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MALEYL-α-LACTALBUMIN AS LACTOSE SYNTHETASE SPECIFIER PROTEIN

B. KITCHEN* and T. E. BARMAN

National Institute for Research in Dairying, Shinfield, Reading, RG2 9AT (Great Britain)

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SUMMARY

We have studied the effects on the function and conformation of bovine α-lactalbumin after reaction with the protein-dissociating reagent maleic anhydride.

The 13 amino groups of α-lactalbumin were accessible to the reagent and when all of these were maleylated a highly acidic and more expanded protein resulted. Despite these differences in physical properties, maleyl-α-lactalbumin was as effective as native α-lactalbumin in the lactose synthetase reaction. On the other hand, when α-lactalbumin was treated with trinitrobenzenesulphonic acid, inactivation occurred.

INTRODUCTION

The enzyme lactose synthetase (EC 2.4.1.22) is made up of two different protein components, lactose synthetase A protein and α-lactalbumin. α-Lactalbumin itself is devoid of catalytic activity and its sole function appears to be to change the acceptor substrate specificity of the A protein from *N*-acetylglucosamine to glucose[1].

The mechanism of action of lactose synthetase and, in particular, that of the interaction of α-lactalbumin with the A protein is poorly understood. It is thought that the presence of a substrate is required for the interaction and that the lactose synthetase complex contains one molecule each of the A protein and α-lactalbumin[2,3]. In previous communications[4,5] it was suggested that three of the four tryptophan residues of α-lactalbumin (Trp-26, Trp-104 and Trp-118) are located within a single hydrophobic region on the α-lactalbumin surface and that this region is involved in the interaction of the A protein with α-lactalbumin. Another area[6] which could be concerned in the interaction is the stretch Gly-100–Lys-108: this is the longest stretch conserved in the three α-lactalbumins of known sequences (bovine, human and guinea pig)[6,7]. This area includes Trp-104 (which may be functionally important)[5] and His-107 (which is probably functionally unimportant)[8]. It also includes a lysine residue (Lys-108): another lysine is close (Lys-98 in bovine α-lactalbumin and Lys-99 in human and guinea pig α-lactalbumins). α-Lactalbumin has a high lysine content: α-lactalbumin from bovine milk, for instance, has 10 lysine residues per

Abbreviations: TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNP, 2,4,6-trinitrophenyl group.

* Present address: Otto Madsen Dairy Research Laboratory, Department of Primary Industries, Brisbane, Australia.

100 amino acid residues whereas the "average protein" has only 6 (ref. 9). All the lysine residues of bovine α-lactalbumin are accessible to trinitrobenzenesulphonic acid (TNBS)[10] and it could be that one or more of these is involved in the α-lactalbumin–A protein interaction.

We have modified the amino groups of bovine α-lactalbumin by reaction with maleic anhydride (a reagent which replaces the positively charged amino group by the small but acidic maleyl group) and with TNBS [which results in the introduction of the bulky but neutral trinitrophenyl group (TNP)]. Our results show that maleylation — a method much used to dissociate protein subunits[11,12] — has little effect on the interaction between α-lactalbumin and the A protein whereas trinitrophenylation abolishes the interaction.

EXPERIMENTAL

α-Lactalbumin was prepared as described previously[13]. TNBS was a product of Pierce Chemical Company and maleic anhydride of British Drug Houses Ltd. [2,3-$^{14}C_2$]Maleic anhydride (26.8 Ci/mole) was obtained from the Radiochemical Centre, Amersham, Bucks, Great Britain. All other products were obtained from commercial sources and were used without further purification.

Analytical methods

The concentrations of solutions of native or modified α-lactalbumins were estimated as described previously[4]. The molecular weight of α-lactalbumin was taken to be 14176 and its total number of amino groups 13 (ref. 14). The TNP group was estimated at 367 nm (ref. 15) and the maleyl group at 250 nm (ref. 11). The unreacted amino groups in maleylated α-lactalbumin were determined by treating the modified α-lactalbumin with a 100-fold molar excess of TNBS (see below).

Radioactive samples (1 ml) were diluted with 10 ml scintillation fluid and counted on a Philips liquid scintillation analyser (Model PW 4510/0) in which ^{14}C is counted with an efficiency of 64%.

