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The Effect of Modification and Fragmentation of α-Lactalbumin on Lactose and Lactosamine Synthase Reactions

M M MITRANIC and M A MOSCARELLO*

Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

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In the presence of the modifier protein α-lactalbumin, bovine milk galactosyltransferase transfers galactose to glucose forming lactose instead of transferring to *N*-acetylglucosamine forming *N*-acetyllactosamine. At low concentrations of α-lactalbumin, the lactosamine synthase activity is stimulated by α-lactalbumin and decreases when the lactose synthase activity develops along a sigmoidal curve. The observation suggests that different interactions between α-lactalbumin and enzyme were responsible for the modulating effect of the α-lactalbumin in the lactose and lactosamine synthase reactions.

To study the nature of the protein-protein interactions, α-lactalbumin was both modified and cleaved chemically. Reduction and alkylation with iodoacetic acid, iodoacetamide or 4-vinylpyridine abolished the ability of the α-lactalbumin to induce lactose synthase activity but stimulated lactosamine synthase activity 7- to 12-fold.

A peptide fragment corresponding to residues 26-60 of α-lactalbumin isolated from a 2-(2-nitrophenylsulphenyl)-3-methyl-3'-bromo-indolene (BNPS-skatole) fragmentation of the molecule was active in the lactosamine but not lactose synthase reaction. We concluded that, whereas lactose synthase required α-lactalbumin in the native conformation, lactosamine synthase activity was stimulated by a linear sequence of amino acids in peptide 26-60.

In the absence of α-lactalbumin, milk galactosyltransferase transfers galactose from UDP-galactose to *N*-acetylglucosamine, forming *N*-acetyllactosamine. In the presence of α-lactalbumin, the same enzyme transfers galactose from UDP-galactose to glucose forming lactose. Therefore α-lactalbumin has a unique role to play in the modulation of the activity of milk galactosyltransferase. The mechanism of this modulation has been studied extensively [1-11].

Abbreviations: MES, 4-*N*-morpholinoethanesulfonic acid; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UDP-Gal, uridinediphosphogalactose; BNPS-skatole, 2-(2-nitrophenylsulphenyl)-3-methyl-3'-bromo-indolene; EDTA, ethylene diamine tetra acetic acid.

*Author for correspondence.

It is generally agreed that the modifying activity of α-lactalbumin is exerted through specific protein-protein interactions between α-lactalbumin and galactosyltransferase. Neither the peptide sequences involved nor the nature of the interaction is understood. Chemical modification of α-lactalbumin has implicated certain amino acid residues which might be important in the interaction between α-lactalbumin and the enzyme. Thus a role for tryptophan has been suggested [12]. In addition, two lysyl residues have been implicated at or near the interaction site [9, 13]. By the use of a UDP-galactose photoaffinity analogue, 4-azido-2-nitrophenyluridylyl pyrophosphate, the protein-protein interaction site in the region of lactose synthetase was studied [14], and it was concluded that α-lactalbumin did not bind to galactosyltransferase at the UDP-galactose binding domain. In an NMR study the close proximity of Ile 95, Tyr 103, Trp 60 and Trp 104 has been demonstrated [15] from which the existence of a hydrophobic box was postulated, similar to the hydrophobic box postulated for lysozyme. On the basis of competitive inhibition studies with a series of glucosides, the α-lactalbumin interaction site has been placed near the monosaccharide binding site [16].

Although it is generally agreed that *N*-acetyllactosamine synthase activity of milk galactosyltransferase is inhibited at concentrations of α-lactalbumin which support maximal lactose synthase activity, we have observed consistently that *N*-acetyllactosamine synthase activity is stimulated at low concentrations of α-lactalbumin. At maximum stimulation of *N*-acetyllactosamine little lactose synthase activity was observed, implying a requirement for different protein-protein interactions for the two enzymatic activities of galactosyltransferase. This observation has led us to study the effect of peptide fragments of α-lactalbumin on the two enzymatic activities of galactosyltransferase. The first studies in this area are presented in this manuscript.

