Cartwright, S. J., & Waley, S. G. (1983) *Med. Res. Rev. 3*, 341-382.
Charnas, R. L., & Knowles, J. R. (1981) *Biochemistry 20*, 2732-2737.
Citri, N., & Pollock, M. R. (1966) *Adv. Enzymol. Relat. Areas Mol. Biol. 28*, 237-323.
Citri, N., Samuni, A., & Zyk, N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1048-1052.
Easton, C. J., & Knowles, J. R. (1982) *Biochemistry 21*, 2857-2862.
Eggers, S. H., Kane, V. V., & Lowe, G. (1965) *J. Chem. Soc.*, 1262-1270.
Faraci, W. S., & Pratt, R. F. (1984) *J. Am. Chem. Soc. 106*, 1489-1490.
Ghuysen, J.-M., Frere, J.-M., Leyh-Bouille, M., Coyette, J., Dusart, J., & Nguyen-Disteche, M. (1979) *Annu. Rev. Biochem. 48*, 73-101.
Gorman, M., & Ryan, C. W. (1972) in *Cephalosporins and Penicillins* (Flynn, E. H., Ed.) pp 544-569, Academic Press, New York.
Hamilton-Miller, J. M. T., Newton, G. G. F., & Abraham, E. P. (1970a) *Biochem. J. 116*, 371-384.
Hamilton-Miller, J. M. T., Richards, E., & Abraham, E. P. (1970b) *Biochem. J. 116*, 385-396.
Hermann, R. B. (1973) *J. Antibiot. 26*, 223-227.
Indelicato, J. M., Norvilas, T. T., Pfeiffer, R. R., Wheeler, W. J., & Wilham, W. L. (1974) *J. Med. Chem. 17*, 523-527.
Knowles, J. R. (1983) *Antibiotics (N.Y.) 6*, 90-107.
Newton, G. G. F., Abraham, E. P., & Kuwabara, S. (1968) *Antimicrob. Agents Chemother.*, 449-455.
O'Callaghan, C. H., Kirby, S. M., Morris, A., Waller, R. E., & Duncombe, R. E. (1972) *J. Bacteriol. 110*, 988-991.
Pain, R. H., & Virden, R. (1979) in *Beta-Lactamases* (Hamilton-Miller, J. M. T., & Smith, J. T., Eds.) pp 141-180, Academic Press, London.
Sabath, L. D., Jago, M., & Abraham, E. P. (1965) *Biochem. J. 96*, 735-751.
Sassiver, M. L., & Lewis, A. (1977) in *Structure-Activity Relationships among the Semisynthetic Antibiotics* (Perlmann, D., Ed) pp 87-160, Academic Press, New York.
Schindler, P., & Huber, G. (1980) in *Enzyme Inhibitors* (Brodbeck, U., Ed.) pp 169-176, Verlag Chemie, Weinheim.
Stirling, C. J. M. (1979) *Acc. Chem. Res. 12*, 198-203.
Sykes, R. B., & Bush, K. (1982) in *Chemistry and Biology of β-Lactam Antibiotics* (Morin, R. B., & Gorman, M., Eds.) Vol. 3, pp 155-207, Academic Press, New York.
Virden, R., Bristow, A. F., & Pain, R. H. (1978) *Biochem. Biophys. Res. Commun. 82*, 951-956.
Waley, S. G. (1974) *Biochem. J. 139*, 789-790.
Waller, R. E. (1973) *Analyst (London) 98*, 535-541.
Waxman, D. J., & Strominger, J. L. (1982) in *Chemistry and Biology of β-Lactam Antibiotics* (Morin, R. B., & Gorman, M., Eds.) Vol. 3, pp 209-285, Academic Press, New York.
Webber, J. A., & Ott, J. L. (1977) in *Structure-Activity Relationships among the Semisynthetic Antibiotics* (Perlmann, D., Ed.) pp 161-237, Academic Press, New York.
Wick, W. E. (1972) in *Cephalosporins and Penicillins* (Flynn, E. H., Ed.) pp 496-531, Academic Press, New York.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324-332.

