# THE ROLE OF α-LACTALBUMIN AND THE A PROTEIN IN LACTOSE SYNTHETASE: A UNIQUE MECHANISM FOR THE CONTROL OF A BIOLOGICAL REACTION*

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*Communicated by Philip Handler, November 30, 1967*

Lactose synthetase catalyzes the final step in the biosynthesis of lactose in the mammary gland by the reaction

$$\text{UDP-D-galactose} + \text{D-glucose} \rightarrow \text{lactose} + \text{UDP}. \quad (1)$$

A soluble, partially purified form of the enzyme, found in bovine milk, can be separated by gel filtration into two protein components, designated the A and B proteins. Neither component will catalyze reaction (1) separately, but when they are combined, lactose synthesis is obtained. More recently, the B protein has been found to be identical with the familiar milk protein, α-lactalbumin.[2, 3]

Work in our laboratory has shown that the amino acid sequence of bovine α-LA is very similar to that of hen egg-white lysozyme.[4] This homology in primary structure suggests that the structural genes for lysozyme and α-LA have evolved from a relatively recent, common ancestor. The structural similarities also suggest that α-LA may have a conformation quite similar to that established for lysozyme.[5] It is possible to fit the side chains of bovine α-LA to the lysozyme polypeptide backbone, and thereby generate a structure which retains the major structural features of the lysozyme molecule.[6]

The studies reported here have been designed to define the roles of the A protein and α-LA in lactose synthetase. The separated A protein has been found to be a UDP-galactose:*N*-acetylglucosamine galactosyltransferase which catalyzes the following reaction:

$$\text{UDP-galactose} + \text{N-acetyl-D-glucosamine} \rightarrow \textit{N}\text{-acetyllactosamine} + \text{UDP}. \quad (2)$$

Under normal assay conditions, α-LA inhibits this reaction and allows synthesis of lactose in the presence of glucose by reaction (1). Thus, the α-LA modifies the substrate (acceptor) specificity of a galactosyltransferase from NAG to glucose. This appears to be a role hitherto not ascribed to a protein and α-LA may be termed a "specifier" protein. The mechanism by which it effects a change in substrate specificity appears to be complex, but it is possible that it represents a new type of molecular control of a biological reaction.

*Experimental Methods.—Assay of UDP-galactose:glucose β1→4 galactosyltransferase (lactose synthetase) and UDP-galactose:N-acetylglucosamine β1→4 galactosyltransferase (N-acetyllactosamine [NAL]synthetase):* Assays were performed by a modification of the method of Babad and Hassid.[1] NAL synthetase was determined by incubating 100-μliter mixtures containing 5 μmoles Tris-HCl, pH 7.4, 4 μmoles $MnCl_2$, 2 μmoles NAG, 63 mμmoles [$C^{14}$] UDP-galactose (10,000 cpm) (Calbiochem), and enzyme for 5–20 min at 37°C. Appropriate controls were included to correct for the small amount of nonspecific hydrolysis of UDP-galactose.