Materials and Methods

Reduction and Alkylation of α-Lactalbumin

Three aliquots of 10 mg each of commercial α-lactalbumin (Sigma Chemical Co., St. Louis, MO, USA) in 1.0 ml 0.1 M MES buffer pH 8.8 (previously purified on G-100 Sephadex) were flushed with nitrogen and 25 μl of 2-mercaptoethanol were added to each followed by incubation at room temperature for 1 h. To the first aliquot 120 mg of iodoacetic acid was added, to the second 120 mg iodoacetamide and to the third 57 μl of 4-vinylpyridine. In all samples the pH was maintained at 9.0 by addition of 1 N NaOH. After 3 h at room temperature each sample was dialysed against 100 ml of 0.05 M MES buffer pH 8.8 for 1 h followed by dialysis against 1 l of water overnight.

The loss of cystine was monitored by amino acid analyses in a Water's Pico tag system after hydrolysis in 5.7 N HCl for 24 h.

BNPS-Skatole Cleavage

To 200 mg of reduced and alkylated α-lactalbumin in 10 ml of 70% acetic acid was added 12.8 mg tyrosine as scavenger. To 2 ml of 100% acetic acid was added 200 mg BNPS-skatole. The BNPS-skatole solution was combined with the α-lactalbumin solution and allowed to react at 37°C, 4 ml of H_2O_2 was added followed by 30 ml of ethyl acetate. The phases were separated by centrifuging for 10 min. The aqueous phase was extracted two

more times with ethyl acetate and then lyophilized. Excess tyrosine was removed by extraction with 10 ml of acetone. The method is essentially that described by Martenson *et al.* [17].

The resulting peptides were applied to a Sephadex G-50 column (1 × 20 cm) in 0.05 M ammonium bicarbonate containing 2 M urea. This ensured that the fragments were separated from free tyrosine and any remaining reagents. The fragments were dialysed against water in dialysis tubing with a 3500 molecular weight cut off, lyophilized, dissolved in 0.01 M Tris-HCl buffer pH 8.2 and applied to a Sephadex A-50 column (1 × 10 cm). Elution was carried out with a gradient of 0-0.5 M NaCl in Tris buffer pH 8.2. The gradient was determined by measuring the conductivity. Peak 3 eluting at approximately 100 mS (S = Siemens where 1 S = 1 Ohm × m^{-1}) was isolated, dialysed and lyophilized. The yield of peptide was 21.4% on a dry weight basis. This material corrresponded to residues 26-60 of α-lactalbumin. It was characterized by gel electrophoresis, amino acid analysis and microsequencing in a Beckman 890-M sequencer.

Bovine milk galactosyltransferase was obtained from Sigma. *N*-Acetyllactosamine and lactose synthases were assayed as reported previously [18]. The incubation mixture for *N*-acetyllactosamine synthase activity contained 100 mM MES buffer pH 7.4, 10 mM $MnCl_2$, 20 mM *N*-acetylglucosamine, 1 mM UDP-galactose containing 10^5 dpm UDP-[^{14}C]galactose (New England Nuclear, Boston, MA, USA; Sp.act. 302 mCi/mmol). The incubation mixture for lactose synthase activity was the same except 20 mM D-glucose replaced the *N*-acetylglucosamine and α-lactalbumin was added.

For both activities, the incubation was done at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 1 mM EDTA and the mixture was passed through a Dowex 1-X8 column in the Cl^- form prepared in Pasteur pipettes. The columns were washed with 3 ml distilled water which was collected directly into scintillation vials. After addition of scintillant, the sample was counted in a Tracor Betatrac liquid scintillation counter. The results are given as nmoles of galactose transferred in 30 min at 37°C. Each assay was done in duplicate. An appropriate endogenous acceptor activity was subtracted from each incubation.

Results

The Effect of Low Concentrations of α-Lactalbumin on Lactose and Lactosamine Synthase Reactions

At low concentrations of α-lactalbumin, the lactose synthase activity is sigmoidal (Fig. 1A). No activity was detected until the molar ratio of α-lactalbumin/enzyme was 25 (75 pmol α-lactalbumin). However, *N*-acetyllactosamine synthase activity increased immediately upon addition of α-lactalbumin to the enzyme and increased linearly to a maximum stimulation of about 500% at an α-lactalbumin to enzyme ratio of 25. At this concentration of α-lactalbumin lactose synthase was barely detectable but increased rapidly thereafter. As the lactose synthase activity increased, the lactosamine synthase activity decreased (Fig. 1B). We concluded that the interaction which stimulated lactosamine synthesis was different from that required for lactose synthase and possibly involved different peptide segments. The separation of the peptide sequences responsible for these activities was explored by chemical fragmentation of α-lactalbumin.