Association–Dissociation Modulation of Enzyme Activity: Case of Lactose Synthase†

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ABSTRACT: Lactose synthase was found to show anomeric preference for β-D-glucose. This information was utilized in the design of methyl, ethyl, propyl, butyl, and pentyl *N*-acetyl-β-D-glucosaminides, which were subsequently demonstrated to be substrates for galactosyltransferase with apparent K_m values in the low millimolar range. α-Lactalbumin competitively inhibits the transferase activity against these *N*-acetylglucosamine derivatives. This pattern of inhibition has also been observed when the dimer, trimer, and tetramer of *N*-acetylglucosamine and ovomucoid served as the galactose acceptor. The data suggest that the binding of α-lactalbumin and the *N*-acetylglucosamine derivatives is mutually exclusive. This assertion is further supported by the inability of methyl and butyl *N*-acetyl-β-D-glucosaminides to facilitate retention of galactosyltransferase on a column of α-lactalbumin immobilized onto Sepharose. Free *N*-acetylglucosamine, on the other hand, does cause retention of the transferase under the same conditions. Thus, α-lactalbumin must bind to a region on galactosyltransferase in close proximity to the monosaccharide binding site and exert its substrate-specifying action through competitive and mutually exclusive binding with the *N*-acetylglucosamine analogues accompanied by an increased affinity for glucose. In short, our substrate analogue studies have revealed that the association–dissociation modulation of galactosyltransferase activity is effected through a topographical blockade of glycoprotein binding by α-lactalbumin.

Association–dissociation modulation of enzymatic activity represents another form of enzyme regulation. In this process, the activity of an enzyme is increased or decreased or its substrate specificity modified upon association or dissociation of its heterosubunits. The activation of cAMP-dependent protein kinase in the presence of cAMP is an example of inhibition through association of heterosubunits (Gill & Garren, 1970; Brostrom et al., 1970). On the other hand, the

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increased activity of cyclic nucleotide phosphodiesterase on binding of calmodulin represents activation through association (Cheung, 1980). In this paper, we discuss the case of lactose synthase in which the substrate specificity is modified through the association–dissociation process.

Lactose synthase (EC 2.4.1.22) is a complex consisting of two dissimilar proteins: galactosyltransferase and α-lactalbumin (Brew et al., 1968). Galactosyltransferase itself catalyzes the transfer of galactose from UDP-galactose to terminal GlcNAc[1] residues of the carbohydrate portions of glycoproteins. Free GlcNAc can also serve as the galactose acceptor to form *N*-acetyllactosamine (Hill et al., 1968; Brew et al., 1968). Glucose, on the other hand, is a very poor substrate with a K_m above 2 M (Khatra et al., 1974). However, in the presence of α-lactalbumin, glucose becomes an efficient galactose acceptor while *N*-acetyllactosamine synthesis is drastically inhibited (Brew et al., 1968; Fitzgerald et al., 1970). Thus, the association and dissociation of α-lactalbumin and galactosyltransferase modulates lactose biosynthesis and carbohydrate elongation in glycoproteins, respectively.

Although various experiments have demonstrated the association of galactosyltransferase and α-lactalbumin to form a 1:1 complex (Ivatt & Rosemeyer, 1972; Klee & Klee, 1972; Brew et al., 1975), relatively little evidence has been presented with regard to the topographical location of the protein–protein interactions. O'Keeffee et al. (1980) have used fluorescence quenching techniques to measure various distances in lactose synthase and proposed that the cleft region of α-lactalbumin is too far away to be involved in the acceptor substrate binding. We have recently shown that the binding of concanavalin A to the carbohydrate moiety of galactosyltransferase does not inhibit the formation of the lactose synthase complex nor affect the activity of the enzyme. Therefore, we speculated that the active site and the protein–protein interaction site might overlap (Wong et al., 1983). Using photoaffinity labeling, we have demonstrated that α-lactalbumin does not interact at the UDP-galactose binding domain (Lee et al., 1983). It was then suspected that α-lactalbumin might bind in the vicinity of the galactose acceptor region. In an effort to further localize the protein–protein interaction site, we have synthesized a series of alkyl-GlcNAc in order to test the effect of increasing alkyl chain length on the binding of α-lactalbumin. In this paper, we present evidence that suggests that α-lactalbumin, indeed, binds to galactosyltransferase in a region adjacent to the galactose acceptor site.