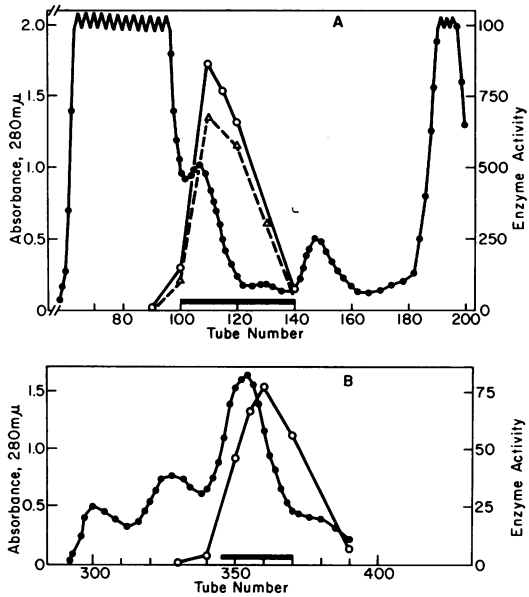


FIG. 1.—Separation of the A protein of lactose synthetase on Sephadex G-100. (*A*) Separation of an ammonium sulfate fraction from raw, skim milk. (*B*) Rechromatography of A protein from (*A*). The fractions indicated by the bar were pooled and rechromatographed on the same column as (*A*). The columns were developed at 5° at a flow rate of 20 ml/hr. Those fractions indicated by the shaded line were pooled. Each fraction contained 10 ml. —●—, Protein concentration; —○—, NAL synthetase activity; —▲—, lactose synthetase activity.

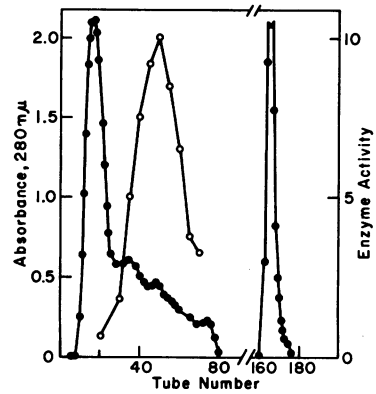


FIG. 2.—Chromatography of A protein on Sephadex C-50. A column (2 × 30 cm) of Sephadex C-50 was equilibrated at 5° with 0.02 *M* Tris-Cl buffer, pH 7.25, at 25°, containing 0.02 *M* NaCl, 0.01 *M* $MgCl_2$, and 0.001 *M* mercaptoethanol. A solution of concentrated A protein (4.5 ml) previously purified on Sephadex G-100 (*shaded line*) was applied to the column and the column was developed at 5° with the same buffer at a flow rate of 12 ml/hr. Each fraction contained 2 ml. At tube number 120, the elution was begun with buffer containing 0.5 *M* NaCl.

The reaction mixtures were kept in tubes in an ice bath until the reaction was started by warming to 37°C. The reaction was stopped by placing the tubes in ice. Water (0.5 ml) was then added and the mixture transferred to 0.5 × 3-cm columns of AG-1 × 8 (Calbiochem) in the chloride cycle, prepared in Pasteur pipettes. The reaction mixture was immediately forced into the column by gentle air pressure to ensure removal of unreacted UDP-gal. The reaction tubes were washed with 0.5 ml of water which was then transferred to the columns. The column was eluted finally with 1 ml of water. The column eluates were collected directly in scintillation vials to which 16 ml of POP/PPOP/toluene/Triton-X scintillation fluid (8 gm PPO, 0.2 gm POPOP, 1 liter Triton-X, dried with silica gel, dissolved in 2 liters toluene) were added. The vials were then counted in a liquid scintillation spectrometer (Packard Tri-Carb or Beckman).

Lactose synthetase was determined in the same manner except that glucose was substituted for NAG in the reaction mixture, and α-LA was added. The final results are expressed as mμmoles of galactosyl moiety transferred per minute of incubation.

*Partial purification of the A protein of lactose synthetase:* A modification of the method of Brodbeck and Ebner[2] was used for the initial purification of the A protein from cow's milk. Raw, skim milk was made 0.03 *M* in $MnCl_2$, 0.02 *M* in Tris-Cl, pH 7.4, and 0.001 *M* in mercaptoethanol, and then fractionated with ammonium sulfate. The fraction precipitating between 35 and 85% saturation was collected, dissolved in a small volume of 0.02 *M* Tris buffer, pH 7.4, containing 0.01 *M* $MgCl_2$ and 0.001 *M* mercapto-

ethanol (buffer A), dialyzed against the same buffer, and concentrated in a protein concentrator (Amicon Corp., Cambridge, Mass.). The concentrated solution was then applied to a 4.0 × 140-cm column of Sephadex G-100 equilibrated with buffer A. A typical elution pattern from this column is shown in Figure 1*A*. In addition to separating the A and B proteins of lactose synthetase, this method separates the A protein from the bulk of other milk proteins. Lactose synthetase activity was assayed in aliquots from the tubes by the addition of α-LA (200 μg per assay mixture).

