Studies on β-Lactoglobulins A, B, and C. II. Preparation of Modified Proteins by Treatment with Carboxypeptidase A*

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ABSTRACT: Modified crystalline proteins have been prepared by reacting β-lactoglobulins A, B, and C with carboxypeptidase A. From previous examination of this reaction, the modified proteins should be lacking 2 moles each of carboxyl-terminal isoleucine and penultimate histidine per molecule (36,000 mw). Polyacrylamide and agar-gel electrophoreses at *p*H 8.6 indicate the purity of the preparations and suggest incomplete removal of histidine with modified β-lactoglobulin C. Examination of ultraviolet spectra and optical rotation data suggests that the modified proteins retain a configuration similar to that of the native materials, and that the isoleucine and histidine are located on the external surface of the molecule. Amino acid composition studies on the modified proteins yield residue numbers identical to the native with the exception of isoleucine and histidine, and confirm the incomplete removal of histidine with modified β-lactoglobulin C. Reaction of the modified proteins with carboxypeptidase showed rates of release of amino acids similar to those found with the native β-lactoglobulins.

During the investigation of the carboxyl-terminal sequence of the genetic β-lactoglobulin variants A, B, and C, using carboxypeptidase A, it was observed that the C-terminal isoleucine and penultimate histidine are released at a much more rapid rate than the subsequent amino acids (Kalan *et al.*, 1965). Davie *et al.* (1959) treated mixed β-lactoglobulin from pooled milk with carboxypeptidase A and performed some preliminary experiments in which modified proteins lacking the C-terminal isoleucine, or histidine and isoleucine, were crystallized.

Since histidine is one of the pair of differing amino acids between β-B[1] and β-C (Kalan *et al.*, 1964, 1965) modified proteins containing two fewer histidine residues per molecule would be helpful in the search for the location of the amino acid substitution. Each β-lactoglobulin molecule (36,000 mw) consists of two identical chains with molecular weights of 18,000 (Townend *et al.*, 1960a). This two-chain structure explains why two residues of isoleucine and two residues of histidine per molecule are involved.

These modified β-lactoglobulins can also be utilized in the further examination of several structural properties such as the tetramerization mechanism as described by Townend *et al.* (1960b), Townend and Timasheff (1960), and Timasheff and Townend (1961), the failure of the variants to hybridize (Townend *et al.*, 1961), and the decreased rate of carboxypeptidase A hydrolysis demonstrated by β-C (Kalan *et al.*, 1965).

Accordingly, detailed experiments were undertaken to prepare modified β-A, β-B, and β-C produced by the action of carboxypeptidase A. These were isolated, purified, crystallized, and examined for several physical characteristics such as electrophoretic mobility, optical rotation, and ultraviolet absorption.

To confirm the removal of the expected residues and to determine whether any other compositional changes might have taken place, the amino acid composition of the three modified proteins was examined. It was also of interest to hydrolyze these materials with carboxypeptidase to confirm the site of removal as the C-terminal end of the native proteins. This latter experiment could possibly contribute further knowledge concerning the sequence near the carboxyl-terminal region of the β-lactoglobulin molecules.

Experimental

Preparation of Modified Proteins. The starting materials, native β-A, β-B, and β-C, were prepared as described previously (Kalan *et al.*, 1965). Their purity was examined by polyacrylamide-gel electrophoresis at *p*H 8.6, where a single band was obtained for each protein. DEAE-cellulose column chromatography, performed under conditions suitable for separation of the variants (Kalan *et al.*, 1965), revealed only one peak for each preparation.

Carboxypeptidase A was obtained from Worthington Biochemical Corp.[2] as a water suspension of three-times-recrystallized material. It was treated with DFP prior to use.

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[1] Abbreviations used in this work: β-A, β-B, and β-C, β-lactoglobulins A, B, and C, respectively.

[2] It is not implied that the U.S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

β-A. Preparative experiments were undertaken with 5.0 g of β-A and 50 mg of carboxypeptidase. The digestion was carried out at *p*H 8.0 in an unbuffered system at 37° for 3 hours. At the end of this time the enzyme, most of which remained undissolved, was removed by centrifugation and the supernatant was adjusted to *p*H 5.5 with 0.5 N HCl. The crystalline suspension, which formed immediately upon lowering of the *p*H, was transferred to a dialysis sac and allowed to dialyze overnight at 4°. After removal of this supernatant, the precipitate was taken up in 0.2 M NaCl solution. A portion of the precipitate remained undissolved in the presence of salt and probably consisted of denatured protein. This material was removed by centrifugation, and the supernatant solution was dialyzed overnight, whereupon the protein derivative crystallized without difficulty. The modified β-A was recrystallized three more times from an NaCl solution. The final recrystallized preparation was lyophilized and stored at room temperature at a constant humidity of 50%. The yield of four-times-recrystallized protein was about 0.7 g. The NaCl-insoluble fraction contained 0.5 g. The remainder of the material was located in the supernatant fractions from the recrystallization steps. It is obvious that, although the modified protein is less soluble than the native β-A, the volumes on recrystallization must be kept low to effect more complete crystallization and increase the yield of the purified material.