Materials and Methods

Materials. Bovine milk galactosyltransferase and α-lactalbumin were purified as described earlier (Wong et al., 1983). Pyruvate kinase (type I), UDP-galactose, α-D-glucose and β-D-glucose, NADH, and phosphoenolpyruvate were obtained from Sigma, and GlcNAc was from Pfanstiehl. Worthington Biochemicals supplied hen ovomucoid. The dimer, trimer, and tetramer of GlcNAc were generous gifts of Dr. John Rupley, Department of Biochemistry, University of Arizona. α-lactalbumin–Sepharose was prepared as described earlier (Lee et al., 1983).

Methyl-, ethyl-, propyl-, butyl-, and pentyl-GlcNAc were prepared by the Koenigs–Knorr method [Knoenigs & Knorr, 1901; for review, see Bochkov & Zaikov (1979)]. Basically, 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride was synthesized according to the procedures of Horton (1966) and reacted with the corresponding alcohol in chloroform in the presence of silver oxide until the test for chloride ion by silver nitrate was negative (Noller & Rockwell, 1938). The tetraacetylated product was crystallized from chloroform by the addition of petroleum ether and deacetylated by sodium methoxide to give the corresponding alkyl-GlcNAc (Noller & Rockwell, 1938). The products were purified by silica gel column chromatography and recrystallization from ethyl acetate. The purity of these compounds was followed by silica gel TLC plates with ethanol–chloroform (3:2) as solvent. Overall yields were between 20 and 50%. The melting points corresponded to that reported in literature (Kuhn & Kirschenlohr, 1953; Leaback & Walker, 1957), and the structures were verified by nuclear magnetic resonance spectroscopy (Hall, 1964).

Enzyme Kinetics. Galactosyltransferase and lactose synthase activities were followed spectrophotometrically as described earlier (Wong et al., 1983). Inhibition kinetics were performed by varying the concentration of the GlcNAc substrate analogues at various fixed concentrations of α-lactalbumin. The data were analyzed by plotting the reciprocals of initial velocity vs. substrate concentration. Kinetic constants were obtained by fitting the data to the competitive inhibition equation for one substrate reaction.

Detection of Lactose Synthase Complex Formation. Galactosyltransferase was applied to a column of 0.3 mL of α-lactalbumin–Sepharose CL-4B equilibrated with 50 mM *N*-ethylmorpholine buffer, pH 8.0, containing 2 mM $MnCl_2$, 1 mM 2-mercaptoethanol, and either 10 mM GlcNAc or one of the alkyl analogues. The column was washed and finally eluted with the same buffer containing 2 mM EDTA but neither monosaccharide nor manganese chloride. The flow rate was maintained at 45 mL/h, and 0.5-mL fractions were collected. The eluates were assayed for galactosyltransferase activity. The assayed activity was normalized to account for differences in the specific activity of the enzyme preparations used in these experiments.

Results

Anomeric Preference of Lactose Synthase. Earlier studies by Schanbacher & Ebner (1970) have indicated that β-glucosides are better substrates for galactosyltransferase than the corresponding α analogues. For the purpose of designing alkyl-GlcNAc derivatives that would also serve as competent substrates for galactosyltransferase, we have investigated the anomeric specificity of lactose synthase for D-glucose. As shown in Figure 1, when β-D-glucose was used as the galactose acceptor, there was an initial fast rate of reaction that decreased to a constant value after approximately 7 min. On the other hand, when α-D-glucose was used, the initial reaction rate was slow but progressively increased to a final value that was identical with that observed for β-D-glucose. This indicates that an equilibrium between the two anomeric forms must be established such that after 7 min the compositions of the two assay solutions are essentially identical. From the data of Hudson (1907), the half-life of mutarotation of D-glucose at 25 °C and pH 8.5 was estimated to be 0.68 min. The time required for the reaction velocities of the two glucose anomers to coincide is about 10 times this value. Thus, the data strongly suggest that β-D-glucose is the preferred anomer for lactose synthase. If this is the case, then galactosyltransferase might be expected to show a similar preference for the β anomer of GlcNAc.