The A protein separated in this way was rechromatographed on G-100 (Fig. 1*B*) after concentration of the peak from the first run. This product was used for most of the experiments described here.

The A protein could be further purified by chromatography on CM-Sephadex (Fig. 2) which removes large quantities of the contaminating protein. This treatment, however, caused no further change in the enzymatic properties of the protein.

*Identification of NAL and lactose:* The product formed on reaction of A protein with $C^{14}$-UDP-gal and NAG was identified as NAL as follows. The reaction mixture was passed through a column of AG-1 × 8. The $C^{14}$-neutral sugar fraction from this column behaved as a disaccharide on Sephadex G-10, had an identical $R_f$ to NAL on paper chromatography (*n*-PrOH:$H_2O$:ethylacetate, 7:2:1) and on degradation with β-galactosidase gave $C^{14}$-galactose. Lactose was identified as the product of reaction of the sonicated rat liver particulate fraction with $C^{14}$-UDP-gal, glucose, and α-LA in a similar manner. The $C^{14}$-neutral sugar fraction from this reaction mixture behaved as a disaccharide on Sephadex G-10, had an identical $R_f$ to lactose in the above paper chromatographic system, and gave $C^{14}$-galactose after degradation with β-galactosidase.

*Experimental Results.—The substrate (acceptor) specificity of A protein and lactose synthetase:* Babad and Hassid[1] reported earlier that NAG was approximately 25 per cent as effective as glucose when used as an acceptor for a partially purified preparation of lactose synthetase. Accordingly, the acceptor specificity of partially purified A protein, α-LA, and a mixture of the two proteins (lactose synthetase) was tested. α-LA was completely devoid of enzymic activity, but the A protein (Table 1) was found to be a potent UDP-galactose : *N*-acetylglucosamine galactosyltransferase (NAL synthetase). Glucose was about 1.5 per cent as effective as NAG for the A protein. In contrast, A protein plus α-LA (lactose synthetase) showed the expected specificity for glucose and *N*-acetylglucosamine.[1] These data suggest that the transferase activity for the mixture of A protein and α-LA with NAG as acceptor was produced by A protein alone and that α-LA inhibited this activity. In a separate experiment, the inhibitory effect of α-LA on the NAL synthetase activity of A protein was found to be a function of α-LA concentration (Fig. 3), just as the lactose synthetase activity is dependent on α-LA concentration.

TABLE 1. *Specificity of A protein of lactose synthetase in presence and absence of α-lactalbumin.*

| Substrate | Activity | | Substrate | Activity | |
|---|---|---|---|---|---|
| | No α-LA | 25 μg α-LA | | No α-LA | 25 μg α-LA |
| *N*-acetylglucosamine | 98 | 30 | *N*-acetylgalactosamine | — | — |
| Glucose | 1.5 | 100 | Galactose | — | — |
| Glucosamine | — | 1.2 | Galactosamine | — | — |
| *N*-acetylmannosamine | — | 1.4 | *N*-acetylneuraminic acid | — | — |

All assays were carried out in the presence of 0.02 *M* acceptor substrate apart from glycogen, which was 1% w/v. The activities are expressed as a per cent of the recombined lactose synthetase activity, that is, with glucose and α-LA. It should be noted that the α-lactalbumin concentration is not saturating (see Fig. 3). Only those activities above 1% were considered significant.

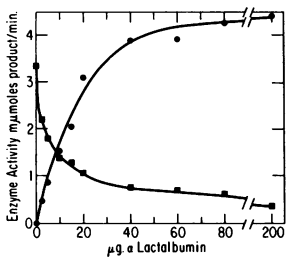


FIG. 3.—The effect of α-LA on the NAL synthetase activity and the lactose synthetase activity of A protein. Each activity was measured as described in the *Methods* section. The concentration of α-LA is given in μg per 100 μliters of the assay mixture.