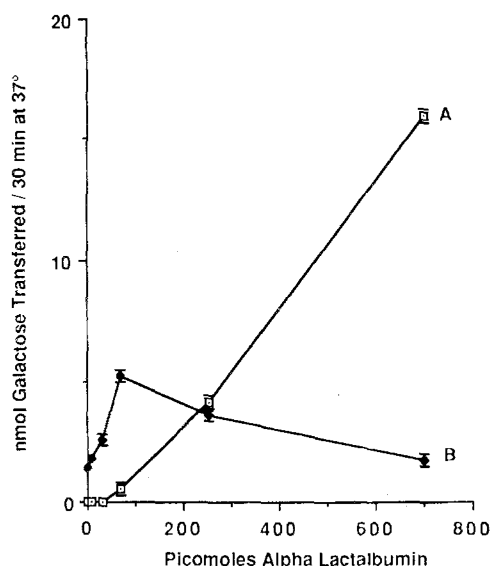

Figure 1. The effect of low concentrations of α-lactalbumin on milk galactosyltransferase activity. A, Lactose synthase; B, *N*-Acetyllactosamine synthase.

Reduction and Alkylation of α-Lactalbumin

α-Lactalbumin was reduced and alkylated as described in the Methods section with three alkylating agents, iodoacetamide, iodoacetic acid and 4-vinylpyridine. The results are shown in Table 1. The unmodified α-lactalbumin showed an increased in *N*-acetyllactosamine synthase activity at low concentrations followed by a gradual decrease to the level of the activity observed in the absence of any α-lactalbumin. The increase at low α-lactalbumin concentrations was observed routinely. When reduced and alkylated with either iodoacetic acid, iodoacetamide or 4-vinylpyridine, an increase in *N*-acetyllactosamine synthase activity was observed. Since the charge imparted by the different alkylating reagents varied from negative (iodoacetic acid) to positive (4-vinylpyridine), the activity was not affected by the additional charge on the cysteinyl residues. On the other hand alkylation with iodoacetamide (an apolar group) yielded an α-lactalbumin which had the greatest stimulatory effect on the lactosamine synthase reaction. None of the alkylated forms of α-lactalbumin supported lactose synthase activity.

Tryptophanyl Cleavage of Reduced and Alkylated α-Lactalbumin

Cleavage of α-lactalbumin, reduced and alkylated with iodoacetamide, with BNPS-skatole yielded a mixture of fragments (Fig. 2, lane B). The ability of this mixture to support both lactose and lactosamine synthase activities is shown in Fig. 3. As expected, no lactose synthase activity was detected (Fig. 3, curve B) when compared to the effect of

Table 1. The effect of reduction and alkylation of α-lactalbumin on *N*-acetyllactosamine synthase activity of bovine milk galactosyltransferase.

α-Lactalbumin added (μg)	Enzyme activity (nmol galactose transferred/30 min at 37°C)[a]			
	Native	Alkylating reagent used		
		Iodoacetic acid	iodoacetamide	4-vinylpyridine
0	2.14	2.14	2.14	2.3
2	4.3	9.7	16.7	7.7
25	2.5	18.2	19.7	14.4
50	2.0	16.1	23.2	15.6

[a] The amount of enzyme in each assay was 3.2 pmoles.

native α-lactalbumin (Fig. 3, curve A). Lactosamine synthase activity was not supported by native α-lactalbumin except at low concentrations (Fig. 3, curve C) but was stimulated in the presence of the BNPS-skatole generated fragments (curve D).