β-B. The preparation of modified β-B was carried out in a similar fashion to β-A with regard to amounts of protein and enzyme and reaction conditions, but minimal volumes of solution were employed throughout. After removal of the enzyme by centrifugation, and upon lowering of the *p*H to 5.5, a crystalline precipitate was again immediately formed. The crystals of modified β-B formed at this stage are shown in Figure 1. The material during all subsequent recrystallizations formed square, flat crystals similar to those of modified β-A, and only at this one stage formed diamond-shaped ones. The suspension was dialyzed for several days at 4°. After centrifugation the precipitate was taken up in 0.2 M NaCl and the insoluble fraction was removed. The supernatant containing the modified protein was dialyzed overnight and crystallized without difficulty. The yield of four-times-recrystallized material was 1.7 g. The NaCl-insoluble fraction contained 0.3 g and the remainder of the material was again found in the supernatant fractions from the recrystallization steps.

β-C. The preparation of modified β-C required some modification of the basic procedure. Previous work (Kalan *et al.*, 1965) showed that the penultimate histidine of native β-C was not completely liberated at 3 hours in contrast to the other β-lactoglobulin variants, and that the solubility of β-C greatly exceeded β-A or β-B. Therefore a lengthened digestion time of 4 hours and still smaller solution volumes were employed with 5.0 g of β-C and 50 mg of carboxypeptidase. The conditions of *p*H and temperature remained unchanged. After removal of the enzyme and adjustment of the *p*H to 5.5, a flocculent amorphous precipitate was formed. No crystals were apparent at this stage. After dialysis at 4° for several days, the precipitate was removed and the supernatant was dialyzed further in an attempt to promote crystallization of the material, the bulk of which was obviously still in solution. When all such attempts proved unsuccessful, the supernatant was lyophilized.

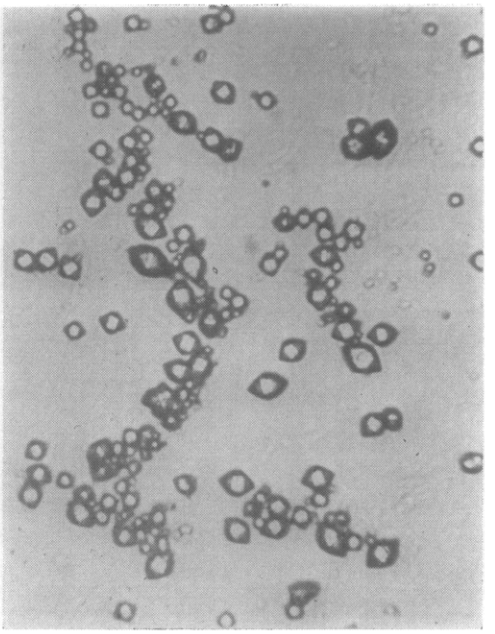

FIGURE 1: Photomicrograph of crystals of modified β-B formed by removal of two residues each of isoleucine and histidine by carboxypeptidase. Material from initial crystallization at *p*H 5.5.

The amorphous precipitate which came down at *p*H 5.5 was taken up in water and treated with NaCl to bring as much as possible into solution. After removal of insoluble material by centrifugation and dialysis of the supernatant for 3 days at 4°, crystalline material was obtained. The modified β-C protein was recrystallized four times as described previously with a yield of 0.45 g. The supernatant of the original digestion mixture contained 3.0 g and the NaCl-insoluble fraction another 0.4 g. The remaining material was found in the supernatant fractions of the recrystallization steps. The modified β-C crystals tend to dissolve at room temperature and, when placed on a slide, melt into a glassy film.

Examination of Purity. The protein derivatives were examined for purity by electrophoresis on polyacrylamide and agar gels at *p*H 8.6. Nitrogen was determined by the Kjeldahl method on dried samples of the modified β-A, β-B, and β-C preparations.

