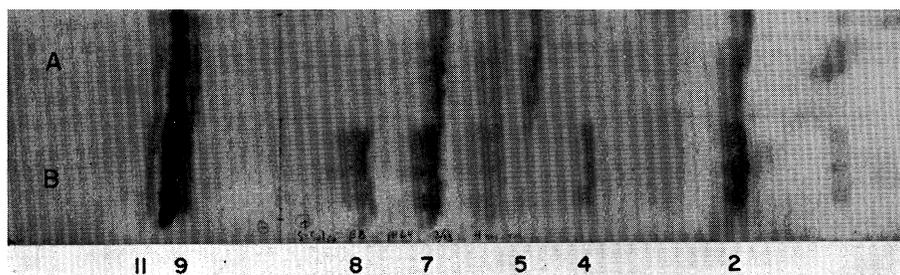


FIG. 2. *S*-Sulfonated β -A and β -B tryptic peptides. Vertical line is point of application. Conditions same as Fig. 1. Time of run 4 hours.

disulfide bonds is incomplete or that the cleavage is slowly reversed upon storage of the product in the lyophilized state. Also, a significantly larger amount of precipitate, insoluble at pH 6.4, remained after tryptic digestion.) Tryptic digestion of the *S*-sulfonated derivatives gave essentially the same pattern as the oxidized derivative. The anionic portion of the ionogram of the *S*-sulfonated derivative is shown in Fig. 2. A major difference between the performic acid-oxidized and *S*-sulfonated derivatives can be seen in the presence of a second β -B difference-band, numbered B_8 , the most slowly moving anionic band shown in this figure. This band has proven very difficult to purify, but seems to contain a large peptide with the residues of B_4 plus several other amino acids. But because of the absence of tryptophan and tyrosine damage associated with performic acid oxidation, and the more clear-cut separation of the peptide bands in the *S*-sulfonated derivative, the latter was used for the following purification and analysis.

Parallel paper strips were loaded with tryptic digest equivalent to about 20 mg of protein, and a 4-hour ionophoresis at pH 6.4 was done to duplicate Fig. 2. A guide strip was stained with ninhydrin, and peptides B_4 and A_5 were eluted with 1 *M* acetic acid, concentrated under vacuum, and re-run in a pH 3.5 pyridine-acetic acid buffer. It was possible to identify the peptides in question on the purification runs since B_4 and A_5 give a test for tryptophan with Ehrlich's reagent, and the neighboring pH 6.4 bands and the band at B_5 do not. The tryptophan-containing bands were again eluted, concentrated, and re-run at pH 1.9. The major ninhydrin and tryptophan-positive band observed on the final guide strip was eluted and hydrolyzed 24 hours at 110° C with 6.7 *M* HCl in sealed capillary tubes. Analysis of the liberated amino acids was done on commercial instruments of the type developed by Spackman *et al.* (16). Results are given in Table I. The four columns represent two duplicate sets of analyses on peptides prepared from different batches of *S*-sulfonated β -lacto-

TABLE I
ANALYSIS OF DIFFERENCE PEPTIDES; 24-HOUR HYDROLYSES

Amino acid	Peptide A ₈ (μmoles)				Av. ratio (rounded)
Asp	0.105 (2.00) ^a	0.19 (2.00)	0.41 (2.00)	0.12 (2.00)	2
Glu	0.15 (2.86)	0.27 (2.84)	0.64 (3.12)	0.20 (3.33)	3
Gly	0	0	0.06 (0.29)	0.02 (0.33)	6
Ala	0.05 (0.95)	0.10 (1.05)	0.20 (0.98)	0.05 (0.83)	1
½ Cys	—	—	—	—	—
Val	—	—	0.08 (0.39)	0.06 (1.00)	1 (?)
Ileu	—	—	—	—	—
Leu	—	—	0.12 (0.59)	0.07 (1.17)	1
Lys	N.D. ^d	0.21 (2.21)	N.D.	0.10 (1.67)	2
Tryp ^e	+	+	+	+	[1]

Peptide B ₄					
Asp	0.065 (1.00)	0.06 (1.00)	0.12 (1.00)	0.20 (1.00)	1
Glu	0.187 (2.88)	0.179 (2.98)	0.37 (3.08)	0.57 (2.85)	3
Gly	0.06 (0.92)	0.055 (0.92)	0.12 (1.00)	0.19 (0.95)	1
Ala	0.06 (0.92)	0.055 (0.92)	0.09 (0.75)	0.17 (0.85)	1
½ Cys	—	—	0.03 (0.25)	0.08 (0.40)	0
Val	—	—	0.07 (0.58)	0.07 (0.35)	1 (?)
Ileu	—	—	0.03 (0.25)	0.03 (0.15)	0
Leu	0.007 (0.11)	0.052 (0.87)	0.12 (1.00)	0.10 (0.50)	1
Lys	N.D.	0.057 (0.95)	N.D.	0.19 (0.95)	1
Tryp ^b	+	+	+	+	[1]

^a Columns 1 and 2 are aliquots of the same peptide preparation analyzed in Dr. Ingram's laboratory; columns 3 and 4 were prepared and analyzed in our laboratory. Numbers in parentheses are ratios with respect to aspartic acid content.

^b Peptide gives positive test with Ehrlich's reagent before hydrolysis. One residue is assumed (see text).

^c This value was obtained on the same sample as in column 2. Since it appears to be in error, it is omitted from the calculation of the average.

^d Not Determined for this aliquot.

globulins and analyzed in different laboratories. (The poorly reproducible values for valine might be expected in view of the known difficulty of hydrolyzing peptide bonds involving this residue (17). Lack of purified material precluded a series of hydrolyses.) It seems clear that this small peptide pair does contain the key aspartic acid-glycine substitution sought and previously found in the pair of chymotryptic difference peptides. It is striking that almost one-half of the residues found (5 out of 11 in β-A) are carboxylic amino acids. The high negative mobility of this peptide pair at pH 6.4 indicates that most, if not all, of these residue are free carboxylic acids, not amides.