Separation of BNPS-Skatole Generated Fragments

BNPS-Skatole fragments were prepared and fractionated as described in the Methods section. A single peptide was isolated (Fig. 2C). The amino acid composition of this peptide is given in Table 2 along with the theoretical values of peptide 26-60. The correspondence between the values expected and found is good for most residues although Thr is low and Leu is high. The low Thr is probably the result of partial destruction while the high Leu is due to the elution of breakdown products of BNPS-skatole, some of which elute in this area of the chromatogram. Although tyrosine was added as scavenger, some destruction of Tyr is not unexpected, accounting for the low Tyr value. Further identification of this peptide was done by automated sequencing of the first nine amino acids on a Beckman microsequencing apparatus which yielded Val-Cys-Thr-Thr-Phe-His-Thr-Ser-Gly. From these data we concluded that the peptide had the sequence Trp-Val-Cys-Thr-Thr-Phe-His-Thr-Ser-Gly-Tyr-Asx-Thr-Glx-Ala-Ile-Val-Glx-Asx-Asx-Glx-Ser-Thr-Asp-Tyr-Gly-Leu-Phe-Glx-Ile-Asx-Asx-Lys-Ile from the sequence published by Castellino and Hill [21].

Effect of Fragment 26-60 on Lactose and Lactosamine Synthetase Activities of Bovine Milk Galactosyltransferase

The effect of fragment 26-60 on the activity of bovine milk galactosyltransferase in both lactose and lactosamine synthase reactions is shown in Fig. 4. The expected stimulation of lactose synthase activity was observed with native α-lactalbumin (Fig. 4A). Little activity was obtained with fragment 26-60, compared with native α-lactalbumin (Fig. 4B). The effect of native α-lactalbumin on the lactosamine synthase activity is shown in Fig. 4C. The effect of peptide 26-60 on the lactosamine synthase is shown in Fig. 4D. A large increase in activity was observed to a maximum of 17-fold over the activity in the absence of α-lactalbumin.

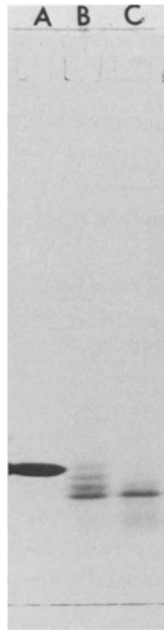

Figure 2. Polyacrylamide gel electrophoresis of BNPS-skatole cleavage of α-lactalbumin.
A, α-lactalbumin; B, mixture of fragments; C, isolated BNPS-skatole generated fragment (26-60). The separating gel was 7.5-15% polyacrylamide.

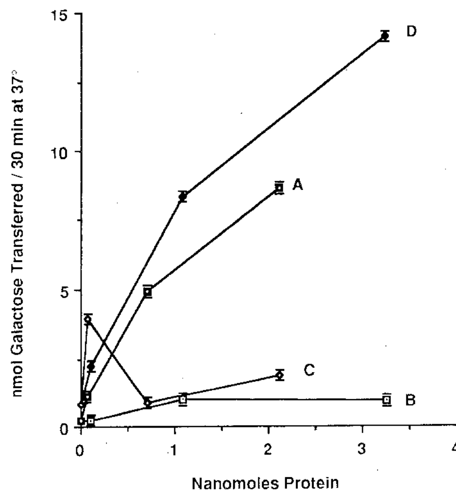

Figure 3. The effect of BNPS-skatole cleavage on lactose and lactosamine synthases of bovine milk galactosyltransferase.
Lactose synthase: (A) in the presence of native α-lactalbumin; (B) in the presence of BNPS-skatole fragments of α-lactalbumin. Lactosamine synthase: (C) in the presence of native α-lactalbumin; (D) in the presence of BNPS-skatole fragments.

Table 2. Amino acid analysis of peak 3 from the Sephadex A-50 column.

Amino acid	Residues/34 amino acids	
	Found	Theoretical
Asp	6.1	6
Thr	2.6	5
Ser	2.7	2
Glu	4.4	4
Pro	—	—
Gly	3.2	2
Ala	1.6	1
Val	1.4	2
1/2Cys	—	—
Met	—	—
Ileu	1.4	3
Leu	4.0	1
Tyr	0.7	2
Phe	1.0	2
Lys	1.7	1
His	0.9	1
Arg	—	—

Discussion

The interaction between α-lactalbumin and milk galactosyltransferase has been studied extensively. However, little is known about the sites of interaction between these two proteins. Using a photoaffinity analogue of UDP-galactose, 4-azido-2-nitrophenyluridylyl pyrophosphate, it was shown that α-lactalbumin does not bind at the UDP-site [14]. In other studies the binding between the two proteins is thought to involve a cluster of apolar amino acids [21, 22] some of which may contribute to the formation of a hydrophobic box [15]. X-ray crystallographic data is not yet available to confirm these conclusions.