Substrate Activity of GlcNAc Derivatives. In accordance with the anomeric preference exhibited by lactose synthase,

[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; methyl-, ethyl-, propyl-, butyl-, and pentyl-GlcNAc, corresponding alkyl *N*-acetyl-β-D-glucosaminides; $(GlcNAc)_n$, *n* β-1,4-linked *N*-acetylglucosamine units; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

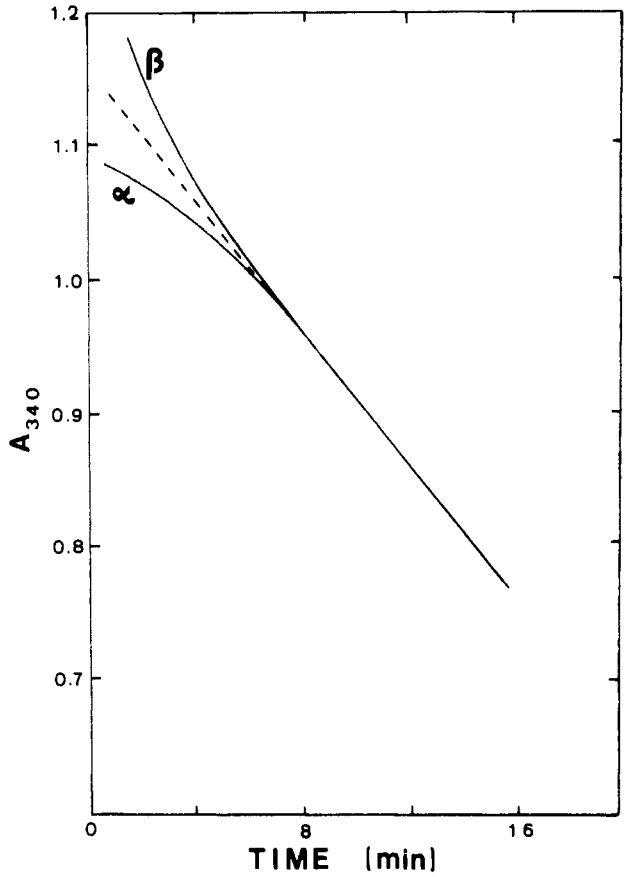

FIGURE 1: Assay tracings of lactose synthase. The assay conditions were those described under Materials and Methods. The reactions were initiated by dissolving a solid sample of either α- or β-glucose anomer in the assay mixture to give a final concentration of 20 mM. The dash line (--) indicates the assay tracing when a standard solution of D-glucose was used to initiate the reaction.

we have synthesized a series of alkyl *N*-acetyl-β-D-glucosaminides of increasing alkyl chain length with the objective of investigating their effects on the association of galactosyltransferase and α-lactalbumin. All of the alkyl derivatives served as substrates for the transferase with apparent K_m values in the low millimolar range (Table I). As it can be seen, the apparent K_m values are slightly smaller than that for GlcNAc, despite the fact that they are in the same order of magnitude. With the exception of the ethyl analogue, there seems to be a slight decreasing trend in the apparent K_m values with increasing alkyl chain length. While the significance for such a trend is not certain, it may reflect hydrophobic interaction. The V_{max} values, on the other hand, remained constant and were similar to that for GlcNAc.

We have also investigated (Table I) the substrate activities of the β-1,4-linked dimer, trimer, and tetramer of GlcNAc as well as hen ovomucoid, a glycoprotein with terminal *N*-acetyl-β-D-glucosaminyl residues (Yamashita et al., 1983). Other compounds containing β-anomeric GlcNAc residues at the nonreducing end of carbohydrate chains have also been found to be substrates for galactosyltransferase (Geren et al., 1976; Geren et al., 1977).

α-Lactalbumin Inhibition. As mentioned earlier, α-lactalbumin inhibits the transfer of galactose from UDP-galactose to GlcNAc, either free or as the terminal residues of glycoproteins, but facilitates the transferase to glucose. Kinetically, this change in substrate specificity is effected by a 1000-fold decrease in the K_m for glucose to the millmolar range (Klee & Klee, 1970; Fitzgerald et al., 1970). α-lactalbumin acts as an uncompetitive inhibitor against free GlcNAc (Morrison & Ebner, 1971; Andrews, 1972). Like free GlcNAc, the substrate activity of the GlcNAc derivatives is subject to inhibition by α-lactalbumin. However, in sharp contrast to its behavior toward free GlcNAc, α-lactalbumin acts as a competitive inhibitor against these GlcNAc analogues as depicted in Figure 2 and summarized in Table I.

