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Bovine Galactosyltransferase: Interaction with α-Lactalbumin and the Role of α-Lactalbumin in Lactose Synthase†

Evelyn T. O'Keeffe, Tom Mordick, and J. Ellis Bell*

ABSTRACT: Bovine α-lactalbumin has been dansylated to give an enzymatically fully active, highly fluorescent derivative. This derivative is uniquely labeled on the N-terminal glutamic acid residue of α-lactalbumin. This fluorescent derivative of α-lactalbumin has been covalently cross-linked to galactosyltransferase by using dimethyl pimelimidate. Resonance energy transfer measurements using cobalt bound to the transferase as the acceptor of energy transfer from the dansyl group on the α-lactalbumin indicate that the dansyl group is 32 Å from the cobalt on the transferase. A model of the active site of the transferase and its interaction with α-lactalbumin is proposed on the basis of these and previous studies.

Lactose synthase (EC 2.4.1.22) is made up of two protein components, galactosyltransferase (EC 2.4.1.38) and the milk protein α-lactalbumin. Alone, the galactosyltransferase catalyzes the transfer of galactose from UDP[1]-galactose to *N*-acetylglucosamine, either free monosaccharide or the terminal residue of a glycosyl side chain, forming a β1→4 linkage. α-Lactalbumin enables the transferase to use glucose as acceptor, giving the milk sugar, lactose. While galactosyltransferase alone will utilize glucose, the Michaelis constant in the absence of α-lactalbumin (~2 M) prohibits effective use. In the presence of α-lactalbumin the K_m for glucose is reduced to the low millimolar range. While the kinetic effects of α-lactalbumin have been established (Bell et al., 1976), little is known concerning the nature of the physical interaction between the two proteins. Because of its extensive homology with lysozyme, it has been suggested that α-lactalbumin may function by providing a binding site for glucose, thereby increasing the affinity of lactose synthase for glucose. To examine this possibility, we have performed several chemical modification studies of α-lactalbumin directed at modification of the so-called cleft region which would, by the lysozyme analogy model, provide the binding residues for glucose. However, none of these (Bell et al., 1975; Prieels et al., 1979) have given any evidence for the involvement of this cleft region in lactose synthase. While the three-dimensional structure of the galactosyltransferase is unknown, several studies (O'Keeffe et al., 1980; L. H. Berliner, M. E. Davis, K. E. Ebner, T. A. Beyer, and J. E. Bell, unpublished experiments) have given a detailed picture of the various binding sites of the transferase. On the other hand, the detailed structure of α-lactalbumin is known from a combination of approaches (Browne et al., 1969; Warme et al., 1974). In this paper we present the results of studies involving chemical modification of α-lactalbumin using a fluorescent probe, chemical cross-linking of the fluorescently labeled α-lactalbumin and native galactosyltransferase, and fluorescence resonance energy transfer measurements between the introduced fluorophore and cobalt bound to the transferase.

Because of the relatively weak interaction between α-lactalbumin and the transferase (Bell et al., 1976), the resonance energy transfer measurements can only be made after chemical cross-linking of α-lactalbumin and the transferase, allowing one to work with a one to one stoichiometry of dansylated α-lactalbumin and the transferase. These studies have allowed an extension of the active site mapping of lactose synthase reported in the preceding paper (O'Keeffe et al., 1980) and give greater insight into the interaction of α-lactalbumin with the transferase and its role in lactose synthase. A preliminary account of this work has been presented (Bell, 1979).

Experimental Procedures

Materials. Galactosyltransferase was isolated essentially as described previously (Barker et al., 1972). α-Lactalbumin was purchased from Sigma Chemical Co. and chromatographed on DEAE-Sephadex A-25 (Pharmacia) prior to use, as described previously (Bell et al., 1976). Dimethyl pimelimidate, used in the cross-linking studies, and [^{12}C]dansyl chloride were from Sigma. [^{14}C]Dansyl chloride was from New England Nuclear. All other materials were as described by O'Keeffe et al. (1980).

Methods. Lactose or *N*-acetyllactosamine synthesis was followed as described previously (O'Keeffe et al., 1980).

† From the Department of Biochemistry, University of Rochester Medical Center, Rochester, New York 14642. *Received October 2, 1979.* This study was supported in part by a U.S. Public Health Service BRSG grant to J.E.B.