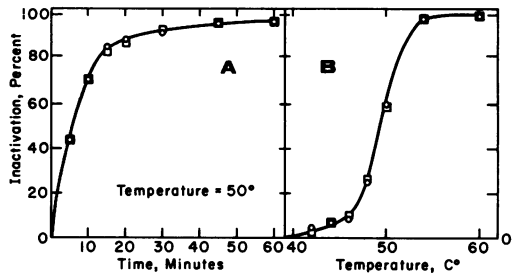


FIG. 4.—Heat denaturation of the NAL synthetase and lactose synthetase activities of A protein. (*A*) A solution of A protein was incubated at 50° in 0.02 *M* Tris-Cl, pH 7.5, containing 0.01 *M* $MgCl_2$ and 0.001 *M* β-mercaptoethanol. Aliquots were removed at intervals, cooled to 0°, and assayed. (*B*) Aliquots of A protein were incubated in the same buffer at each temperature indicated for 10 min, cooled to 0°, and assayed. —○—, Lactose synthetase; —□—, NAL synthetase.

*The identity of A protein in lactose synthetase with UDP-galactose:N-acetylglucosamine galactosyltransferase:* The NAL synthetase activity of partially purified A protein appears to be very similar and perhaps identical to that found by McGuire *et al.*[7] in bovine colostrum and a particulate fraction of a number of rat tissues. This transferase may normally incorporate galactose into the growing oligosaccharide side chains of glycoproteins that contain terminal β-*N*-acetylglucosamine. There are several lines of evidence which indicate that NAL synthetase is identical to the A protein and is required for lactose formation by lactose synthetase. These may be listed as follows.

(1) The two activities of A protein, that is, galactosyl transfer to glucose in the presence of α-LA, and transfer to NAG in the absence of α-LA, were always associated with one another on purification of the A protein. For example, lactose synthetase and NAL synthetase activities were in the same fractions from the Sephadex G-100 column (Fig. 1).

(2) The two activities were lost at the same rate on heat denaturation at a single temperature for different lengths of time (Fig. 4*A*), or at different temperatures for a set length of time (Fig. 4*B*).

(3) Both activities were specifically affected by α-LA (Fig. 3).

(4) The activities were not separated by electrophoresis on a starch column at pH 8.6, nor by chromatography on CM-Sephadex (Fig. 2).

(5) A sonicated particulate preparation from rat liver, which possessed galactosyltransferase activity with NAG as acceptor (NAL synthetase) but no activity with glucose as acceptor (lactose synthetase), was capable of synthesizing lactose from UDP-galactose if bovine α-LA and glucose were added to the particles (Table 2). We believe that these results indicate that the UDP-galactose:NAG galactosyltransferase of McGuire *et al.*[7] is identical to the A protein of lactose synthetase. These workers purified this enzyme from bovine colos-

TABLE 2. *Effect of α-lactalbumin on the NAL synthetase of a sonicated liver particulate fraction.*

| Substrates | Counts per minute | Substrates | Counts per minute |
|---|---|---|---|
| UDP-gal + NAG | 446 | UDP-gal + glucose + 20γ α-LA | 303 |
| UDP-gal + glucose | 0 | UDP-gal + glucose + 30γ α-LA | 379 |
| UDP-gal + glucose + 10γ α-LA | 111 | | |

Four gm of rat liver were homogenized in 0.1 *M* Tris-Cl, pH 7.4 containing 0.01 *M* $MgCl_2$ and 0.001 *M* mercaptoethanol in an all glass homogenizer and then centrifuged at 50,000 × *g* for 90 min. The resulting insoluble fraction was suspended in 10 ml of buffer A (see *Methods*) and sonicated for ten 30-sec periods at 0° with intervals for cooling. Twenty μliters of homogenate were used in the usual assays for NAL synthetase and lactose synthetase. The counts per minute of $C^{14}$ in the neutral sugar fraction were corrected for acceptor-independent hydrolysis of UDP-gal.

trum by procedures which are very similar to those used here for A protein. It was also noted that the transferase was present in many tissues. The enzyme in homogenates of liver was found in greatest concentrations in the microsome fraction of the cells. This result is exactly the same as that obtained for the distribution of lactose synthetase A protein in the lactating rat and bovine mammary gland.[8] The correlation of these results would seem to suggest that the A protein of lactose synthetase is a widely distributed enzyme which is active in most tissues in the synthesis of glycoproteins, including the mammary gland in late pregnant animals. In the lactating mammary gland, its specificity could be modified by α-LA in some way so that it will then catalyze the lactose synthetase reaction.

