the simple occupation of the active site of 20β-HSD by an alkylator does not always (and more likely rarely) lead to covalent bond formation.

The 3α and 20β activities were lost simultaneously and at an identical rate during cortisone reductase inactivation by OPA, both when it was incubated directly and when it was enzymatically generated from β-HPA. That this coidentity persisted during inactivation in different pH buffers, in the presence of competing substrate steroids, in the presence of nonprotective steroids, and in 24-h incubations offers persuasive evidence that the 3α and 20β activities result from catalysis within the same active site on a single protein and further justifies naming this enzyme 3α,20β-hydroxysteroid dehydrogenase.

These studies do not indicate whether the active site performs dissimilar stereospecific oxidation/reduction reactions because (1) two cofactor sites, one adjacent to the A ring and one adjacent to the D ring, could proximate an active site wherein all steroids identically align or (2) a single steroid binding site might proximate a single cofactor region and the androstane steroids undergo "wrong way binding" (i.e., the steroid is reversed and rotated 180°) relative to pregnene steroids within the active center. This question may be answered by studies designed to map the amino acids which proximate affinity alkylating analogues of androstane and pregnene steroids as they align within the active site. Finally, the observed inactivation may arise from two mechanisms when β-HPA in the presence of cofactor is enzymatically oxidized to OPA. The enzyme-generated alkylating product could covalently bond to a nucleophilic amino acid within the active site, or the alkylating steroid could modify the cofactor and thereby prevent dissociation of either from the binding region. The synthesis of radiolabeled β-HPA would be one approach to answer this question.

References

Betz, G. (1968) Ph.D. Thesis, University of Kansas, Lawrence, KS, pp 63–65.
Betz, G., & Warren, J. C. (1968) *Arch. Biochem. Biophys. 125*, 745–752.
Blomquist, C. H. (1973) *Arch. Biochem. Biophys. 159*, 590–595.
Covey, D. F. (1979) *Steroids 34*, 199–206.
Edwards, C. A. F., & Orr, J. C. (1978) *Biochemistry 17*, 4370–4376.
Gibb, W., & Jeffery, J. (1971) *Eur. J. Biochem. 23*, 336–342.
Gibb, W., & Jeffery, J. (1972) *Biochim. Biophys. Acta 268*, 13–20.
Gibb, W., & Jeffery, J. (1973) *Biochem. J. 135*, 881–888.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Pocklington, T., & Jeffery, J. (1968) *Eur. J. Biochem. 7*, 63–67.
Strickler, R. C., Sweet, F., & Warren, J. C. (1975) *J. Biol. Chem. 250*, 7656–7662.
Sweet, F. (1976) *Steroids 27*, 741–749.
Sweet, F., & Samant, B. R. (1980) *Biochemistry 19*, 978–986.
Sweet, F., Arias, F., & Warren, J. C. (1972) *J. Biol. Chem. 247*, 3423–3433.
Sweet, F., Strickler, R. C., & Warren, J. C. (1978) *J. Biol. Chem. 253*, 1385–1392.

Active Site of Bovine Galactosyltransferase: Kinetic and Fluorescence Studies†

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ABSTRACT: Bovine galactosyltransferase has been shown to have two metal binding sites. The functional properties of these metal binding sites have been established by using kinetic, spectroscopic, and affinity chromatographic approaches. Metal site I, which is involved in maintaining the structural integrity of the protein, must be liganded prior to other substrates binding and prior to a second metal binding to site II, which is shown to be associated with UDP-galactose binding. Both metal sites can bind a variety of metals; however, calcium and its fluorescent analogue europium bind only to site II. Fluorescent resonance energy transfer measurements between europium in site II and cobalt in site I indicate a distance of 18 ± 3 Å between the two sites. Chemical modification studies with *S*-mercuric-*N*-dansylcysteine indicate that one (of a total of three exposed sulfhydryl groups) can be specifically dansylated and that this sulfhydryl group is in or near the UDP-galactose binding site. Resonance energy transfer measurements between this introduced sulfhydryl group and cobalt in metal site I give a distance of 19 ± 3 Å between these points, consistent with the interpretation that the UDP-galactose binding site, which is associated with metal site II, is located some distance from the structural metal site (site I).