[1] Abbreviations used: dansyl, 8-dimethylamino-1-naphthalenesulfonyl; TEA, triethanolamine; UDP, uridine diphospho or phosphate.

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Metal-free transferase or cross-linked complex was obtained by dialysis against Chelex 100 (O'Keeffe et al., 1980). Analysis of kinetic data was as described previously (Bell et al., 1976).

Dansylation of α-Lactalbumin. Bovine α-lactalbumin was dansylated by using dansyl chloride. α-Lactalbumin (60 mg) was dissolved in 60 mL of 0.1 M sodium bicarbonate, pH 8.5. A dansyl chloride suspension was prepared by adding 0.1 mL of acetone to 4 mg of dansyl chloride containing [^{14}C]dansyl chloride to give a specific activity of 1.45×10^5 cpm/μmol. The two solutions were mixed and incubated for 4 h in the dark. At the end of the incubation, the mixture was centrifuged at 20000*g* for 20 min, dialyzed vs. 0.1 M KCl (1 L) with two changes of buffer, and chromatographed on Sephadex G-100 (2 × 40 cm column) with 0.1 M KCl. Fractions were collected and assayed for absorbance at 280 nm, for activity in lactose synthase by using 20 mM glucose as acceptor, and for radioactivity. Active fractions with ~1 mol of dansyl/mol of α-lactalbumin eluted first from the column; at later times α-lactalbumin with much higher amounts of dansyl incorporated and markedly decreased activity was eluted. Those fractions containing fully active α-lactalbumin (as judged by using a nonmodified standard) and ~1 mol of dansyl/mol of α-lactalbumin were pooled and freeze-dried.

Cross-Linking of Dansylated α-Lactalbumin and Galactosyltransferase. Galactosyltransferase and dansylated α-lactalbumin were chemically cross-linked with dimethyl pimelimidate by using a procedure adapted from that described by Brew et al. (1975). Galactosyltransferase solution (0.6 mg/mL) was made 0.2 M in triethanolamine hydrochloride, pH 8.1, by chromatography on Sephadex G-25 (2 × 20 cm column) equilibrated with TEA. A 15-mL sample of this transferase solution was incubated with 1 mL of dansylated α-lactalbumin, containing 90 mg of dansylated α-lactalbumin, 1 mL of dimethyl pimelimidate (containing 17 mg), 0.2 mL of 50 mM UDP, 0.2 mL of 1 M $MnCl_2$, and 0.2 mL of 1 M *N*-acetylglucosamine. After a 2-h incubation at room temperature, the reaction mixture was loaded onto a Sephadex G-100 column (2 × 60 cm) and eluted with 50 mM cacodylate, pH 7.4. Fractions were assayed for lactose synthase activity with 20 mM glucose as acceptor. Active fractions were pooled, made 5 mM with respect to *N*-acetylglucosamine and 20 mM in $MnCl_2$, and run through a small column of α-lactalbumin–Sepharose (1.5 × 25 cm) to remove un-cross-linked transferase, which adheres to the affinity resin under these conditions. The material which flowed through the affinity resin was assayed for both lactose and *N*-acetyllactosamine synthase activities. This material, designated as the cross-linked complex showed considerable lactose synthase activity and barely detectable *N*-acetyllactosamine synthase activity (<3% that observed with glucose as acceptor).

Results

Characterization of Dansylated α-Lactalbumin. The first fractions of dansylated α-lactalbumin eluting from Sephadex G-100 (Figure 1) were characterized by having a single dansyl group incorporated per mole of α-lactalbumin as judged from their protein concentration and incorporated radioactivity. A portion of this material was dialyzed against water exhaustively, freeze-dried, hydrolyzed with hydrochloric acid, and used for amino acid mapping, by using thin-layer chromatography on silica gel (Brenner & Niederwieser, 1967). The resultant two-dimensional maps indicated that dansyl glutamate was the only modified amino acid residue. Since bovine α-lactalbumin has an N-terminal glutamic acid residue (Brew et al., 1970), it is apparent that in the dansylated α-lactalbumin