Table I: Kinetic Constants for *N*-Acetyl-β-D-glucosaminyl Derivatives[a]

substrates	app K_m (mM)	K_I (μM α-lactalbumin)	pattern of inhibition
GlcNAc	4.4	1.9	uncompetitive
methyl-GlcNAc	3.2	28.4	competitive
ethyl-GlcNAc	1.4	13.8	competitive
propyl-GlcNAc	2.0	45.7	competitive
butyl-GlcNAc	1.3	56.5	competitive
pentyl-GlcNAc	0.8	ND[b]	ND[b]
$(GlcNAc)_2$	1.2	48.0	competitive
$(GlcNAc)_3$	1.8	86.7	competitive
$(GlcNAc)_4$	1.4	54.7	competitive
ovomucoid	0.4	66.7	competitive

[a] The data were obtained from kinetics analysis as shown in Figure 2. [b] Not determined.

Effect of Alkyl-GlcNAc Derivatives on Lactose Synthase Complex Formation. The observation of competitive inhibition by α-lactalbumin against the GlcNAc derivatives suggests that the binding of these species to galactosyltransferase is mutually exclusive. If this conclusion is correct, then formation of a ternary complex between galactosyltransferase, α-lactalbumin, and the GlcNAc derivatives in the presence of Mn^{2+} should not occur. This is precisely what has been observed. Galactosyltransferase, when applied to a column of α-lactalbumin–Sepharose in Buffer devoid of substrates and Mn^{2+}, is not retained to any significant extent (Figure 3). When applied in the presence of GlcNAc and Mn^{2+}, retention is facilitated and elution does not occur unless GlcNAc and Mn^{2+} are excluded from the buffer, a procedure employed in the purification of the enzyme (Trayer & Hill, 1971). However, if the transferase is applied in the presence of methyl-GlcNAc and Mn^{2+}, no retention is observed. In fact, the GlcNAc derivative seems to prevent any interaction between the two proteins as the enzyme is excluded from the column slightly earlier in its presence than in its absence. Identical results were also obtained with butyl-GlcNAc.

DISCUSSION

The observed competitive inhibition kinetics (Figure 2) can be best accounted for by the fact that α-lactalbumin and β-1-GlcNAc derivatives cannot simultaneously bind to galactosyltransferase. However, such kinetic pattern may be obtained if α-lactalbumin causes a conformational change on galactosyltransferase. This possibility is ruled out by the inability of the β-1-GlcNAc derivatives to facilitate the interaction between the two proteins as shown in Figure 3. Thus, the mutually exclusive binding must be due to the presence of the substituted group at the anomeric carbon, which overlaps with the α-lactalbumin binding region or extends into a region otherwise occupied by α-lactalbumin, creating a direct steric interference. Since a derivative as small as the methyl group is capable of preventing the binding of α-lactalbumin, the latter must bind to galactosyltransferase at a region adjacent to the monosaccharide binding domain. An estimation from the bond lengths suggests that α-lactalbumin is in close approximation to the anomeric carbon of the galactose acceptor as proposed by O'Keeffee et al. (1980). The location of this interactiuon site is consistent with our earlier contention that α-lactalbumin does not bind to the sugar nucleotide binding domain (Lee et al., 1983) and the report that its binding does not involve UDP

