Reductive Methylation of β-Lactoglobulin

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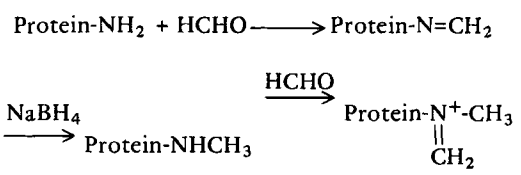
ABSTRACT

The effect of reductive methylation on the properties of β-lactoglobulin was studied with preparations in which 6, 32, or 80% of the amino groups were methylated. Content of sulfhydryl groups was not changed and little difference in electrophoretic mobility of the protein was evident on polyacrylamide gel electrophoresis. Thin-layer isoelectric focusing from pH 4 to 6 indicated that protein in which 80% of the amino groups were modified showed an increase in isoelectric point of no more than .1 pH unit for each electrophoretic component, compared with untreated β-lactoglobulin (A plus B variants). Reductive methylation with [carbon-14] formaldehyde at low reagent concentration yielded a product which contained approximately one mole of [carbon-14] methyl groups for each mole of β-lactoglobulin. Tracer [carbon-14] β-lactoglobulin added to skim milk exhibited a heat-denaturation curve similar to that described for unmodified β-lactoglobulin. Such a radioactively labeled derivative will be useful for studying protein interactions in milk.

INTRODUCTION

Free amino groups in proteins may be methylated by treatment, under mild conditions, with formaldehyde and sodium borohydride (11), as in Equation [1]:



Received July 19, 1978.

$$\text{Protein-N(-H)-CH}_3 \xrightarrow{\text{NaBH}_4} \text{Protein-N(-CH}_3\text{)-CH}_3 \qquad [1]$$

N-Terminal amino groups, and the ε-amino group of lysine, may form both mono and dimethyl derivatives. This procedure, with [^{14}C] HCHO, has been used to prepare radioactively labeled proteins (13, 15). The technique has been applied to caseins (12), and in this report we describe the reductive methylation of β-lactoglobulin and suggest the usefulness of [^{14}C] β-lactoglobulin as a tool for studying protein interactions in milk.

MATERIALS AND METHODS

Chemicals were purchased from the indicated suppliers: β-lactoglobulin, three times crystallized and containing the A and B variants, and sodium borohydride, from Sigma Chemical Co. (St. Louis, MO); formalin (37% aqueous formaldehyde wt/wt) from J. T. Baker Co. (Phillipsburg, NJ); [^{14}C] HCHO (250 μCi, 44 μCi/μmole) and Aquasol® scintillation fluid from New England Nuclear Co. (Boston, MA); and ampholytes, pH 4 to 6 narrow range, from Bio-Rad Laboratories (Richmond, CA).

Protein concentration was estimated from absorbance at 278 nm with untreated β-lactoglobulin as a standard. The absorptivity at 278 nm for a 10 g/liter solution (.1 M NaCl) of β-lactoglobulin A and B was 9.6 (1). Protein also was measured by the method of Lowry (9). Content of free amino group in protein solutions was measured with trinitrobenzenesulfonic acid (6), and free sulfhydryls were measured by the Ellman reaction (4), as modified by Habeeb (7).

Reductive methylation followed the method of Means and Feeney (11) but with three concentrations of reagent to modify the extent of reaction. Twenty milligrams of β-lactoglobulin (which contained .8 μmole of lysine per milligram protein, (5)) were dissolved in 4 ml .2 M borate buffer, pH 9.0, and cooled to 0 C. Then sodium borohydride solution, freshly prepared in cold borate buffer, and formalin

were added as indicated as the reaction mixture was maintained at 0 C:

Procedure A (slight derivation): 10 μl of .29 M sodium borohydride were added, followed by .5 μl formalin and another 10 μl of sodium borohydride; or

Procedure B (moderate derivation): five additions were made over 20 min of 11 μl .29 M sodium borohydride and .5 μl formalin; or

Procedure C (extensive derivation): ten additions were made over 30 min of 10 μl .4 M sodium borohydride and 1 μl formalin.

Thirty minutes after the last addition of reagent, a drop of octanol was added to minimize foaming, and the solution was adjusted to pH 5 with 20% acetic acid to decompose the borohydride. Unless otherwise stated, the protein solution then was dialyzed against water at 5 C and analyzed. Certain extensively modified samples were purified further after methylation by precipitation from 3.1 M ammonium sulfate solution, dialysis, and lyophilization.

To prepare [^{14}C]β-lactoglobulin, Procedure A was used, except that 250 μCi [^{14}C] HCHO (44 μCi/μmole) were used instead of formalin. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter, and counts were corrected for quenching by external standardization.