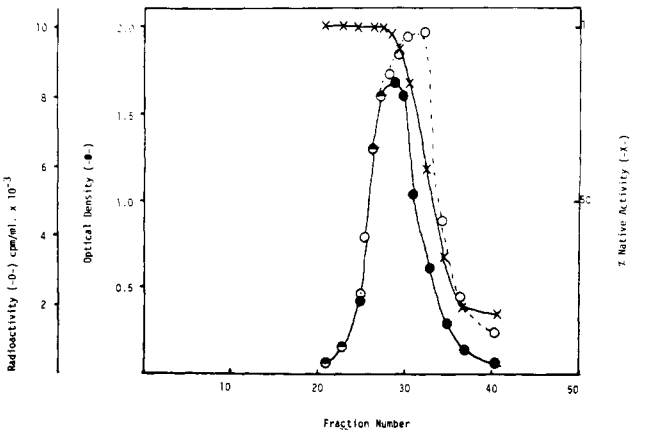

FIGURE 1: Elution of dansylated α-lactalbumin from Sephadex G-100. Eluent was monitored for radioactivity (○), protein concentration (●), and activity relative to native α-lactalbumin (×). Conditions were as described in the text.

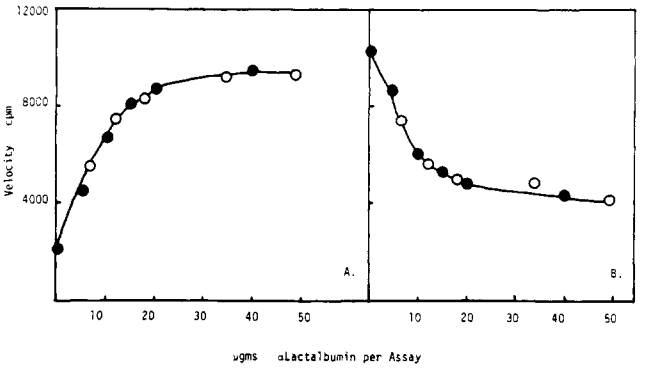

FIGURE 2: Comparison of native and dansylated α-lactalbumin. Both forms of α-lactalbumin were assayed for lactose synthase activation (A) and *N*-acetyllactosamine synthase inhibition (B). Conditions: 50 mM cacodylate, pH 7.4, 20 mM $MnCl_2$, 0.2 mM UDP-galactose and 20 mM *N*-acetylglucosamine or glucose. Native α-lactalbumin (●); modified α-lactalbumin (○).

prepared here, a single dansyl group is incorporated uniquely on the N-terminal glutamic acid residue.

This dansylated α-lactalbumin has the full activity of native α-lactalbumin as evidenced (Figure 2) by its ability to both activate lactose synthesis (Figure 2A) and inhibit *N*-acetyllactosamine synthesis (Figure 2B).

Kinetic Studies with Cross-Linked Lactose Synthase. When the rate of lactose synthesis by the cross-linked complex was studied with either varied concentrations of UDP-galactose or glucose at fixed concentrations of the other substrates, intersecting patterns were observed in Lineweaver–Burk plots (Figure 3). From these plots apparent K_m values for glucose (75 μM) and UDP-galactose (35 μM) were obtained.

With apo-cross-linked complex, prepared as described previously (O'Keeffe et al., 1980), and varied Mn^{2+} concentrations at fixed concentrations of UDP-galactose (200 μM) and glucose (20 mM), biphasic Lineweaver–Burk plots were obtained, giving two apparent K_m values for Mn^{2+} of 4.5 μM and 130 μM (Figure 4). The effects of cobalt on the Mn^{2+}-supported activity were examined by varying the cobalt concentration at two different, fixed Mn^{2+} concentrations of 20 μM and 2 mM (Figure 5A). Cobalt was inhibitory at either Mn^{2+} concentration. The K_I for cobalt with respect to the high-affinity Mn^{2+} site was determined by using varied Mn^{2+} concentrations in the presence and absence of 5 μM $CoCl_2$ (Figure 5B). From these data a K_I of 6 μM for $CoCl_2$ was calculated. Co^{2+} is clearly a competitive inhibitor with respect to the high-affinity Mn^{2+} site.