*The effects of α-lactalbumin on the lactose and NAL synthetase activities of A protein:* When increasing amounts of α-LA were added to A protein, an increasing rate of lactose synthesis was observed. The increase in rate is not linear with respect to α-LA concentration but appears to follow Michaelis-Menton kinetics at two different concentrations of A protein. Graphical analysis of the data gives an apparent $K_m$ for α-LA of $3.8 \times 10^{-5}$ *M*/liter.

The effect of α-LA on the NAL synthetase activity of A protein is more complex. The inhibition of NAL synthetase by α-LA does not parallel the stimulation of the lactose synthetase activity (Fig. 3). In addition, α-LA does not affect the NAL synthetase activity in the same manner at all concentrations of NAG (Figs. 5 and 6). At low concentrations of NAG (<3 mM), α-LA stimulates the NAL synthetase activity, whereas at higher concentrations (>3 mM) marked inhibition is observed. The enhancement of the synthetase activity at low concentrations is difficult to understand at present but may possibly be an aspect of the same effect as the stimulation of the lactose synthetase activity by α-LA.

*Discussion.*—The work described here shows that the actual enzymatic activity of lactose synthetase resides in the A protein and appears to be identical to the NAL synthetase of McGuire *et al.*[7] In the absence of α-LA, this enzyme catalyzes the formation of NAL, although it possesses a very low but significant lactose-synthesizing activity. In the presence of α-LA, the acceptor specificity of the enzyme changes from NAG to glucose. α-LA does not have a separate enzymatic activity insofar as can be judged by several experiments. For

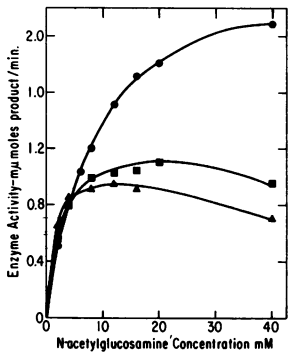


FIG. 5.—Effect of $\alpha$-LA on NAL synthetase at different NAG concentrations. The symbols correspond to: —●—, no $\alpha$:LA; —■—, 5 $\mu$g $\alpha$-LA per assay mixture; —▲—, 10 $\mu$g $\alpha$-LA per assay mixture.

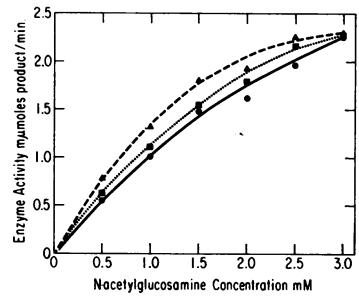


FIG. 6.—Effect of $\alpha$-LA on NAL synthetase at low NAG concentrations. The A protein concentration is three times that in Fig. 5. The symbols are the same as in Fig. 5.

example, it does not exchange the NAG of NAL for glucose. There is also no diffusible intermediate in the reaction, as judged experimentally in an equilibrium dialysis cell. Glucose, $C^{14}$-UDP-gal, Tris-buffer, and $MnCl_2$ were placed on both sides of the dialysis membrane at the concentrations employed in the assay system for lactose synthetase. $\alpha$-LA was placed on one side of the membrane and A protein on the other. After incubation of the cell at 40° for 16 hours and removal of unchanged UDP-gal on columns of Dowex 1 × 8, the eluates showed no significantly greater radioactivity than those from a similar cell containing no glucose.