Discontinuous polyacrylamide gel electrophoresis was by the method of Davis (3), with a 9% separating gel at pH 8.9. Proteins were detected with Coomassie blue (2). Isoelectric focusing used granular Sephadex® G-75 Superfine and 2% pH 4 to 6 ampholytes. Proteins were detected by staining a paper print with Coomassie blue. The protein standards, and their isoelectric points by this technique, were: bovine serum albumin (pH 4.84 and 4.71, for the two major components); β-lactoglobulin (pH 5.14 and 5.3 for A and B variants); and conalbumin (pH 5.88) (14).

Heat denaturation was studied essentially as described by Larson and Rolleri (8). A tracer amount of [^{14}C]β-lactoglobulin (1 μCi) was added to 60 ml raw skim milk, and 3.0-ml aliquots of the mixture were dispensed into tubes. The tubes were incubated, in triplicate, for 30 min at several temperatures between 66 and 82 C; an additional 3 min were allowed for the milk to reach bath temperature. After heating, the tubes were cooled rapidly, and each aliquot was adjusted to pH 4.6 with 10% acetic acid. This precipitated casein and denatured whey proteins. After centrifugation, the amount of ^{14}C from [^{14}C]β-lactoglobulin remaining in the supernatant was measured. Concentration of undenatured [^{14}C] β-lactoglobulin was expressed as percent of the concentration in the pH 4.6 supernatant from unheated milk, as determined by measurement of radioactivity.

RESULTS AND DISCUSSION

Reductive methylation of β-lactoglobulin caused increased absorbance in the Lowry protein assay, so use of this method, based on standardization with untreated β-lactoglobulin, may yield spurious results. Therefore, comparisons of free sulfhydryl and amino group content in β-lactoglobulin and methylated β-lactoglobulin were based on protein concentration, as determined by absorbance of the protein solutions at 278 nm.

Even extensive reaction (Table 1), which resulted in derivation of 80% of the amino groups, caused no change in content of free sulfhydryl groups. β-Lactoglobulin contains one sulfhydryl group and two disulfide linkages for each monomer of 18,000 molecular weight (10). Means and Feeney reported no loss of

TABLE 1. Content of sulfhydryl and amino groups following reductive methylation of β-lactoglobulin.

Protein	Relative content of: -SH	-NH_2
Untreated β-lactoglobulin	1.00	1.00
Reductively methylated β-lactoglobulin		
Procedure A		
Sample 1	1.01	.94
Sample 2	.99	.95
Procedure B		
Sample 1	1.03	.68
Sample 2	.95	.69
Procedure C		
Sample 1	1.00	.19
Sample 2	1.05	.20

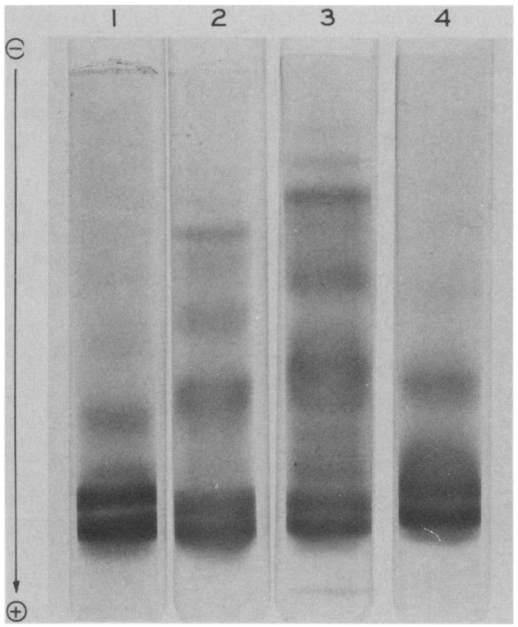


FIG. 1. Disc gel electrophoresis of unmodified and reductively methylated β-lactoglobulin: 1) unmodified β-lactoglobulin; 2) reductively methylated β-lactoglobulin, 32% of amino groups modified; 3) and 4) two preparations of reductively methylated β-lactoglobulin, 80% of amino groups modified. The protein of gel 4) had been purified after methylation by precipitation from 3.1 M ammonium sulfate solution.