Galactosyltransferase catalyzes the transfer of galactose from UDP[1]-galactose to *N*-acetylglucosamine, which may be either the free monosaccharide or in glycosidic linkage in glycoproteins, forming β1→4 galactosides (Hill & Brew, 1975). The transferase, in the presence of α-lactalbumin, will also utilize glucose as an acceptor substrate producing lactose. Together the transferase and α-lactalbumin form lactose synthase (Brew & Hill, 1975).

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[1] Abbreviations used: dansyl, 8-dimethylaminonaphthalene-1-sulfonyl chloride; GlcNAc, *N*-acetylglucosamine; UDP, uridine diphospho or phosphate; EDTA, ethylenediaminetetraacetic acid.

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Galactosyltransferase has been purified to homogeneity from bovine colostrum (Trayer & Hill, 1971), and previous studies have shown that the substrates of the transferase reaction add in a random-order equilibrium manner to an enzyme–Mn^{2+} complex (Bell et al., 1976). The report (Powell & Brew, 1976) that the metal requirements of the transferase were more complex than originally suggested stimulated the detailed kinetic studies reported here with a variety of metals to explore the functions of these two metal binding sites. We have shown that a variety of metals, including Co^{2+}, can replace Mn^{2+}, the presumed native metal, in one site and that once Mn^{2+} or one of its analogous metals has bound to the first site, a second class of metals could bind to the second site causing activation. Included in this second group of metals are the fluorescent lanthanides Eu^{3+} and Tb^{3+}.

Several groups have examined the effects of sulfhydryl reagents such as *p*-(hydroxymercuri)benzoate (Magee & Ebner, 1974) and *N*-ethylmaleimide (Kitchen & Andrews, 1974) on the activity of the transferase and found that modification by such sulfhydryl reagents results in considerable loss of activity. While these studies showed that the nucleotide donor substrate and analogues would protect against inactivation, they did not establish the numbers or types of sulfhydryl groups reacted.

Detailed knowledge of the metal specifities for the two metal binding sites on galactosyltransferase and the introduction of a fluorescent derivative on a specific sulfhydryl group in the transferase have permitted measurement of the distances between the two metal binding sites, as well as a sulfhydryl group near the UDP-galactose binding site and metal binding site I, by means of resonance energy transfer measurements between the $Eu^{3+} \rightarrow Co^{2+}$ and the dansyl $\rightarrow Co^{2+}$ couples. These measurements have given a clearer understanding of the spatial relationships in the active site of galactosyltransferase. The distances so obtained are consistent with suggestions based on kinetic measurements concerning the role of the two metal sites in galactosyltransferase.

Experimental Procedures

Materials and Methods. Galactosyltransferase was isolated from bovine colostrum by affinity chromatography on immobilized UDP and immobilized α-lactalbumin (Barker et al., 1972). Apoenzyme was prepared from the holoenzyme and characterized as described. In all the studies reported here enzymatic activity was measured by using the *N*-acetyllactosamine synthesis assay (Bell et al., 1976). [2-^{14}C]EDTA, UDP-[$^{14}C_1$]galactose, and *N*-ethylmaleimide, uniformly labeled with ^{14}C, were obtained from New England Nuclear. Unlabeled UDP-galactose and bovine serum albumin were from Sigma Chemical Co. *N*-Acetylglucosamine was from Pfanstiehl.

Chelex 100 (100–200 mesh) was obtained from Bio-Rad Laboratories. Manganese, zinc, and cobalt were obtained in solution from Scientific Products and were spectrographically pure. Calcium chloride and aluminum ammonium sulfate were from Baker Analyzed Reagents. Europium and terbium chlorides were from Alfa Inorganics. In the studies to be described, no differences were observed between the spectrographically pure metal solutions obtained from Scientific Products and the same metals from other sources. With zinc salts, no differences were observed between zinc chloride and zinc sulfate, suggesting that, at the concentrations used, the anion is of little significance.