Physical Measurements. The optical rotation measurements were carried out in 0.15 M KCl solution at *p*H 6.5 using a polarimeter equipped with a sodium lamp, while the rotatory dispersion measurements were performed in a Rudolph Model 200S spectropolarimeter.[2]

Both the direct and difference spectra of the proteins were examined in 0.15 M KCl using the Cary recording spectrophotometer, Model 14.[2]

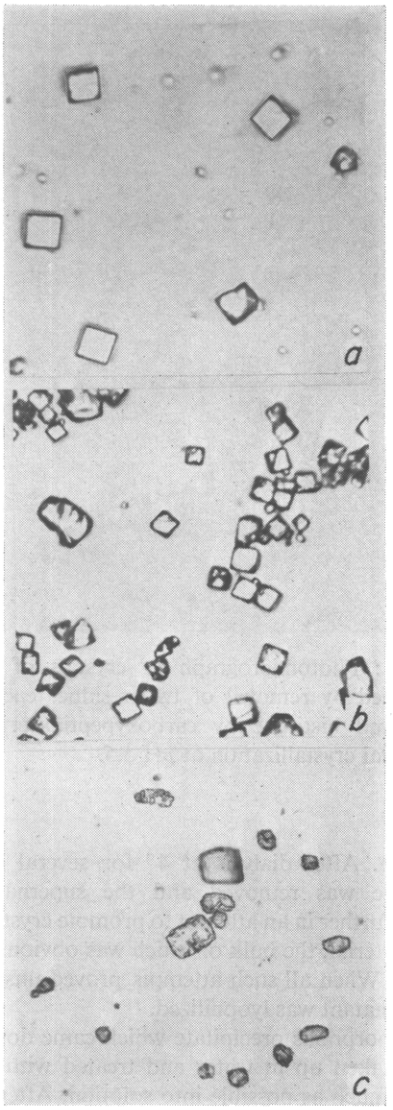

FIGURE 2: Photomicrograph of crystals of four-times-recrystallized modified β-lactoglobulins formed by reaction with carboxypeptidase. (a) Modified β-A; (b) modified β-B; (c) modified β-C.

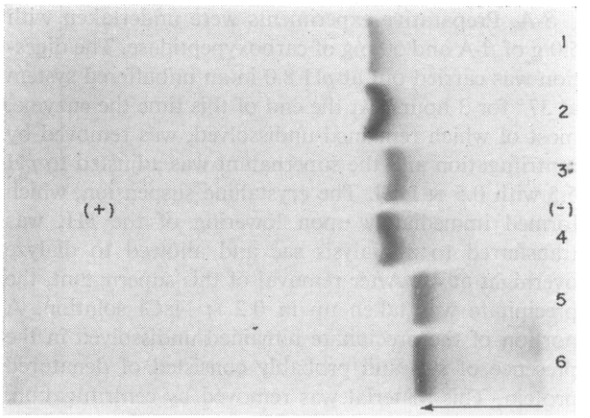

FIGURE 3: Polyacrylamide-gel (5%) electrophoresis at *p*H 8.6 in Veronal buffer (0.025 M) at 16 v/cm for 2 hours. (1) Native β-A; (2) modified β-A; (3) native β-B; (4) modified β-B; (5) native β-C; (6) modified β-C.

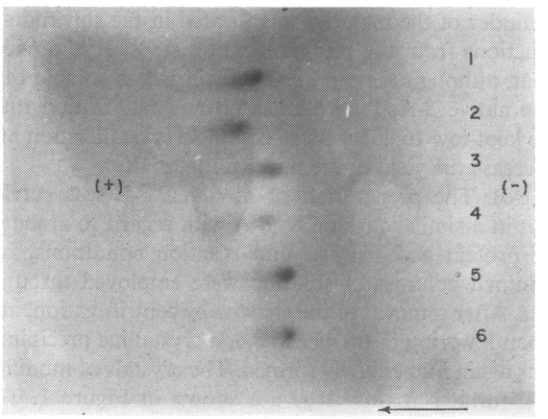

FIGURE 4: Agar-gel (1%) electrophoresis at *p*H 8.6 in Veronal buffer (0.025 M) at 25 v/cm for 2 hours. (1) Native β-A; (2) modified β-A; (3) native β-B; (4) modified β-B; (5) native β-C; (6) modified β-C.

Amino Acid Analysis. For the determination of the amino acid composition, samples of each protein were hydrolyzed in 6 N HCl in evacuated sealed tubes at 110° for 24, 72, and 96 hours. The analyses were carried out using the automatic recording apparatus described by Piez and Morris (1960). All determinations were run in duplicate.