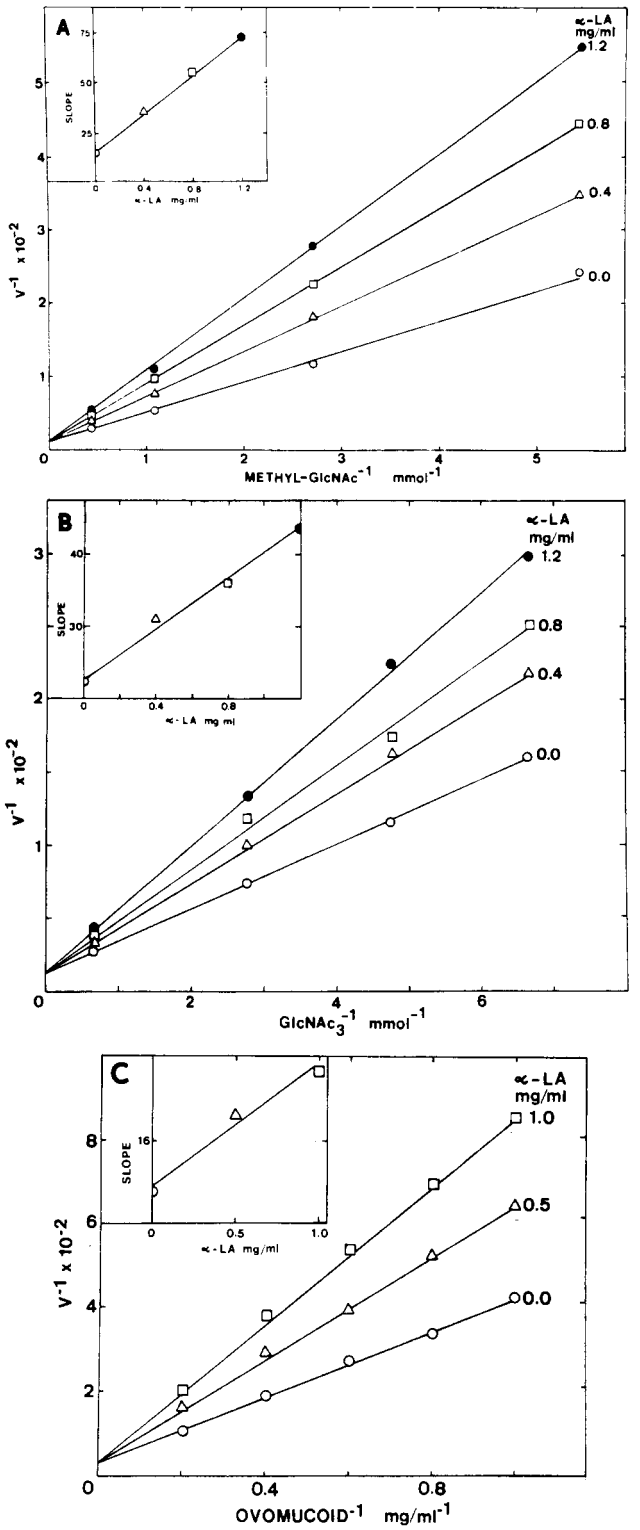

FIGURE 2: Kinetics for α-lactalbumin inhibition: double-reciprocal plots. Assays were carried out by varying the acceptor concentration at various fixed concentrations of α-lactalbumin (α-LA). (Inserts) Secondary plots of slope vs. α-lactalbumin concentration. Variable substrate: (A) methyl-GlcNAc; (B) $(GlcNAc)_3$; (C) ovomucoid.

(Takase & Ebner, 1981) nor has any effect on the binding of Mn^{2+} and UDP-galactose (Khatra et al., 1974; Morrison & Ebner, 1971).

The close proximity of α-lactalbumin to the enzyme-bound monosaccharide raises the possibility that the specifier protein may be involved in the galactose acceptor binding, even though it does not possess monosaccharide binding sites per se (Burkhardt et al., 1975). The spatial relationship between

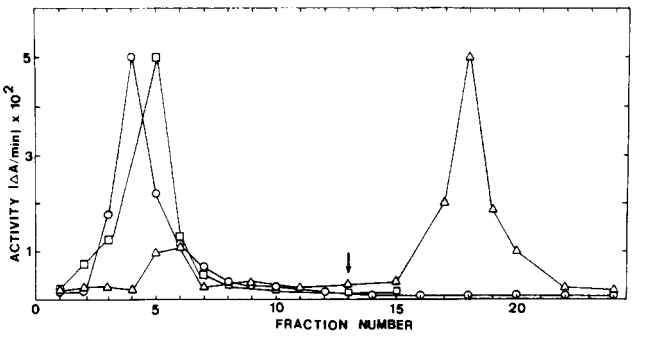

FIGURE 3: Effect of methyl-GlcNAc on the retention of galactosyltransferase on an α-lactalbumin–Sepharose column. Galactosyltransferase was applied to a 0.3-mL column of the immobilized α-lactalbumin equilibrated with buffer alone (□), buffer + Mn^{2+} + 10 mM GlcNAc (Δ), or buffer + Mn^{2+} + 10 mM methyl-GlcNAc (○). The column was washed with the same solution with which it was equilibrated and eluted (arrow) with a solution containing buffer and EDTA but neither monosaccharide nor $MnCl_2$.