Sedimentation constants were obtained on the Beckman Model E analytical ultracentrifuge at 44770 rev./min. Two measurements of sedimentation velocity were conducted simultaneously so that native and maleyl-α-lactalbumin could be compared under identical conditions. This was done by using two cells, each with a capillary type synthetic boundary centre piece; one cell had a standard window and the other a positive wedge window.

Reaction of α-lactalbumin with TNBS

Portions (1 ml) of a solution of 0.34 mM α-lactalbumin in 0.5 M $NaHCO_3$ were treated with TNBS (0.36–36 μmoles) dissolved in 1 ml 0.5 M $NaHCO_3$. The mixtures were incubated in the dark at 37 °C for 90 min and the modified proteins recovered after gel filtration and freeze–drying.

Reaction of α-lactalbumin with maleic anhydride

The procedure used was essentially that of Butler *et al.*[11]. Typically, 5.8 μmoles α-lactalbumin were dissolved in 10 ml 0.1 M sodium pyrophosphate buffer (pH 9.0) and the well-stirred solution treated with 156 μmoles maleic anhydride in 0.2 ml dioxane. The pH of the mixture was kept at 9 (1 M NaOH). Excess reagent was

removed by gel filtration and the modified protein recovered by freeze-drying. The product contained an average of 13.9 moles of the maleyl group and 0.6 moles of remaining amino groups per mole of protein: it was homogeneous when subjected to electrophoresis at pH 8.6 (see below). This material will be referred to as maleyl-α-lactalbumin.

α-Lactalbumin was also treated with [2,3-$^{14}C_2$]maleic anhydride. [2,3-$^{14}C_2$]-Maleic anhydride was diluted with carrier anhydride in dioxane to a specific activity of 0.49 Ci/mole and this material was used to maleylate 2.9 μmoles of α-lactalbumin following the procedure described above. The [2,3-$^{14}C_2$]maleyl-α-lactalbumin thus obtained had a spec. act. of 5.9 Ci/mole and contained 13.7 maleyl groups per mole of protein.

RESULTS

Accessibilities of the amino groups of α-lactalbumin

When α-lactalbumin was treated with a 26-fold molar excess of maleic anhydride, 13.9 maleyl groups were incorporated. A molar excess of TNBS of about 110 was required for the incorporation of 12.5 TNP groups into α-lactalbumin. Since the remaining amino group content of maleyl-α-lactalbumin was found by reaction with TNBS to be about 0.6, reaction with the hydroxy amino acids of α-lactalbumin had probably occurred[16]. Our results are in good agreement with those of Habeeb and Atassi[10] and they indicate that the amino groups of α-lactalbumin are accessible to maleic anhydride and TNBS.

The effect of the modification of the amino groups on the specifier protein activity of α-lactalbumin

As indicated in Fig. 1, the specifier protein activity of α-lactalbumin was progressively lost when the molar ratio of TNP to protein was increased with 50%

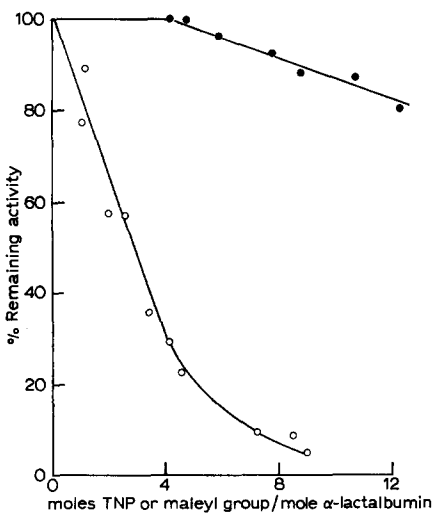

Fig. 1. The effect of the incorporation of the TNP group (○—○) or the maleyl group (●—●) into α-lactalbumin on its specifier protein activity in the lactose synthetase reaction. The concentration of native or modified α-lactalbumin in each reaction mixture was 1 μM. For other details see Table I and the text.

inactivation at a molar ratio of 3. On the other hand, 4 moles of the maleyl group could be introduced into α-lactalbumin without affecting its specifier protein activity and even after the introduction of 13.9 groups, the K_m of α-lactalbumin in the lactose synthetase reaction was increased by only 50% (Table I). The maleyl group of maleyl-α-lactalbumin was not eliminated during the course of the lactose synthetase reaction (Fig. 2).