Lysine-Lysine Linkage

The presence of two lysine residues in peptide A was unexpected as a product of tryptic digestion. However, a lysine-lysine linkage is known to be somewhat trypsin-resistant (17), and, in addition, it has been reported that lysine residues peptide-bonded through their α-amino group to an acidic residue (17) or, more pertinently, to an aspartic acid residue (18, 19), become quite resistant to tryptic cleavage. If the substitution of the β-A aspartic acid residue for the β-B glycyl residue has occurred on the N-terminal side of a lysyl-lysyl pair, the fortuitous combination of both factors could account for the di-lysyl peptide being the

major product of the β -A trypsin digestion. The presence of significant amounts of free lysine in the tryptic digests of both A and B derivatives (the most rapid cationic band) indicates that there are other places in the molecules where basic amino acids occur together. A lysine-lysine sequence in β -A, more resistant to tryptic digestion than its corresponding sequence in β -B, should result in the liberation of comparatively more free lysine upon digestion of the latter. This was tested as follows. Samples of the centrifuged digests of *S*-sulfonated β -A and β -B were lyophilized and carefully weighed out to give exactly 4 mg of each derivative. Each was applied to one-half of a Whatman 3 MM paper strip, and ionophoresis was carried out for 3 hours at 35 V per centimeter and pH 6.4. The strip was dried and was stained with 0.2% ninhydrin in acetone, and several of the better separated bands were measured on an integrating densitometer (Spinco Analytrol) at 500 m μ . Results are given in Table II. It can be seen that, while other peptide bands give an almost identical

color yield from one protein to the other, β -B gives 20% more free lysine than β -A, supporting the above conclusion that a trypsin-resistant lysine-lysine linkage occurs in β -A. A further strong indication that the two lysines of peptide A₅ are adjacent was given by 4- and 24-hour carboxypeptidase B digestions of aliquots of a solution of this peptide, each calculated to contain 0.005 μ mole of alanine, which occurs as a single residue (Table I). Digestion conditions were: pH approximately 8 with 0.1 M NH₄HCO₃ buffer; 37°; enzyme substrate ratio 1:50. At both digestion times, somewhat more than 0.01 μ mole of lysine was released and no other amino acids. These amino acid analyses were performed by ionophoresis at pH 1.9 on 3 MM paper followed by ninhydrin staining and quantitation on the densitometer, after the method of Atfield and Morris (20). Reagent grade amino acids were run on the same paper strip as colorimetric standards.

DISCUSSION

From the above data, the richness of the primary structure of the difference peptides in carboxyl groups may be seen. A reasonable deduction of the immediate environment of the substitution amino acid may be made by comparison of the chymotryptic and tryptic difference peptides. This is illustrated in Table III. That the substituent residue is indeed in the site or region of intermolecular contact (10) upon octamer

TABLE II

Peptide band No.	Integrated optical density	
	β A	β B
2	79	73
7	61	59
14	130	131
15	70	68
19 (Free lysine)	74	89

TABLE III

PROPOSED ENVIRONMENT OF SUBSTITUENT AMINO ACIDS^a.

	β -A Locus	
Tryptic-A ₅	(leu tryp)	(val ala glu ₃ asp) <i>asp</i> lys* lys
Chymotryptic-A ₂		(val ala glu ₃ asp) <i>asp</i> lys lys (glu ala ₂ lys ₂ thr pro $\frac{1}{2}$ cys ileu ₂ phe)
		β -B Locus
Tryptic B ₄	(leu tryp)	(val ala glu ₃ asp) <i>gly</i> lys + lys
Chymotryptic-B ₁		(val ala glu ₃ asp) <i>gly</i> lys lys (glu ala ₂ lys ₂ thr pro $\frac{1}{2}$ cys ileu ₂ phe)

^a Genetically substituted residues are shown in italics. Arrows show points of enzymic attack; asterisk marks resistant lysine-lysine linkage (see text).

^b Composition of chymotryptic peptides taken from Ref. (13).

formation is a most plausible conclusion when the physical chemistry and geometry of the reaction is taken into account, along with the overwhelming similarity of β -A and β -B (3-5) and their difference in the ability to form octamers (9).

The burial of approximately 30 carboxyl side chains (12) when a molecule of octamer is formed is nearly equivalent to burial of four free carboxyls per 18,000 molecular weight monomer. The presence of five carboxylic residues in the β -A undecapeptide would be more than sufficient to account for these titration results.

The tryptic digestion method provides additional evidence that the 18,000 molecular weight subunits (3) are identical (21). The arginine content per 36,000 molecular weight (7, 8) is six residues. Three peptide bands (2, 9, and 15) give a positive color reaction with 8-hydroxyquinoline. Histidine content is four residues; peptides 6 and 15 give a red color with the Pauly diazotized sulfanilic acid reagent. Eight tyrosine residues are found by analysis; peptides, 2, 3, 9, and 11 give reaction with acidic α -nitroso β -naphthol. Two peptide bands, 9 and 5 (in β -A), are positive for tryptophan. In the reported analyses (7, 8) the colorimetric method of Spies and Chambers (22) gives five residues per mole. This method uses free tryptophan as a standard. However, the analysis of Brand (23) gives 3.7 residues when reduced to the above molecular weight, and recent work in this laboratory (T. T. Herskovits, unpublished data) with different tryptophan-containing model compounds as spectrophotometric references shows that four residues per mole is the best value. This fact preserves the concept of subunit identity.

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Mention of specific products or manufacturers in this publication does not imply endorsement

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