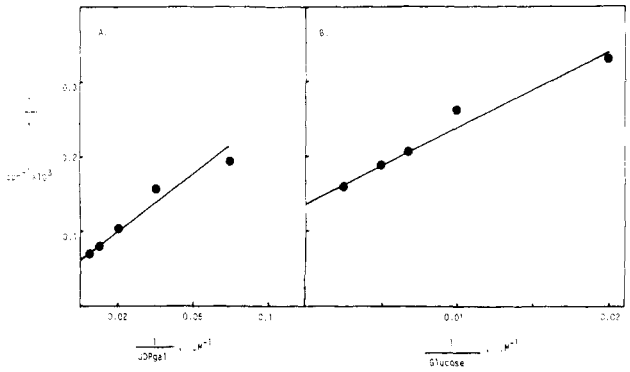

FIGURE 3: Kinetic properties of cross-linked complex. The rate of lactose synthesis was followed with varied concentrations of (A) UDP-galactose, fixed concentrations of glucose (20 mM) and $MnCl_2$ (20 mM), and (B) glucose, fixed concentrations of UDP-galactose (0.4 mM) and $MnCl_2$ (20 mM).

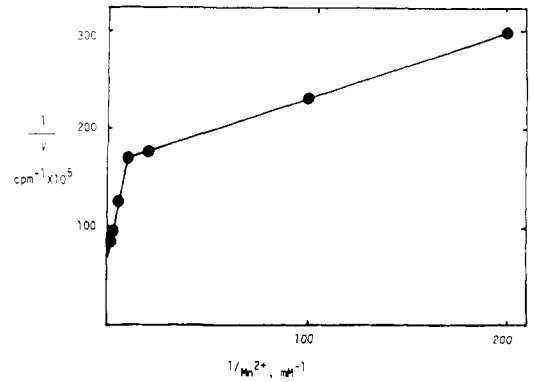

FIGURE 4: Effects of Mn^{2+} concentration. The rate of lactose synthesis was followed with varied concentrations of Mn^{2+} and with fixed concentrations of glucose (20 mM) and UDP-galactose (0.2 mM).

Fluorescence Studies with Dansyl-α-lactalbumin–Galactosyltransferase Cross-Linked Complex. The fluorescence excitation and emission spectra of the dansyl-α-lactalbumin–galactosyltransferase cross-linked complex are shown in Figure 6. The emission spectrum is a typical blue-shifted dansyl emission spectrum indicating a nonpolar environment for the fluorophore. The excitation spectrum is characterized by maxima at 290 and 350 nm, indicating that there is probably considerable energy transfer from protein tryptophan residues to the introduced dansyl group in addition to direct excitation of the dansyl chromophore. As was discussed in the preceding paper (O'Keeffe et al., 1980), the absorption spectrum of the cobalt-substituted enzyme overlaps the fluorescence emission spectrum of the introduced dansyl group. The effects of the addition of cobalt to the apo-cross-linked complex on the fluorescence emission of the dansyl group were studied. Figure 7A shows that as micromolar concentrations of cobalt are added to the cross-linked complex, a small, but quite distinct decrease in the quantum yield of the dansyl fluorescence is observed, reaching a maximum quenching of 15.1%. When the quenching is plotted in the form of a double-reciprocal plot of $1/\Delta F$ vs. $1/[Co^{2+}]$ (where ΔF is the difference between the fluorescence in the absence of Co^{2+} and in the presence of any particular cobalt concentration), a linear plot is obtained (Figure 7B), giving an apparent dissociation constant for Co^{2+} of 1.5 μM.

Discussion

The work described in this paper was initiated with the aim of providing a better understanding of how α-lactalbumin and galactosyltransferase interacted to form lactose synthase. To