TABLE I

THE EFFECT OF THE CONCENTRATION OF SPECIFIER PROTEIN (NATIVE OR MODIFIED α-LACTALBUMIN) IN THE LACTOSE SYNTHETASE REACTION

Maleyl-α-lactalbumin was prepared as described in the text. Lactose synthetase activity was determined by the spectrophotometric method of Andrews[2].

Specifier protein	*V (relative)*	*K_m (μM)*
Native α-lactalbumin	1.0	1.0
Maleyl-α-lactalbumin	1.0	1.5

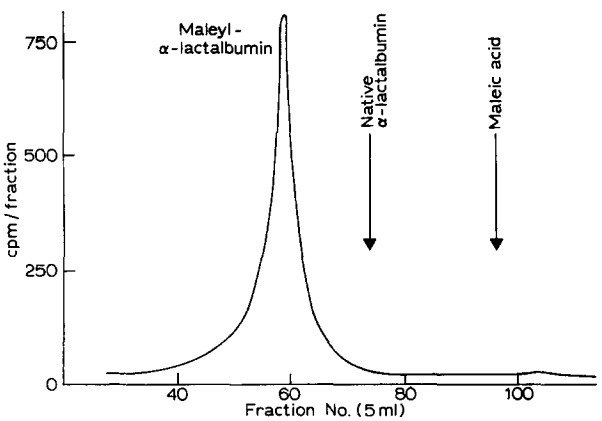

Fig. 2. The recovery of [2,3-$^{14}C_2$]maleyl-α-lactalbumin from a lactose synthetase reaction mixture containing [2,3-$^{14}C_2$]maleyl-α-lactalbumin as specifier protein. 1.08 nmoles [2,3-$^{14}C_2$]maleyl-α-lactalbumin (containing 13.7 maleyl groups per mole of α-lactalbumin, see text) was added to a lactose synthetase reaction mixture (1 ml) containing 3 μg lactose synthetase A protein and the reaction followed spectrophotometrically at 340 nm (ref. 2). The initial rate was 73% of that obtained with 1.0 nmole of native α-lactalbumin as the specifier protein (also see Fig. 1). After incubation for 10 min, the reaction mixture was subjected to gel filtration on a column of Sephadex G-100 (500 mm × 36 mm) in 0.05 M NH_4HCO_3 and 1-ml portions from each fraction counted. The elution volume of maleic acid was obtained by subjecting to gel filtration a sample (0.036 μmole) of [2,3-$^{14}C_2$]maleyl-α-lactalbumin which had been incubated in 2 ml 0.17 M acetic acid, pH 2.7, at 37 °C for 4 h. Under these conditions 12% of the maleyl group was eliminated.

Investigation of conformational changes

Some of the physical properties of native and maleyl-α-lactalbumin are summarised in Table II together with their immunological properties.

Maleyl-α-lactalbumin has a lower sedimentation coefficient than the native

TABLE II

PHYSICAL AND IMMUNOLOGICAL PROPERTIES OF NATIVE AND MALEYL-α-LACTALBUMIN

Maleyl-α-lactalbumin was prepared as described in the text. Sedimentation constants were determined at a protein concentration of 10 mg/ml; for other details see the text. Stokes radii were determined by gel filtration on a column of Sephadex G-100 (500 mm × 36 mm) in 0.2 M NH_4HCO_3 containing 1 M KCl, pH 8.5. Under these conditions the elution volumes for native α-lactalbumin and maleyl-α-lactalbumin were 358 ml and 336 ml, respectively (see refs 5 and 17). Electrophoretic mobilities were determined by thin-layer electrophoresis at pH 8.6 (ref. 13). The reaction of maleyl-α-lactalbumin with rabbit antiserum to native α-lactalbumin was carried out by the interfacial ring test[18].