Carboxyl-Terminal Study. Hydrolysis of the modified proteins with carboxypeptidase A was carried out at *p*H 8.0 in an unbuffered system at 37° with a weight ratio of enzyme to protein of 1:100. Aliquots of the digestion mixture were withdrawn at specific time intervals ranging from 3 to 72 hours. These were precipitated with trichloroacetic acid (final concentration 6%) and centrifuged in the cold, and the supernatants were decanted and frozen until analyzed. The amino acids released were identified and quantitated using the aforementioned automatic recording chromatographic equipment.

Results

Photomicrographs of the four-times-recrystallized modified β-A, β-B, and β-C are shown in Figure 2. The modified β-C crystals tended to dissolve at room temperature and the heat generated by the illumination necessary for the photographic exposure hastened their dissolution. Hence these crystals have irregular surfaces because they have begun to go into solution. Modified β-A and β-B appear as square plates while the β-C protein seems to exist in a somewhat more oblong shape.

The polyacrylamide-gel electrophoresis pattern is

shown in Figure 3. Modified β-A and β-B (Figure 3, bands 2 and 4) have a slightly faster mobility than their respective native proteins (Figure 3, bands 1 and 3). This is to be expected if two weakly basic histidine residues per molecule have been removed. The two bands of β-C proteins (Figure 3, bands 5 and 6) have approximately the same mobility, suggesting the possibility of incomplete or total lack of removal of the penultimate histidine residues. The agar gel, Figure 4, reveals the same information. Modified β-A and β-B (Figure 4, bands 2 and 4) again have mobilities slightly different from the respective native proteins (Figure 4, bands 1 and 3) while the β-C spots (Figure 4, bands 5 and 6) exhibit no difference.

The results of the optical rotation measurements are given in Table I. The modified β-lactoglobulins have values only slightly higher than those of the native materials. This implies that the modified materials are not denatured and retain a configuration similar to the original proteins.

TABLE I: Specific Rotation $[\alpha]_D^{20}$ of β-Lactoglobulins in Degrees.

	β-A	β-B	β-C
Native	27.4	31.5	35.6
Modified	34.1	35.0	37.0

Table II summarizes the rotatory dispersion measurements. The values for the a_o and b_o parameters of the native β-lactoglobulins are those presented by Townend *et al.* (1964). A comparison of the data obtained for the modified proteins with these parameters again suggests that the native and modified proteins do not differ appreciably in secondary structure. The interpretations, for the native proteins, of a folded structure with a fair amount of order yet little or no helix (Townend *et al.*, 1964) may be applied equally well to the modified β-lactoglobulins.

TABLE II: Rotatory Dispersion Parameters of the Native and Modified β-Lactoglobulins.

Parameter[a]	Native[b]			Modified		
	A	B	C	A	B	C
$-a_o$	162	162	182	207	217	182
$-b_o$	83	87	81	90	90	75

[a] Conditions of measurement: 0.03 M NaCl-HCl, pH 2.70, 25°. [b] Data taken from Townend *et al.* (1964).

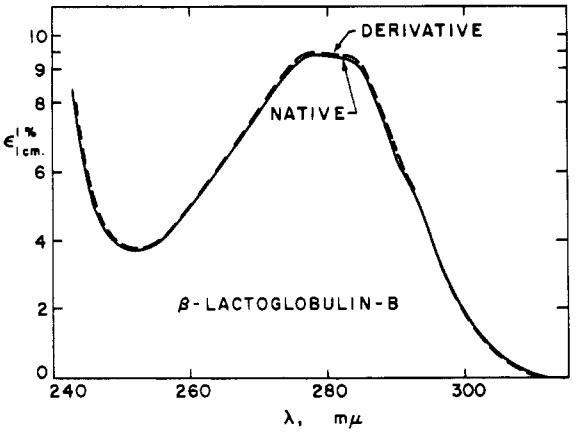

FIGURE 5: Ultraviolet spectra of native and modified β-B.