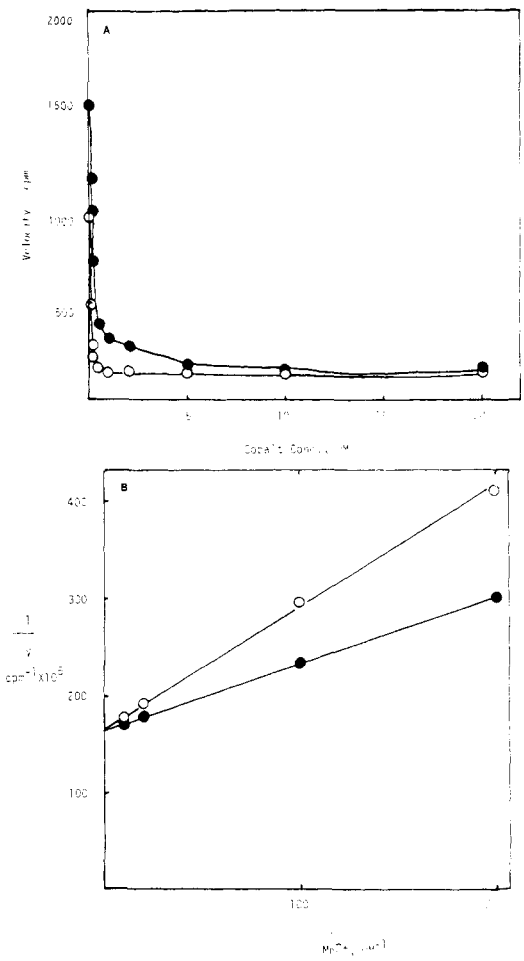

FIGURE 5: The effects of cobalt on lactose synthesis. (A) The effects of varied concentrations of Co^{2+} on the rate of lactose synthesis were followed at Mn^{2+} concentrations of 0.02 mM (○) and 2 mM (●). (B) The rate of lactose synthesis was followed with varied concentrations of Mn^{2+} in the absence (●) and presence (○) of 5 μM Co^{2+}. In both experiments UDP-galactose was 0.2 mM and glucose was 20 mM.

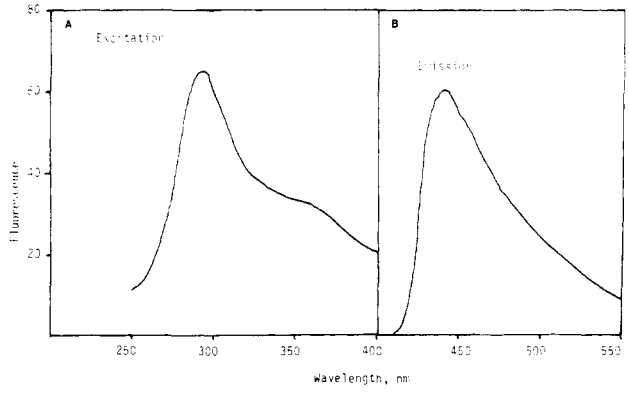

FIGURE 6: Fluorescence properties of the dansylated α-lactalbumin–galactosyltransferase cross-linked complex. (A) Fluorescence excitation spectrum with emission monitored at 440 nm. (B) Fluorescence emission spectrum with excitation at 300 nm.

this aim, we have chemically modified a unique residue on bovine α-lactalbumin, the N-terminal glutamic acid residue, with dansyl chloride, introducing a fluorophore which has been used in resonance energy transfer experiments to give a distance between the N-terminal glutamic acid of α-lactalbumin and one of the two metal binding sites on galactosyltransferase. These experiments have allowed an extension of the active site mapping of lactose synthase described earlier (O'Keeffe et al.,

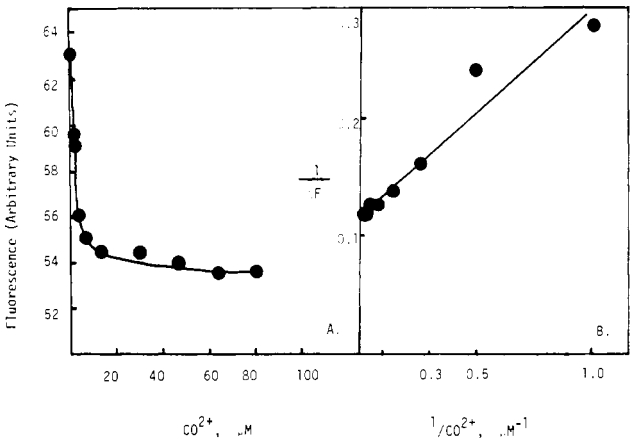

FIGURE 7: Effects of Co^{2+} on the fluorescence of the cross-linked complex. (A) Quenching of the dansyl fluorescence by cobalt. (B) Double-reciprocal plot of the data from part A. Conditions: 50 mM cacodylate buffer, pH 7.4, 3.2 μM apo-cross-linked complex, excitation at 300 nm, emission intensity obtained by graphical integration of emission spectrum between 390 and 490 nm.