The fluorescent sulfhydryl reagent *S*-mercuric-*N*-dansylcysteine was initially obtained through the generosity of Dr. Sherwin Lehrer. Preliminary studies with this sample indicated its usefulness with galactosyltransferase. Subsequently *S*-mercuric-*N*-dansylcysteine was synthesized as described by Leavis & Lehrer (1974).

Preparation of Apoenzyme. Initially, metal was removed from galactosyltransferase by dialysis against EDTA, followed by exhaustive dialysis against buffer, as described previously (Bell et al., 1976). These apoenzyme preparations gave hyperbolic saturation curves for velocity vs. Mn^{2+} concentrations rather than the sigmoidal curves reported by Powell & Brew (1976). Sigmoidal saturation curves could, however, be obtained by the addition of low concentrations of EDTA (5–10 μM), as would indeed be expected. This raised the question as to whether or not all of the EDTA had been removed by the methods used to prepare apoenzyme (Powell & Brew, 1976). To answer this point, we prepared apoenzyme as described previously (Powell & Brew, 1976), but, after dialysis against EDTA, we added ^{14}C-labeled EDTA to the protein in order to follow the removal of EDTA from the protein during dialysis against buffer. The dialysis tubing was changed on each buffer change to avoid problems due to ^{14}C-labeled EDTA adhering to the dialysis membrane. After three changes of buffer, 8 μM EDTA was found inside the dialysis sac, suggesting that the "apoenzyme" preparations used in previous studies (Powell & Brew, 1976) could have contained significant amounts of EDTA. Further dialysis decreased the concentration of EDTA, but, even after exhaustive dialysis (six changes), EDTA was present in an approximately twofold molar excess over the enzyme concentration, suggesting that activity may be inhibited by tightly bound EDTA rather than metal removal. Atomic absorption studies (kindly performed by Dr. Premakumar) showed that this final "apoenzyme" was in fact a complex containing enzyme, metal, and EDTA in the ratio of 1.0:0.6:2.0.

Metal-free apoenzyme was prepared by a combination of a high pH treatment (which precipitates much of the Mn^{2+}) and dialysis against Chelex 100. The pH of an $\sim$10 μM solution of galactosyltransferase in 50 mM cacodylate was raised to pH 9.0–9.5 by addition of sodium hydroxide and left at 0 °C for 30 min. The solution was clarified by centrifugation and the supernatant was dialyzed against a suspension of Chelex 100 in 50 mM cacodylate at pH 7.4. The resulting apoenzyme was then dialyzed exhaustively against the required buffer. The final apoenzyme preparation showed no detectable Mn^{2+} by atomic absorption and no activity in the absence of added metal. In the presence of 40 mM Mn^{2+}, this apoenzyme preparation had a specific activity of 21.1 μmol (min mg)$^{-1}$, similar to that reported previously for the holoenzyme (Bell et al., 1976).

The rate equation for any three-substrate enzyme mechanism may be considered in the general form (Dalziel, 1969)

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{S_1} = \frac{\phi_2}{S_2} + \frac{\phi_3}{S_3} + \frac{\phi_{12}}{S_1S_2} + \frac{\phi_{13}}{S_1S_3} + \frac{\phi_{23}}{S_2S_3} + \frac{\phi_{123}}{S_1S_2S_3} \quad (1)$$

where, with galactosyltransferase, S_1 is metal, S_2 is UDP-galactose, and S_3 is *N*-acetylglucosamine. Values of each of the ϕ parameters in this generalized rate equation can be obtained experimentally by systematically varying each of the substrates at fixed concentrations of the others. Slopes and intercepts of primary Lineweaver–Burk plots obtained at several concentrations of a second substrate are plotted against the reciprocal of the concentration of the second substrate. Such secondary plots are constructed at several concentrations of the third substrate, and slopes and intercepts of these sec-

ondary plots are replotted against the reciprocal of the concentration of the third substrate. From the slopes and intercepts of the four tertiary plots that are obtained in this manner, values for each of the eight ϕ parameters in eq 1 are obtained. In the results to be described here, ϕ parameters were calculated from experiments by using a 4 × 4 × 4 grid of substrate concentrations. Estimated standard errors of the ϕ parameters obtained from the slopes and intercepts of the appropriate tertiary plots were obtained from the regression analysis by using standard procedures (Draper & Smith, 1968).