α-lactalbumin and the monosaccharide binding domain on galactosyltransferase is not evident from our present data. However, it is possible that α-lactalbumin could provide a hydrogen-bonding residue to at least the anomeric hydroxyl group of the acceptor. This could account for the increased affinity of lactose synthase for glucose and GlcNAc as well as the anomeric preference.

Thus, it seems that α-lactalbumin modulates the substrate specificity of galactosyltransferase through competitive and mutually exclusive binding against GlcNAc-containing compounds, concomitant with an increased affinity for D-glucose. As a consequence of the association of α-lactalbumin and galactosyltransferase, only free monosaccharides such as glucose and GlcNAc are able to bind. This is consistent with our observation that methyl and butyl β-D-glucosides are not substrates of lactose synthase (T. Lee, unpublished observations). Therefore, in the lactating mammary gland, where α-lactalbumin is synthesized in considerable quantities reaching concentrations as high as 5.5 mg/mL in guinea pig milk (Brew & Campbell, 1967) and other mammals (Davies, 1932), the binding of glycoproteins to galactosyltransferase is competitively inhibited, but not completely prohibited, while the affinity for glucose is increased, resulting in the production of lactose.

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REFERENCES

Andrews, P. (1972) *FEBS Lett. 26*, 333–335.

Bochkov, A. F., & Zaikov, G. E. (1979) in *Chemistry of the O-Glycosidic Bond*, Pergamon Press, New York.

Brew, K., & Campbell, P. N. (1967) *Biochem. J. 102*, 258–264.

Brew, K., Vanaman, T. C., & Hill, R. L. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 491–497.

Brew, K., Sharper, J. H., Olsen, K. W., Trayer, I. P., & Hill, R. L. (1975) *J. Biol. Chem. 250*, 1434–1444.

Brostrom, M. A., Reimann, E. M., Walsh, D. A., & Krebs, E. G. (1970) *Adv. Enzyme Regul. 8*, 191–203.

Burkhardt, A. E., Russo, S. O., Rinehardt, C. G., & Loudon, G. M. (1975) *Biochemistry 14*, 5465–5469.

Cheung, W. Y. (1980) *Calcium Cell Funct. 1*, 1–12.

Davies, W. L. (1932) *J. Dairy Res. 4*, 142–148.

Fitzgerald, D. K., Brodbeck, U., Kiyosawa, I., Mawal, R., Colvin, B., & Ebner, K. E. (1970) *J. Biol. Chem. 245*, 2103-2108.
Geren, C. R., Magee, S. C., & Ebner, K. E. (1976) *Arch. Biochem. Biophys. 172*, 149-155.
Geren, C. R., Geren, L. M., Lee, D., & Ebner, K. E. (1977) *Biochim. Biophys. Acta 497*, 128-132.
Gill, G. N., & Garren, L. D. (1970) *Biochem. Biophys. Res. Commun. 39*, 335-343.
Hall, L. D. (1964) *Adv. Carbohydr. Chem. 19*, 51-93.
Hill, R. L., Brew, K., Vanaman, T. C., Trayer, I. P., & Mattock, P. (1968) *Brookhaven Symp. Biol. No. 21*, 139.
Horton, D. (1966) *Org. Synth. 46*, 1-5.
Hudson, C. S. (1907) *J. Am. Chem. Soc. 29*, 1572.
Ivatt, R. J., & Rosemeyer, M. S. (1972) *FEBS Lett. 28*, 195-197.
Khatra, B. S., Herries, D. G, & Brew, K. (1974) *Eur. J. Biochem. 44*, 537-560.
Klee, W. A., & Klee, C. B. (1970) *Biochem. Biophys. Res. Commun. 39*, 833-841.
Klee, W. A., & Klee, C. B. (1972) *J. Biol. Chem. 247*, 2336-2344.
Koenigs, W., & Knorr, E. (1901) *Ber. Dtsch. Chem. Ges. 34*, 957-981.
Kuhn, R., & Kirschenlohr, W. (1953) *Chem. Ber. 86*, 1331-1333.
Leaback, D. H., & Walker, P. G. (1957) *J. Chem. Soc.*, 4754-4760.
Lee, T. K., Wong, L.-J. C., & Wong, S. S. (1983) *J. Biol. Chem. 258*, 13166-13171.
Morrison, J. F., & Ebner, K. E. (1971) *J. Biol. Chem. 246*, 3992-3998.
Noller, C. R., & Rockwell, W. C. (1938) *J. Am. Chem. Soc. 60*, 2076-2077.
O'Keeffee, E. T., Mordick, T., & Bell, J. E. (1980) *Biochemistry 19*, 4962-4966.
Schanbacher, F. L., & Ebner, K. E. (1970) *J. Biol. Chem. 245*, 5057-5061.
Trayer, I. P., & Hill, R. L. (1971) *J. Biol. Chem. 246*, 6666-6675.
Wong, S. S., Malone, T. E., & Lee, T. K. (1983) *Biochim. Biophys. Acta 745*, 90-96.
Yamashita, K., Kamerling, J. P., & Kobata, A. (1983) *J. Biol. Chem. 258*, 3099-3106.