It is well known that *N*-acetylglucosamine is required for the binding of α-lactalbumin to galactosyltransferase. In fact this knowledge is exploited during purification of detergent solubilized galactosyltransferase on α-lactalbumin affinity columns [22]. In the present report we have demonstrated that this binding is complex. Although the transfer to *N*-acetylglucosamine is inhibited at α-lactalbumin concentrations which support good lactose synthase activity, at low α-lactalbumin concentrations the transfer of galactose to *N*-acetylglucosamine is actually stimulated. With increasing amounts of α-lactalbumin the transfer to glucose is preferred but the transfer of galactose to *N*-acetylglucosamine is not eliminated. Instead, it slowly decreases to the level of transfer in the absence of α-lactalbumin. Small changes in structure of *N*-acetylglucosamine, such as methylation of the anomeric carbon, eliminated the interaction between the two proteins [16]. The data suggest that the nature of the interactions which support transfer of galactose to *N*-acetylglucosamine is different from those required for transfer to glucose, although these are not defined.

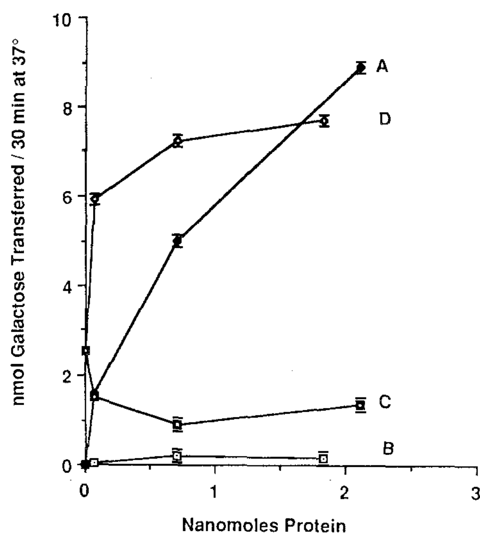

Figure 4. The effect of BNPS-skatole fragment 26-60 on lactose and lactosamine synthase activities of bovine milk galactosyltransferase.
Lactose synthase: (A) in the presence of native α-lactalbumin; (B) in the presence of fragment 26-60. Lactosamine synthase: (C) in the presence of native α-lactalbumin; (D) in the presence of fragment 26-60.

Transfer to glucose can be eliminated by destroying the native conformation of α-lactalbumin by reduction and alkylation. The nature of the alkylating agent matters little. Thus, alkylating with iodoacetamide, iodoacetic acid or 4-vinyl pyridine yielded an α-lactalbumin which did not support lactose synthesis. On the other hand *N*-acetyllactosamine synthesis was stimulated markedly (Table 1). Furthermore, a peptide consisting of amino acids 26-60 obtained after BNPS-skatole cleavage of reduced and alkylated α-lactalbumin was effective in stimulating *N*-acetyllactosamine synthase activity. Therefore, it appears that a particular peptide sequence is sufficient to stimulate *N*-acetyllactosamine synthase activity. On the other hand lactose synthase appears to require an intact folded structure. The hydrophobic box proposed by Koga and Berliner [15] may represent the specific folded structure required for lactose synthase activity. This structure consists of Trp 60, Tyr 103, Ile 95 and Trp 104, which would be destroyed by the chemical treatments used in the communication.

An examination of the primary structure of α-lactalbumin shows that three of the four Trp residues are within one or two residues of Cys (Trp 26, 60, 118). Reduction and alkylation at the Cys residues would be expected to disrupt the three dimensional structure resulting in disruption of the hydrophobic box. Disruption of this structure generates a linear sequence of amino acids which results in stimulation of *N*-acetyllactosamine synthase activity.

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