Fluorescence measurements were made by using a Farrand Mark I fluorometer. Excitation and emission spectra reported here are uncorrected for variation of lamp intensity with wavelength or phototube response. Intensities are relative quantum yields, changes in which are calculated by graphical integration of areas under the appropriate emission spectra. When fluorescence intensities were measured in the presence of potential absorbing species such as UDP-glucose or Co^{2+}, attenuation of incident light was corrected for by using free *S*-mercuric-*N*-dansylcysteine in the presence of the added ligands. Such corrections were always <10% of the measured intensity.

Energy Transfer Calculations. The distance, *R*, between the donor and the acceptor in an energy transfer couple may be calculated from the change in quantum yield of the donor fluorescence as a result of energy transfer (Stryer, 1978) as

$$q = q_0 \frac{1}{1 + (R_0/R)^6} \quad (2)$$

where q is the quantum yield of the donor in the presence of energy transfer, q_0 is the quantum yield of the donor in the absence of energy transfer, and R_0 is the critical distance for resonance energy transfer for a particular donor–acceptor pair. R_0 is the distance between donor and acceptor at which the rate of emission from the donor equals the rate of transfer from donor to acceptor, and it may be calculated for any given donor–acceptor pair from the expression

$$R_0 = (JK^2Q_0{}^{n-4})^{1/6}(9.7 \times 10^3) \quad (3)$$

where n is the refractive index of the medium, Q_0 is the quantum yield of the donor in the absence of energy transfer, and J is the overlap integral of the emission spectrum of the donor and the absorption spectrum of the acceptor and is approximated by the expression

$$J = \frac{\int f(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda}{\int f(\lambda) \, d\lambda} \quad (4)$$

where $f(\lambda)$ is the fluorescence intensity of the donor at wavelength λ (in cm) and $\epsilon(\lambda)$ is the extinction coefficient in cm^{-1} M^{-1} of the acceptor. This integral is obtained graphically as has been described (Weber, 1960). K^2, the orientation factor between the donor and acceptor, may vary between 0 and 4. However, with the assumption of rapid rotation of the two oscillators, the statistical average of $K^2 = {}^2/_3$ is used (Eisinger, 1974).

Results

Kinetic Studies with Metals. When the rate of *N*-acetyllactosamine synthesis is studied as a function of varied Mn^{2+} concentration at fixed *N*-acetylglucosamine (20 mM) and UDP-galactose (1 mM) concentrations, the double-reciprocal plot (Figure 1) is biphasic, giving two apparently linear regions, one extending from 1 to ~30 μM Mn^{2+} and the other extending from 0.1 to 40 mM Mn^{2+}.

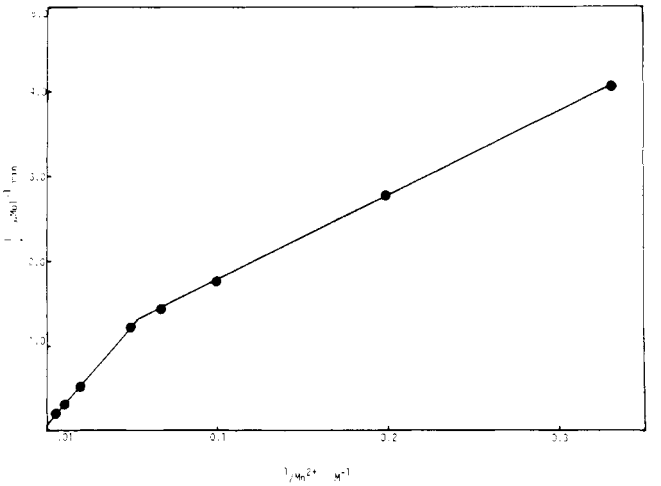

FIGURE 1: Double-reciprocal plot of Mn^{2+} kinetics with apogalactosyltransferase at pH 7.4 with 50 mM cacodylate, 20 mM *N*-acetylglucosamine, and 1 mM UDP-galactose.