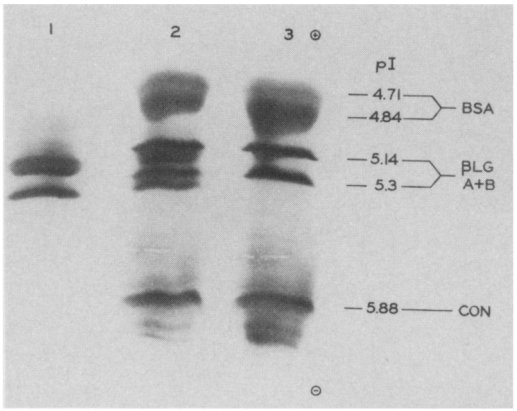


FIG. 2. Isoelectric focusing of reductively methylated β-lactoglobulin: 1) reductively methylated β-lactoglobulin, 80% of amino groups modified, purified after methylation by precipitation from 3.1 M ammonium sulfate solution; 2) a mixture of reductively methylated β-lactoglobulin with protein standards; 3) protein standards: bovine serum albumin (BSA), unmodified β-lactoglobulin (βLG), and conalbumin (CON). The pI for each major component of the standards is shown.

sulfhydryls on reductive methylation of glutathione or chicken ovalbumin (11).

Disc electrophoresis revealed no apparent change in the electrophoretic mobility of the two major bands, corresponding to the A and B variants, due to reductive methylation (Fig. 1). However, there was an increase in concentration of the minor, slower moving protein species, with a shift in the distribution of the minor components. Purification by precipitation from ammonium sulfate solution eliminated most of the minor components. Thin-layer isoelectric focusing of methylated β-lactoglobulin (80% of amino groups derivatized) demonstrated that the two bands in methylated β-lactoglobulin were shifted slightly toward the cathode, with an increase in the isoelectric point of .1 pH unit or less for each component (Fig. 2).

Carbon-14 reductively methylated β-lactoglobulin incorporated 2.7 μCi of the label (.06 μmole [^{14}C] HCHO) for each mg of protein, or approximately one mole of [^{14}C] methyl groups for each mole of β-lactoglobulin. This radiolabeled protein was tested in a milk protein system by adding a tracer amount of [^{14}C] β-lactoglobulin to raw skim milk and subjecting the mixture to heat treatments of 30 min at 66 to 82 C. In Figure 3, the heat-denaturation of [^{14}C] β-lactoglobulin in skim milk is compared with data derived from that reported for heat-denaturation of endogenous β-lactoglobulin in milk (8). In the latter experiment (8), specific protein concentrations were determined by electrophoretic techniques and are recalculated here as the percent of the concentration in the pH 4.6 filtrate from unheated (40 C) milk. The denaturation of [^{14}C] β-lactoglobulin added to skim milk between 66 and 82 C is similar to that described for endogenous β-lactoglobulin in milk. These data indicate that there is little change in β-lactoglobulin as a result of reductive methylation. Nevertheless, reductive methylation may have altered the protein in subtle ways that will become evident only when more sensitive analytical methods are applied.

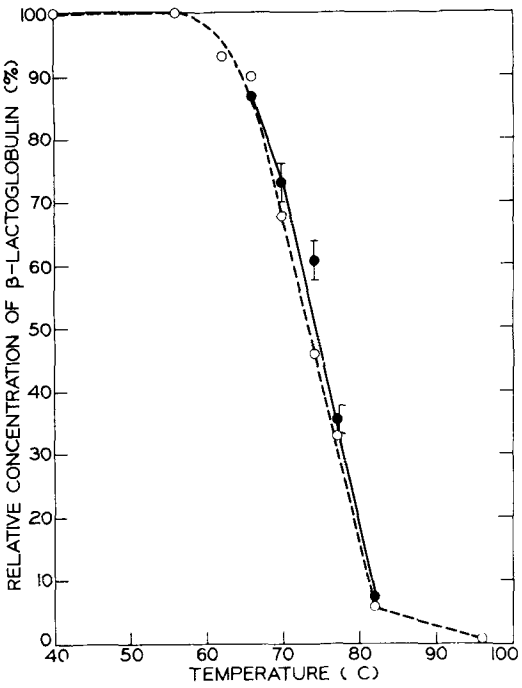


FIG. 3. Denaturation of [^{14} C]β-lactoglobulin added to skim milk, compared with that of endogenous β-lactoglobulin in milk, following 30 min heating at various temperatures: (o- - -o) denaturation of endogenous β-lactoglobulin, derived from the data of Larson and Rolleri (8). (•——•) Denaturation of [^{14}C]β-lactoglobulin added to skim milk; a range for triplicates of more than 2% is indicated by a bar.

ACKNOWLEDGMENTS

This research was supported by a grant from Ross Laboratories (Columbus, OH) and by the College of Agricultural and Life Sciences, University of Wisconsin (Madison, WI).

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