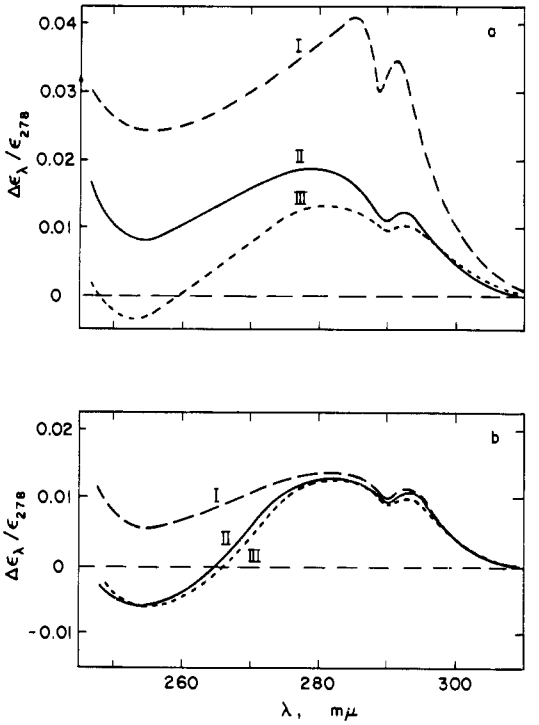

FIGURE 6: Difference spectra using 20% sucrose as perturbant. (a) I, Thioglycolic acid–reduced β-A, 8 M urea, pH 3.4, 0.02 M thioglycolate; II, native β-A, 0.03 M Cl^-, pH 2.9; III, modified β-A, 0.03 M Cl^-, pH 2.9. (b) I, Native β-B, 0.03 M Cl^-, pH 2.9; II, modified β-B, 0.03 M Cl^-, pH 2.9; III, modified β-C, 0.03 M Cl^-, pH 2.9.

The ultraviolet spectra of native β-B and the modified β-B are shown in Figure 5. The two spectra are quite

TABLE III: Amino Acid Composition of Modified β-Lactoglobulins Produced by the Action of Carboxypeptidase A.

	Residues per Mole of Protein								
	A		B		C		Nearest Even Integer		
Amino Acid	Average	95% Confidence Limits[a]	Average	95% Confidence Limits	Average	95% Confidence Limits	A	B	C
Aspartic acid	32.82	0.310	30.40	0.205	30.42	0.281	32	30	30
Threonine	16.7		16.0		16.0		16	16	16
Serine	14.0		13.9		13.2		14	14	14
Glutamic acid	50.25	0.440	49.90	0.300	47.57	0.416	50	50	48
Proline	16.78	0.160	16.97	0.103	16.79	0.155	16	16	16
Glycine	6.19	0.082	8.15	0.053	8.22	0.087	6	8	8
Alanine	28.20	0.271	30.22	0.198	30.14	0.285	28	30	30
Half-cystine[b]	5.66	0.220	5.50	0.180	5.98	0.168	6	6	6
Valine	20.08	0.164	18.16	0.122	18.12	0.159	20	18	18
Methionine	7.82	0.071	7.85	0.183	7.67	0.097	8	8	8
Isoleucine	17.50	0.208	17.25	0.132	17.41	0.195	18	18	18
Leucine	44.04	0.416	44.16	0.278	44.26	0.400	44	44	44
Tyrosine	7.85	0.077	7.75	0.073	7.73	0.091	8	8	8
Phenylalanine	8.00	0.038	8.02	0.060	8.12	0.081	8	8	8
Lysine	29.60	0.316	29.75	0.206	29.87	0.327	30	30	30
Histidine	2.01	0.046	2.01	0.025	5.35	0.065	2	2	5.35[c]
Arginine	6.01	0.063	6.05	0.051	6.12	0.071	6	6	6
Tryptophan	3.9–4.6		3.9–4.6		3.9–4.6		4	4	4

[a] If the experiment were repeated, the average would be expected to be within the computed average ± the 95% confidence limits, 95% of the time. [b] Uncorrected for destruction during hydrolysis. [c] Actual average; not rounded off. See text.

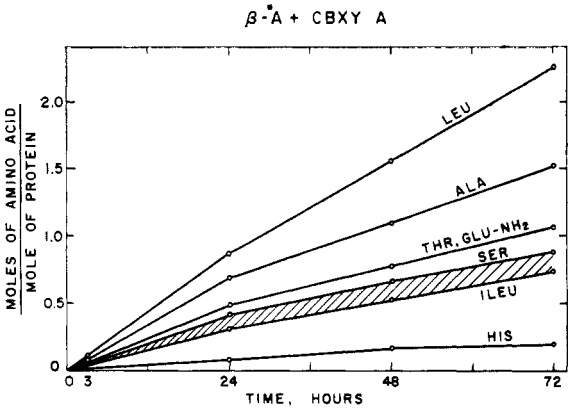

FIGURE 7: Hydrolysis of modified β-A by carboxypeptidase A at *p*H 8.0, 37°, with enzyme-substrate ratio of 1:100. The amino acids found in the shaded area are valine, glutamic acid, and tyrosine.