Table I: Initial Rate Parameters for *N*-Acetyllactosamine Synthesis Obtained at Low and High Mn^{2+} Concentration Ranges

	manganese concn range	
parameter	1–20 μM	0.1–40 mM
ϕ_0	0.150 ± 8%	0.01 ± 8%
ϕ_1	6.01	136 ± 2%
ϕ_2	13.95 ± 4%	0.223 ± 5%
ϕ_3	6856 ± 1%	41.1 ± 7%
ϕ_{12}	0	293 ± 25%
ϕ_{13}	0	(4.2×10^5) ± 2%
ϕ_{23}	(4.1×10^4) ± 6%	1268 ± 3%
ϕ_{123}	(4.4×10^6) ± 1%	(7.5×10^6) ± 4%

The effects of varying both UDP-galactose and *N*-acetylglucosamine concentrations in each of these linear regions of double-reciprocal plots with Mn^{2+} as the varied substrate were studied, and the ϕ parameters of eq 1 were determined by using a 4 × 4 × 4 grid of substrate concentrations at both high and low Mn^{2+} concentration ranges. With both Mn^{2+} concentration ranges, UDP-galactose concentrations were varied in the range of 2–20 μM and *N*-acetylglucosamine concentrations were varied in the range 2–20 mM.

The ϕ parameters obtained from tertiary kinetic plots are given in Table I. In the low Mn^{2+} concentration range, ϕ_{12} and $\phi_{13} = 0$, whereas in the high Mn^{2+} concentration range all eight ϕ parameters of eq 1 have finite values.

Zinc and cobalt were also used as metal cofactors at two different UDP-galactose concentrations, 10 and 200 μM. As the Zn^{2+} concentration is increased the maximum velocity is reached at about 0.6–1.0 mM. The Michaelis constants for Zn^{2+} calculated from these data are 49 μM at 10 μM UDP-galactose and 37 μM at 200 μM UDP-galactose. At higher Zn^{2+} concentrations, however, inhibition is observed and is far more marked at the higher UDP-galactose concentration. Studies with Co^{2+} showed similar results, only the inhibition observed at high Co^{2+} concentrations was less marked than with Zn^{2+}. Michaelis constants for Co^{2+} were 93 μM at 10 μM UDP-galactose and 68 μM with 200 μM UDP-galactose. The Michaelis constants and maximum velocities for Mn^{2+}, Co^{2+}, and Zn^{2+} are compared in Table II.

Studies with Metal "Activators". In addition to the above studies, we have confirmed the recent report (Powell & Brew, 1976) that calcium, which alone does not support *N*-acetyllactosamine synthesis, causes a slight but definite activation in the presence of low concentrations (micromolar range) of

Table II: Michaelis Constants and Maximum Velocities for Metals Supporting Galactosyltransferase Activity

metal	K_m (μM)	V_{max} (μmol/min)
Mn^{2+} (1–20 μM)	15	1.41
Mn^{2+} (0.1–40 mM)	440	21.1
Zn^{2+}	37	3.38
Co^{2+}	68	1.41

Table III: Metal Activation Effects with Site I Saturated with Mn^{2+}

metal	max activation (%)	total Mn act. (%)
calcium	56 (1 mM)[a]	10.4
aluminum	61 (1 mM)	10.8
europium	69 (0.15 mM)	11.3
terbium	63 (0.1 mM)	10.9

[a] Kinetic dissociation constants in parentheses.