Property	*Native α-lactalbumin*	*Maleyl-α-lactalbumin*
Molecular weight, calculated	14176	15450
$s_{20,w}$ (in 0.1 M KCl, pH 7.5)	1.79	1.68
$s_{20,w}$ (in 0.1 M KCl, pH 8.5)	1.81	1.61
Stokes radius (Å), theoretical (ref. 17)	19.4	19.6
Stokes radius (Å), found	19.2	21.3
Electrophoretic mobility (relative)	1	5.8
Reaction with antiserum to native α-lactalbumin	+	−

protein and since the value obtained at pH 8.5 was lower than that at pH 7.5 this is probably a reflection of an isotropic swelling process. Habeeb and Atassi[10] also found that, at pH 7.5, maleylated α-lactalbumin had a lower sedimentation constant than the native protein. It is known that at pH values above neutrality α-lactalbumin exists largely as a monomer: at pH 8.55, for instance, there was little evidence for association[19]. The low sedimentation coefficients obtained for maleyl-α-lactalbumin under our experimental conditions are, therefore, unlikely to be due to dissociation as a consequence of charge repulsion phenomena.

Gel filtration is often used as a method for detecting gross conformational changes in proteins[20]. Highly acidic proteins such as maleyl-α-lactalbumin, however, present a problem in that they can be repelled by the somewhat acidic Sephadex[17] or Biogel media[21]. This effect can often be abolished by the use of buffers of high ionic strengths but this device may prevent the manifestation of the very property under investigation, namely, the repulsive forces leading to expansion of the protein molecule. The Stokes radius of maleyl-α-lactalbumin given in Table II is probably a minimum value since it was obtained in a buffer of high ionic strength; in a buffer of lower ionic strength (0.2 M NH_4HCO_3) a value of 22.6 Å was obtained. These values are considerably lower than the 31 Å obtained by Habeeb and Atassi[10] who, however, conducted their experiments with Biogel P60 and a buffer of low ionic strength.

Maleyl-α-lactalbumin was homogeneous on electrophoresis and it migrated much more rapidly than the native protein. The fact that maleyl-α-lactalbumin did not react with anti-α-lactalbumin could be due to loss of the antigenic sites of the native protein but it could also be caused by charge repulsion between maleyl-α-lactalbumin and anti-α-lactalbumin.

DISCUSSION

When the maleyl group is introduced into a protein molecule, a positively charged amino group is replaced by a negatively charged carboxyl group. Maleylated proteins have, therefore, an increased negative charge at neutral pH and this results in reduced protein–protein interactions. The introduction of a few maleyl groups is often enough to induce dissociation of oligomeric proteins: for example, when 16 of the 107 lysine residues of aldolase were maleylated, the native tetramer dissociated[12]. When single chain proteins are maleylated, expansion of the protein molecule through electrostatic repulsion and increased protein–water interaction would be expected to occur.

Bovine α-lactalbumin has a high content of glutamic and aspartic acid residues and when its 13 amino groups are maleylated a protein results in which, on average, one residue in four has a carboxyl group. The structure of maleylated α-lactalbumin is more expanded than that of the native protein and it may possess few if any of its antigenic sites. In spite of these differences, maleyl-α-lactalbumin was only slightly less effective than native α-lactalbumin as lactose synthetase specifier protein. These results indicate not only that the amino groups of α-lactalbumin are not involved in its interaction with lactose synthetase A protein but also that the α-lactalbumin molecule can undergo large changes in its surface charge properties without appreciably affecting its biological function. It has been proposed that the local conformations of certain regions of the α-lactalbumin molecule are maintained by charge pairs involving 6 lysine residues[22]: our results indicate that these regions are probably not important in the A protein–α-lactalbumin interaction. When α-lactalblumin was modified with TNBS, rapid inactivation occurred. It is clear, therefore, that the α-lactalbumin molecule can tolerate a large increase in its negative charge, but not the introduction of the bulky TNP group without losing its specifier protein activity. It is possible that the region on the α-lactalbumin surface which interacts with the lactose synthetase A protein is relatively stable and remains intact when the remainder of the protein changes its physical properties but that when the TNP group is introduced inactivation occurs due to steric hindrance.

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