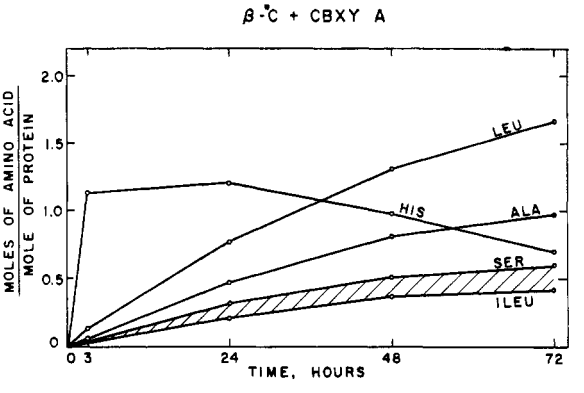

FIGURE 8: Hydrolysis of modified β-C by carboxypeptidase A. The reaction conditions and the amino acids in the shaded area are the same as those in Figure 7 with the addition of threonine.

similar and a specific extinction coefficient of 9.5 was calculated for the carboxypeptidase-treated protein. This is in agreement with the values 9.4–9.6 previously obtained for the native proteins. The other two pairs of proteins produced ultraviolet spectra identical to the ones shown. The similarity of spectra of the native and modified proteins demonstrates the fact that no change in content of tyrosine, cystine, or tryptophan has occurred during the preparation procedure of the modified materials.

The difference spectra of the modified proteins were

examined in aqueous solution with 20% sucrose as a perturbant (Herskovits and Laskowski, 1962). Figure 6a demonstrates the comparison of the difference spectra for reduced unfolded β-A, native β-A, and modified β-A. These data suggest that the numbers of buried tryptophyl and tyrosyl residues of both the native (Townend *et al.*, 1964) and modified proteins are of the same order. From Figure 6b the identical conclusion may be drawn concerning modified β-B and β-C. In this respect, all the carboxypeptidase-treated proteins are similar to one another and to the native materials.

Nitrogen values of 16.0, 16.1, and 16.2% were found for the modified A, B, and C proteins, respectively. These compare favorably with values of 15.8, 15.9, 16.0% calculated from the amino acid composition.

The amino acid composition data are summarized in Table III. Results of six determinations, duplicates of 24-, 72-, and 96-hour hydrolysates, were included in the calculations for all the amino acids with the exception of isoleucine and valine, for which only the 72- and 96-hour values were considered. The residues per mole found in the table are grand averages which were obtained by the following procedure. For each separate analysis of the modified A and B proteins, calculations of the molar ratios were made based on ten different amino acids (aspartic acid, glycine, alanine, methionine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine). Therefore, ten values for each of the six determinations, or sixty values, were then considered to calculate the grand average for each amino acid. From these were eliminated the six values for which the amino acid in question was used as the base to calculate molar ratios and assigned a whole number. The remaining fifty-four values were averaged and the result was reported for the ten amino acids mentioned. In the calculations for the modified C protein, nine amino acids were used as bases to calculate the molar ratios with histidine omitted. For half-cystine and proline, all the values were averaged (60 for A and B and 54 for C). Linear regression analysis by the method of least squares was used to calculate threonine and serine from the data. As mentioned previously (Kalan *et al.*, 1965), cysteine emerges under the proline peak in this analytical system and causes the proline values to be somewhat high. The cystine values are uncorrected for destruction during hydrolysis.

A comparison of the last three columns of Table III with the analyses of the native β-lactoglobulins already reported (Kalan *et al.*, 1965) reveals the changes in composition of the derivatives. The modified β-A and β-B proteins each contain two fewer residues of isoleucine and histidine than the native proteins while the modified β-C protein is lacking two isoleucine residues and less than one residue of histidine. The residue numbers of all other amino acids constituting the modified proteins are identical to the native materials, confirming the fact that no compositional changes have taken place except at the carboxyl-terminal and penultimate positions. The leucine values from this set of data, forty-four residues per mole, agree with those found by Piez *et al.* (1961) for native β-A and β-B, and clarify the slight uncertainty concerning leucine which existed in the recently reported composition of the three native protein variants (Kalan *et al.*, 1965). Tryptophan was calculated in the same manner as that for the native proteins since the extinction coefficients are identical.

The results of the hydrolysis of the modified β-A and β-C proteins with carboxypeptidase A are shown in Figures 7 and 8. Since the results with the modified B protein are essentially identical to those obtained with the modified A, only the latter figure is presented. For the A and B proteins the carboxyl-terminal isoleucine and penultimate histidine of the native materials are no longer present. Leucine is the new C-terminal group released, followed by alanine. The order and rates of release of these amino acids are similar to those found in the hydrolysis of the native proteins with carboxypeptidase (Kalan *et al.*, 1965). The isoleucine released from the modified proteins represents the second residue located near the carboxyl-terminal portion of the chain and is equivalent to the amount released from the native proteins above the 2 eq comprising the C-terminal isoleucine.