We can say, therefore, that $\alpha$-LA has a function which has not been ascribed previously to a protein, although in some respects it has features similar to tryptophan synthetase.[9] It binds to the enzyme moiety, or to an enzyme-substrate complex, and thereby changes the specificity of the system. The effect of $\alpha$-LA on the synthesis of NAL by the A protein appears to be complex. At low NAG concentrations, $\alpha$-lactalbumin stimulates NAL synthesis, and appears to be acting in this respect as a regulatory protein. At higher NAG concentrations, $\alpha$-LA shows an inhibitor effect which increases with increasing NAG concentration.

The fact that the A protein or a closely related enzyme is present in liver and can be made to synthesize lactose on the addition of $\alpha$-LA is particularly significant. Lactose is only found naturally in milk and in a few plant tissues. Evidently in the liver and other tissues, the A protein is fulfilling a different function, possibly catalyzing a step in glycoprotein synthesis.[7] Therefore the actual specificity for the lactose synthetase reaction is partly resident in $\alpha$-LA, which, because of its apparently unique role, may be termed a "specifier" of the lactose synthetase reaction.

An important question which may be considered is whether lactose biosynthesis by the A protein and $\alpha$-LA could be a general type of control mechanism in

growing and differentiating tissues other than the mammary gland. If "specifier" proteins could be synthesized in an organ at some stage of their development and thereby modify the specificity of an enzyme, specific reactions could be catalyzed which are required only transiently during development of the organ. Indeed, this would seem to be the case for the mammary gland, which, except during lactation, does not synthesize lactose. In recent studies[10] we have found that as the gland develops during pregnancy the A protein increases in amount with time, as may be expected for many enzymes in a rapidly differentiating tissue. During this time the $\alpha$-LA content of the gland increases, but at a very low rate compared to that of A protein. However, at parturition, when lactation commences, the level of $\alpha$-LA increases manyfold and provides the proper conditions for lactose synthesis. This differential response of the A protein and $\alpha$-LA to hormonal stimuli was not demonstrated in organ culture. Lactose synthesis does not proceed in other tissues because $\alpha$-LA is synthesized only in the mammary gland in response to specific hormones.

We wish to thank Dr. E. A. Davidson for his interest in this study and his generous gift of *N*-acetyllactosamine.

The abbreviations used in this paper are as follows: $\alpha$-lactalbumin, $\alpha$-LA; uridinediphosphogalactose, UDP-gal; *N*-acetylglucosamine, NAG; *N*-acetyllactosamine, NAL; 2-diphenyloxazole, PPO; 1,4 bis-2 (4-methyl-5-phenyloxazolyl) bezene, POPOP.

* This work was supported by research grants from the National Institutes of Health and the National Science Foundation.

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[1] Babad, H., and W. Z. Hassid, *J. Biol. Chem.*, **241**, 2672 (1966).

[2] Brodbeck, U., and K. E. Ebner, *J. Biol. Chem.*, **241**, 762 (1966).

[3] Brodbeck, U., W. L. Denton, N. Tanahashi, and K. E. Ebner, *J. Biol. Chem.*, **242**, 1391 (1967).

[4] Brew, K., T. C. Vanaman, and R. L. Hill, *J. Biol. Chem.*, **242**, 3747 (1967).

[5] Blake, C. C. F., G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc. (London), Ser. B*, **167**, 365 (1967).

[6] Brew, K., T. C. Vanaman, R. L. Hill, W. J. Browne, A. C. T. North, and D. C. Phillips, manuscript in preparation.

[7] McGuire, E. J., G. W. Jourdian, D. M. Carlson, and S. Roseman, *J. Biol. Chem.*, **240**, PC4113 (1965).

[8] Brodbeck, U., and K. E. Ebner, *J. Biol. Chem.*, **241**, 5526 (1966).

[9] Brody, J., and C. Yanofsky, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 26 (1961), p. 11.

[10] Brew, K., T. C. Vanaman, R. L. Hill, and R. W. Turkington, manuscript in preparation.