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Affinity-Chromatography Studies on the A Protein of Lactose Synthetase

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The terminal step in the biosynthesis of lactose in the mammary gland is catalysed by the enzyme lactose synthetase according to the following reaction:

The synthetase has been resolved into two protein components, the A protein and the milk protein, α -lactalbumin (Brodbeck *et al.*, 1967). In the absence of α -lactalbumin, the A protein has been shown to be a galactosyltransferase that catalyses the incorporation of galactose from UDP-galactose in a β -(1 $\rightarrow$ 4)-linkage with free or protein-bound *N*-acetylglucosamine, and is found in many mammalian tissues (Brew *et al.*, 1968; Hill *et al.*, 1968):

Under these conditions glucose is a very poor acceptor in the transferase reaction and has K_m about 1M, which is decreased about 1000-fold in the presence of α -lactalbumin, effectively allowing the A protein to catalyse reaction (1) (Morrison & Ebner, 1971*a,b,c*). α -Lactalbumin has no effect on the V_{max} of this reaction. Thus the unique ability of mammary gland to synthesize the lactose is dependent on it being the only tissue to synthesize α -lactalbumin.

Although it is membrane-bound in the mammary gland this galactosyltransferase (the A protein) is found soluble in milk, and this material is usually the starting point for its preparation. It is our experience, and apparently that of others (Fitzgerald *et al.*, 1970), that purification of the A protein by 'conventional' protein-separation techniques is both tedious and time-consuming and results in low yields of the final product. The introduction of affinity-chromatography methods has greatly simplified the purification of the A protein. Initial experiments were made by attaching α -lactalbumin covalently by diazotization to amino-cellulose and polyaminostyrene. Better separations were achieved, however, on columns of α -lactalbumin covalently linked to

Sepharose 4B by the CNBr procedure of Axén *et al.* (1967). The bovine A protein was bound to this column in buffers containing glucose, and was specifically eluted from the column in a homogeneous form by omitting glucose from the developing buffer (Trayer *et al.*, 1970; Trayer & Hill, 1971). Andrews (1970) used *N*-acetylglucosamine instead of glucose to effect the binding of human A protein to the α -lactalbumin-Sepharose conjugate. The binding of the A protein to these affinity columns may be very similar to that occurring between the A protein and α -lactalbumin in solution, since the conjugate can replace soluble α -lactalbumin in lactose synthesis. Indeed, the fact that the A protein binds to this conjugate provided the first direct evidence that a complex is formed between the two proteins. More recently (Shaper *et al.*, 1971; R. L. Hill, R. Barker, K. W. Olsen, J. H. Shaper & I. P. Trayer, unpublished work) UDP and *N*-acetylglucosamine have been covalently linked to Sepharose and used as affinity columns in the purification of the A protein. The conjugates were prepared by reaction of the hexanolamine derivatives of UDP and *N*-acetylglucosamine with CNBr-treated Sepharose.

With the UDP-Sepharose columns Mn^{2+} can be used in a manner similar to glucose with the α -lactalbumin-Sepharose columns, i.e. the A protein can be adsorbed on the column in the presence of Mn^{2+} and eluted from it by the removal of this bivalent cation. Elution from the *N*-acetylglucosamine-Sepharose conjugate can be achieved by the aid of borate buffer or 1.5M-urea. When milk whey solutions were passed first down a UDP-Sepharose column and then down an *N*-acetylglucosamine-Sepharose column, preparations of the A protein close to homogeneous form were obtained.

These techniques have also been used in the purification of glycogen synthetase (Shaper *et al.*, 1971), but the general applicability of these methods with other nucleotide diphosphate- and glycosyl-substituted Sepharose derivatives extends their use to the purification of a wide range of enzymes. Preliminary results with an ADP-Sepharose derivative to bind the muscle protein myosin and its biologically active subfragments confirm this.

The galactosyltransferase purified by these techniques is a glycoprotein, and appears to be heterogeneous in size depending on the carbohydrate content. One species containing 12% carbohydrate and having a molecular weight of 42000 has been well characterized (Trayer & Hill, 1971), but species with molecular weights close to 50000 are readily observed by electrophoresis in gels containing sodium dodecyl sulphate. Our understanding of the function of the polysaccharide component of the enzyme is incomplete and awaits further study, but it is noteworthy that the A protein is normally bound to the membranous structures of the Golgi apparatus in

the lactating mammary gland, and few membrane-bound enzymes of this type have been so extensively purified.

Kinetic parameters of the *N*-acetyl-lactosamine synthetase reaction (2) were measured, and the results obtained were consistent with a reaction mechanism in which there was an ordered addition of UDP-galactose and *N*-acetylglucosamine and an ordered release of *N*-acetyl-lactosamine and UDP. These results are in agreement with those obtained by Morrison & Ebner (1971*a,b,c*). The strength of binding of the A protein to the affinity columns was influenced by its substrates and inhibitors in accord with this mechanism.

The binding stoichiometries between the A protein and α -lactalbumin were examined by the method of continuous variation (Job, 1928). In this the total protein concentration was held constant, but the relative concentrations of the A protein and α -lactalbumin were varied. The ability to synthesize lactose was then used as a measure of complex-formation, and the positions of maximum activity indicated the molar ratios of the complexes formed. Two major complexes were formed, corresponding to A protein/ α -lactalbumin molar ratios 1:3 and 1:1. The existence of these complexes in solutions containing the A protein and α -lactalbumin in the presence of glucose was confirmed by the use of a bifunctional cross-linking reagent, dimethyl suberimidate (Davies & Stark, 1970). After reaction with this reagent the incubation mixtures were submitted to analysis by electrophoresis in gels containing sodium dodecyl sulphate. Species were apparent with molecular weights corresponding to those of the A protein, α -lactalbumin and complexes corresponding to 1:1, 1:2 and 1:3 molar ratios respectively of these proteins. An additional complex containing the A protein and α -lactalbumin in 1:2-molar ratio was found by this method. The formation and stability of these complexes, however, will be a function of their equilibrium constants, and it may be that the lactose synthetase assay is not sensitive enough to pick up more than the most active and stable species in the method of continuous variation.

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The Separation of Functional and Non-functional Xanthine Oxidase by Affinity Chromatography

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Since 1949 evidence has accumulated that even the most active preparations of milk xanthine oxidase contain non-functional active sites, even though they possess their full complement of FAD, molybdenum and iron-sulphur chromophores (Lowry *et al.*, 1949; Morell, 1952; Bray *et al.*, 1961; Massey *et al.*, 1970*a*). Recent work on the powerful inhibitory effects of pyrazolo[3,4-*d*]pyrimidines on xanthine oxidase has revealed that these compounds form inactive complexes with the enzyme when the molybdenum component of the latter is in a reduced state, e.g. Mo^{IV} (Massey *et al.*, 1970*a,b*; Spector & Johns, 1970). It was found that only functional enzyme formed such tight complexes; non-functional enzyme has been found to complex only weakly with pyrazolo[3,4-*d*]pyrimidines. We have made use of this property to devise an affinity-chromatography method for the separation of functional and non-functional xanthine oxidase.

6-Amino-1-propyl-3-(1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)hexanoate was synthesized and attached to Sepharose 6B by reaction with CNBr (in the manner described by Cuatrecasas *et al.*, 1968). This was converted into the 6-hydroxy derivative by prolonged aerobic incubation with xanthine oxidase. The structure of the derivative used in affinity chromatography was therefore as shown in formula (I).