Glutamine is not resolved in the analytical system employed and appears as a shoulder on the threonine peak. The value for threonine shown in Figure 7 includes this shoulder, although its contribution appears quite small as judged from the analyzer record. No definite sequence can be established beyond alanine, but it is evident that serine and threonine are located in the immediately adjacent region. The amino acids in the shaded section of Figure 7 are also present in the C-terminal area, but their relative sequence cannot be ascertained from these experiments.

The results for the modified β-C protein present a slightly different picture as shown in Figure 8. As predicted from the electrophoretic mobility and verified by the amino acid composition, the penultimate histidine has been only partially removed. Carboxypeptidase still liberates 1.2 moles of histidine per mole of protein at 24 hours. The decrease of the histidine values from 24 to 72 hours was previously observed in the experiment with the native proteins. No explanation can be offered for this behavior. Leucine and alanine are liberated sequentially at a rate approximately equivalent to the native β-C experiment, but reduced in comparison to the modified β-A and β-B proteins. Leucine does not reach a full 2 eq per mole as it does with the other two modified variants.

The shoulder on the threonine peak ascribed to glutamine with the modified A and B proteins does not appear on the analyzer records of the β-C hydrolysates. In Figure 8, threonine is located in the shaded area between serine and isoleucine.

Discussion

The modified β-lactoglobulins A, B, and C produced by the action of carboxypeptidase A exhibit many interesting properties. Each protein can be crystallized with a crystal habit apparently different from that of the

native materials. The modified β-B protein occurs in two crystal forms, one on initial crystallization from the reaction mixture at *p*H 5.5 and another on subsequent recrystallization from NaCl solution. Davie *et al.* (1959) presented a photograph of β-lactoglobulin AB treated with carboxypeptidase A. In it, two different crystal forms can be observed. One form, diamond shaped, is similar to the modified β-B initial crystals and the other, square plates, shows similarity to the modified β-A crystals. The solubility of the modified A and B proteins is much less than that of the native proteins while the comparison for the modified β-C protein is more difficult to make. (Both crystalline C proteins are quite soluble and the crystals tend to dissolve at room temperature.) From the data presented, the modified β-C preparation is a mixture of at least two different molecules, some with all the isoleucine removed and no histidine, and some with all of both amino acids removed. The ratio of the former to the latter is about 65:35.

As inferred by the optical rotation data, the configuration and tertiary structure of the modified proteins has not been greatly altered from the native proteins. This fact suggests the possibility that the C-terminal isoleucine and histidine are located on the external surface of the β-lactoglobulin molecule, since their removal does not seem to hinder formation of a nativelike tertiary structure. Along these lines, an interpretation of the previous experiments on the action of carboxypeptidase A on the native proteins, where there was noted a sharp break in rates between the histidine-isoleucine and the rest of the amino acids released, leads to a conclusion consistent with the foregoing. Some unfolding of the protein is needed to make this second group of residues accessible to the action of the enzyme while the histidine-isoleucine are released rapidly even at moderately low *p*H values (Davie *et al.*, 1959).

The presence of C-terminal histidine in the modified β-C protein is understandable upon examination of the study of the carboxypeptidase reaction with the native protein. The original decrease of rate of action of the enzyme on native β-C as compared with β-B was ascribed to some change in enzyme accessibility caused by the substitution of histidine for glutamine, the only chemical difference between the variants. One might imagine a small difference in the tertiary structure of β-C in this area which causes the penultimate histidine to be slightly less exposed than the same residue in the other two genetic variants.

The amino acid compositions of the modified crystalline proteins clearly demonstrate again the substitutions among the three proteins. The glycine/aspartic acid and valine/alanine differences between β-A and β-B and especially the glutamine/histidine difference between β-B and β-C are again confirmed. The possibility arises that the treatment of the modified proteins with carboxypeptidase has offered a hint as to the location of this latter amino acid substitution. The failure of glutamine to appear with the modified β-C protein as contrasted to its presence with β-A and β-B might be so interpreted. An alternative and probably more likely explanation is that the reaction with the modified β-C, having a greatly reduced rate compared to the modified A and B proteins, has just not proceeded far enough along the chain to liberate the small amount of glutamine found in the other variants. The definitive answer to this question must await isolation of either the C-terminal peptides from the three proteins or the glu-NH_2/his difference peptides from β-B and β-C.