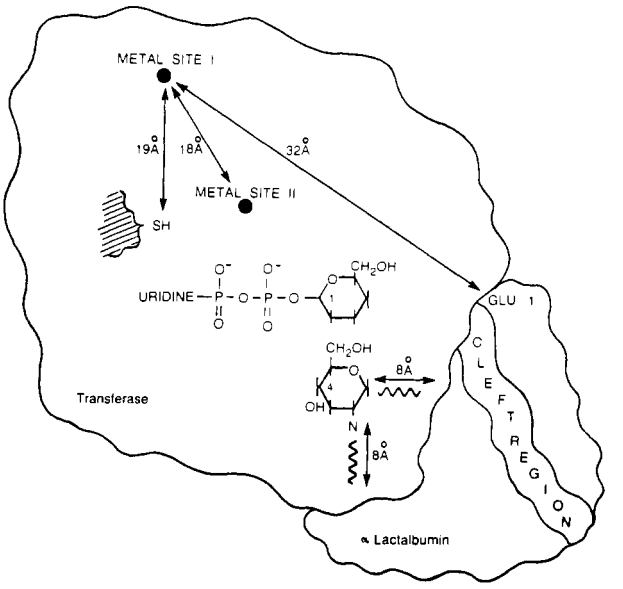

FIGURE 8: Schematic representation of the active site of bovine galactosyltransferase and its interaction with α-lactalbumin.

1980; L. H. Berliner, M. E. Davis, K. E. Ebner, T. A. Beyer, and J. E. Bell, unpublished experiments). In addition, when the results discussed here are examined in the light of current concepts of the three-dimensional structure of α-lactalbumin (Browne et al., 1969; Warme et al., 1974), greater insight into the role α-lactalbumin plays in lactose synthase is obtained.

The modified α-lactalbumin obtained in these experiments is clearly uniquely labeled by dansyl chloride at the N-terminal glutamic acid residue of α-lactalbumin. The species isolated by Sephadex G-100 chromatography has, as judged by incorporation of ^{14}C-labeled dansyl, a single dansyl group per molecule. Two-dimensional thin-layer chromatography of the amino acid hydrolysate of the modified α-lactalbumin shows only glutamic acid as a dansyl derivative, indicating that the site of dansylation is the N-terminal glutamic acid residue. When compared on a mole to mole basis, the dansylated α-lactalbumin functions in both lactose synthesis and *N*-acetyllactosamine synthesis identically with native α-lactalbumin (Figure 2). The chemically modified α-lactalbumin is covalently cross-linked to galactosyltransferase by using essentially those procedures described previously (Brew et al., 1975). The cross-linked complex has similar kinetic properties to those described previously (Brew et al., 1975; Bell et al., 1976) in that it shows high activity with glucose as acceptor and decreased activity with *N*-acetylglucosamine as acceptor. The apparent Michaelis constant for glucose of 75 μM (from Figure 3) is somewhat lower than values previously reported.

The effects of metal ions on the cross-linked α-lactalbumin–galactosyltransferase are complex. As with native galactosyltransferase, Lineweaver–Burk plots with Mn^{2+} varied are biphasic. With the cross-linked complex, K_m values of 4.5 μM and 130 μM can be calculated, compared with values of 15 μM and 440 μM with native transferase (O'Keeffe et al., 1980). The later values were obtained with *N*-acetylglucosamine as acceptor, in the absence of α-lactalbumin. With native galactosyltransferase, a variety of metal ions, including cobalt, have been shown to support activity in the absence of Mn^{2+} (Powell & Brew, 1976; O'Keeffe et al., 1980). However, with the cross-linked complex, we could detect no activity with cobalt alone. Cobalt is, however, an effective competitor with respect to the high-affinity Mn^{2+} ion (Figure 5) with a K_I value of 6 μM.

Cobalt absorption in the dansylated α-lactalbumin–galactosyltransferase complex overlaps the dansyl fluorescence emission. As discussed in the previous paper (O'Keeffe et al., 1980), a distance between the dansyl group and the metal ion can be calculated from the quenching of the dansyl fluorescence by the cobalt ion. The results (Figure 7) indicated that at cobalt concentrations sufficient to saturate site I, 15% of the dansyl fluorescence is quenched. By use of the equations discussed previously (O'Keefe et al., 1980), a distance between the dansyl group on α-lactalbumin and the cobalt ion in metal site I on the transferase of 32 Å can be estimated.