Catabolism of Bis(5′-nucleosidyl) Oligophosphates in *Escherichia coli*: Metal Requirements and Substrate Specificity of Homogeneous Diadenosine-5′,5‴-P^1,P^4-tetraphosphate Pyrophosphohydrolase

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ABSTRACT: Diadenosine-5′,5‴-P^1,P^4-tetraphosphate pyrophosphohydrolase (diadenosinetetraphosphatase) from *Escherichia coli* strain EM20031 has been purified 5000-fold from 4 kg of wet cells. It produces 2.4 mg of homogeneous enzyme with a yield of 3.1%. The enzyme activity in the reaction of ADP production from Ap_4A is 250 s^{-1} [37 °C, 50 mM tris(hydroxymethyl)aminomethane, pH 7.8, 50 μM Ap_4A, 0.5 μM ethylenediaminetetraacetic acid (EDTA), and 50 μM $CoCl_2$]. The enzyme is a single polypeptide chain of M_r 33K, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and high-performance gel permeation chromatography. Dinucleoside polyphosphates are substrates provided they contain more than two phosphates (Ap_4A, Ap_4G, Ap_4C, Gp_4G, Ap_3A, Ap_3G, Ap_3C, Gp_3G, Gp_3C, Ap_5A, Ap_6A, and dAp_4dA are substrates; Ap_2A, NAD, and NADP are not). Among the products, a nucleoside diphosphate is always formed. ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP are not substrates; Ap_4 is. Addition of Co^{2+} (50 μM) to the reaction buffer containing 0.5 μM EDTA strongly stimulates Ap_4A hydrolysis (stimulation 2500-fold). With 50 μM $MnCl_2$, the stimulation is 900-fold. Ca^{2+}, Fe^{2+}, and Mg^{2+} have no effect. The K_m for Ap_4A is 22 μM with Co^{2+} and 12 μM with Mn^{2+}. The added metals have similar effects on the hydrolysis of Ap_3A into ADP + AMP. However, in the latter case, the stimulation by Co^{2+} is small, and the maximum stimulation brought by Mn^{2+} is 9 times that brought by Co^{2+}. Exposure of the enzyme to Zn^{2+} (5 μM), prior to the assay or within the reaction mixture containing Co^{2+}, causes a marked inhibition of Ap_4A hydrolysis. The inhibition is relieved by prolonged incubation of the enzyme with excess EDTA.

The recent interest in bis(5′-adenosyl) tetraphosphate (Ap_4A) arose from the correlation between its intracellular concentration and cell proliferation, its ability to trigger DNA replication, and its involvement in the priming reaction of replication possibly through specific binding to DNA polymerase α [reviewed in Zamecnik (1983) and Grummt (1983)].

In pursuit of the initial observation that bis(5′-adenosyl)

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