The rates of release of the amino acids from the modified proteins upon reaction with carboxypeptidase are identical to the rates exhibited during the same reaction with the native materials. The rates do not approach those obtained on denatured material or on the *S*-sulfo derivatives (Kalan *et al.*, 1965). This fact lends added weight to the observation that the modified proteins possess a nativelike configuration although they are lacking the carboxyl-terminal and penultimate residues.

These modified proteins can be valuable in structure and sequence studies on the β-lactoglobulins. Since the difference in composition between β-B and β-C involves glutamine and histidine, the removal of histidine residues, although incomplete in the case of β-C, and subsequent proteolytic digestion of the derivatives might produce peptide maps with these difference peptides more easily located. Physicochemical examination of the derivatives can provide more information on tertiary structure and the effects of removal of two amino acids on such characteristics as tetramerization and the failure of the genetic variants to hybridize (Townend *et al.*, 1961).

Acknowledgments

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Free Energy of Paired Hydrogen Bonds of 2-Aminopyrimidine*

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ABSTRACT: Complex formation between ϵ-caprolactam and 2-aminopyrimidine in cyclohexane has been demonstrated by ultraviolet absorption spectroscopy. The complex is detected by a shift of the absorption band of 2-aminopyrimidine to longer wavelengths. The equilibrium constant for dissociation of the complex was determined with absorbancy values at a particular wavelength and the slope of a plot of $[A - a_P b(P)_0]/(L)_0$ versus A.

The results are consistent with the formation of a 1:1 complex maintained by paired hydrogen bonds of the type in DNA. The equilibrium constant was used to calculate a $\Delta G°$ of 2.23 kcal mole^{-1} for dissociation of the complex.

An important feature of the structure of DNA is the paired hydrogen bonds between the heterocyclic bases (Watson and Crick, 1953). On the basis of melting experiments with synthetic polynucleotides, Crothers and Zimm (1964) have estimated a free-energy change of 2 kcal mole^{-1} for the paired hydrogen bonds of the adenine-thymine base pair. Although a direct determination of the $\Delta G°$ for the dissociation of these bonds would facilitate the interpretation of experiments on DNA strand separation, measurements of intermolecular hydrogen bonding in polar solvents are seriously complicated by solute-solvent interactions. Attempts to determine $\Delta G°$ values with the purines and pyrimidines of DNA in nonpolar solvents failed because these compounds are not sufficiently soluble to provide stable solutions. On the other hand, simple model compounds may provide valuable thermodynamic data on intermolecular hydrogen bonds.

Hydrogen bonds which are similar to those in DNA should be present in a complex between a pyrimidine derivative of the *cis*-amidine type and a compound of the *cis*-amide type. Preliminary experiments indicated that saturated solutions of 4.3×10^{-3} M 2-aminopyrimidine and 0.14 M ϵ-caprolactam could be prepared in cyclohexane at 25°. The effect of the cyclic lactam on the ultraviolet absorption spectrum of the aminopyrimidine was chosen as a suitable model system for the application of a procedure for the determination of $\Delta G°$ values (Rose and Drago, 1959). This model system is based on the observation that a cyclic lactam in a nonpolar solvent will form dimers which are maintained by paired hydrogen bonds (Tsuboi, 1951; Klemperer *et al.*, 1954). A similar structure has been suggested for the dimer of 2-pyridone (Wigler, 1958; Katritzky and Jones, 1960; Krackow *et al.*, 1965).

Experimental

A sample of 2-aminopyrimidine (Mann Research Laboratories) was purified by vacuum sublimation, mp 126.5–127.0°. The ϵ-caprolactam (Calbiochem Organic Chemicals) was recrystallized from ethyl acetate and dried under vacuum, mp 68.0–68.5°. The cyclohexane (Fisher, reagent grade) was purified by passage through a 6- × 30-cm column of dry silicic acid (Mallinckrodt, A.R.). The solvent was stored over anhydrous sodium sulfate and redistilled from sodium-lead alloy (Baker, Dri-Na) just before use.

The ultraviolet absorption spectra of dilute solutions of 2-aminopyrimidine were determined at 25° in a cylindrical cell of 100 mm light path with a Cary Model 14PM recording spectrophotometer. The spectra of more concentrated solutions were determined with rectangular cells and quartz inserts which provide a minimum light path of 0.5 mm.

Results

The ultraviolet absorption band of 2-aminopyrimidine in cyclohexane is given in Figure 1; two vibronic components are found at 2850 and 2920 A and a minor component is at 3025 A. The spectrum of 2.41×10^{-5} M 2-aminopyrimidine was determined in solutions which contained ϵ-caprolactam at 0.14 M

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