As mentioned in the introduction, there has been much discussion about the three-dimensional structure of α-lactalbumin. This discussion has centered on the sequence homology α-lactalbumin has with hen egg-white lysozyme (Brew et al., 1967). Browne et al. (1969) constructed a model of α-lactalbumin which approximates the known structure of lysozyme (Blake et al., 1967). Warme et al. (1974) have further refined the structure for α-lactalbumin proposed by Browne et al. (1969) by using energy minimization procedures. Through these varied approaches, a detailed picture of the structure of α-lactalbumin has been obtained.

On the basis of what is known about the three-dimensional structure of α-lactalbumin and the work described in the previous paper (O'Keeffe et al., 1980) and by L. H. Berliner, M. E. Davis, K. E. Ebner, T. A. Beyer, and J. E. Bell (unpublished experiments), together with the results obtained here with a covalently cross-linked complex of dansylated α-lactalbumin and galactosyltransferase, a detailed model of the active site of galactosyltransferase and the interaction of the transferase with α-lactalbumin is presented (Figure 8). The transferase contains two metal binding sites (Powell & Brew, 1976; O'Keeffe et al., 1980) which have been shown to be ~18 Å apart. One of these sites, termed site I, binds Mn^{2+} (and a variety of other metals) with high affinity. The second, termed site II, binds metals with lower affinity and is associated with the binding of UDP-galactose (O'Keeffe et al., 1980). In or near the UDP-galactose binding site lies a sulfhydryl residue (one of three in the transferase) which can be modified by *S*-mercuric-*N*-dansylcysteine (Bell, 1979). This sulfhydryl lies some 19 Å from metal site I. The acceptor substrate must of course bind with its 4-hydroxyl close to the P–O–C bond of UDP-galactose. Studies with acceptor substrate analogues (Bell et al., 1976; Berliner et al., 1980) have shown that the 1 position or the 2 position of the acceptor can

be blocked with quite bulky substituents without affecting the interaction of α-lactalbumin with the transferase. When these substituents are greater than ~8 Å, the interaction of α-lactalbumin with the transferase is blocked.

While it has been suggested that α-lactalbumin may provide binding residues for the acceptor substrate, thus increasing its affinity, the present studies suggest that the "cleft" region, which in the lysozyme analogy model should be involved in this role, is probably located some distance from the acceptor binding site. According to the model proposed (Browne et al., 1969; Warme et al., 1974) for α-lactalbumin, the α-amino group of Glu-1 interacts with the side-chain carboxyl oxygens of Asp-37 and Glu-39. These two residues are located at the entrance to the cleft region of α-lactalbumin, thereby placing Glu-1 in a nearby position. It is the amino-terminal Glu-1 which is dansylated in the studies reported here. The energy transfer measurements reported here place this dansyl group some 32 Å from metal site I, making it highly unlikely that this region of α-lactalbumin is involved in acceptor substrate binding. It is likely that the interaction of α-lactalbumin with the transferase involves a hydrophobic region on α-lactalbumin containing Tyr-36, Phe-31, Trp-118, and His-32 (Warme et al., 1974). Evidence for the involvement of Trp-118 and His-32 in the binding of α-lactalbumin has come from a variety of chemical modification studies (Bell et al., 1975; Prieels et al., 1979). The fact that *N*-acyl derivatives of the acceptor substrate *N*-acetylglucosamine bind more tightly than *N*-acetylglucosamine provides some evidence that the transferase has a "sticky" hydrophobic region (L. H. Berliner, M. E. Davis, K. E. Ebner, T. A. Beyer, and J. E. Bell, unpublished experiments), which, since substrate analogues with a hydrocarbon chain greater than six carbons block α-lactalbumin binding, presumably extends into the α-lactalbumin binding site of the transferase.

On the basis of the above discussion, it seems likely that α-lactalbumin exerts its influence on galactosyltransferase either by inducing a conformational change in the transferase or by a purely equilibrium effect of increasing the concentration of reactive complexes in the kinetic mechanism as has been proposed previously (Morrison & Ebner, 1971; Bell et al., 1975; Bell et al., 1976).

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