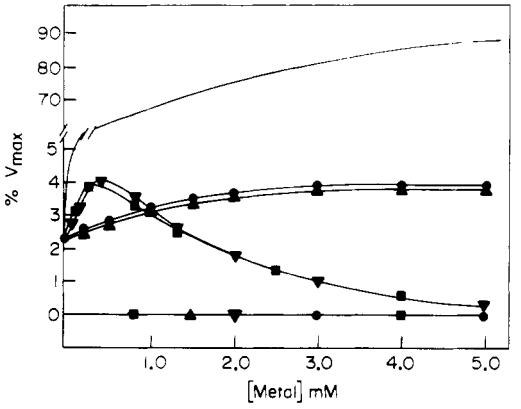

FIGURE 2: Activation effects of a second metal. Activation of *N*-acetyllactosamine synthesis by addition of calcium (●), aluminum (▲), europium (▼), and terbium (■) in the presence of 10 μM Mn^{2+}, 200 μM UDP-galactose, and 20 mM *N*-acetylglucosamine at pH 7.4 in 50 mM cacodylate buffer, 37 °C. The line at 0% V_{max} shows results obtained in the absence of Mn^{2+}. The solid line shows the Mn^{2+} saturation curve for comparison.

Mn^{2+}. The maximum velocity obtained at saturating Ca^{2+} concentrations and a fixed Mn^{2+} concentration of 10 μM is, however, very much less than the maximum velocity obtained at saturating Mn^{2+} concentrations, as shown in Figure 2. In addition to Ca^{2+}, several other metals were found to mimic the effects of calcium, in that they do not support activity in the absence of Mn^{2+} but stimulate activity at low Mn^{2+} concentrations. These results, in terms of the percentage activation, the "kinetic" dissociation constant for the activator metal (defined as the concentration of metal required to produce half the maximal activation observed), and the relative maximum velocity extrapolated to saturation of site I with Mn^{2+} and saturating concentrations of the second metal, are shown in Figure 2 and Table III. For comparison, this relative maximal velocity is expressed by taking the maximum velocity in the presence of saturating Mn^{2+} concentrations as 100. As can be seen, in addition to Ca^{2+}, aluminum produces a similar activation with a similar "kinetic" dissociation constant. Two lanthanides, terbium and europium, also produced significant activation; though at higher concentrations a marked inhibition was observed with both metals. The results with Tb^{3+} were obtained at pH 6.0, since this metal is insoluble at higher pH values. However, comparative studies with Mn^{2+}, Ca^{2+}, and Eu^{3+} showed that these metals produced similar effects at pH 6.0 as they did at pH 7.4.

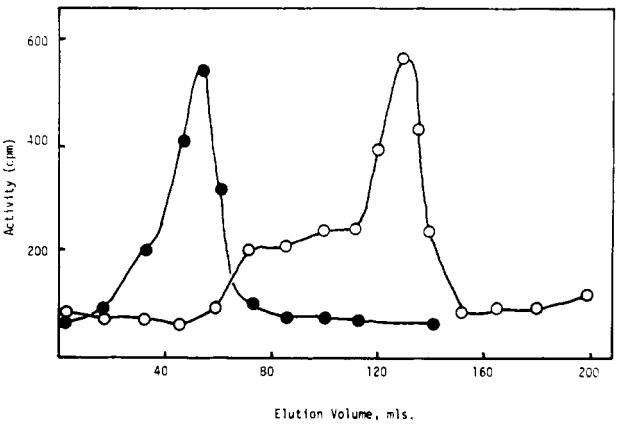

FIGURE 3: Elution of galactosyltransferase from α-lactalbumin–Sepharose. The effects of Mn^{2+} concentration. Activity was eluted by exclusion of *N*-acetylglucosamine at 0.1 mM Mn^{2+} (○) or 25 mM Mn^{2+} (●).

All of these "activation" effects were also observed by using low concentrations (10 or 20 μM) of zinc or cobalt in place of the manganese. With either of these alternate site I metals, the activation effects observed with calcium, aluminum, and the two lanthanides were qualitatively and quantitatively similar to those observed with manganese in site I.

Affinity Chromatography Studies. In addition to the kinetic studies reported above, we have examined the affects of both metal concentration and the nature of the divalent metal ion on the ability of various affinity resins to retard apogalactosyltransferase. With apogalactosyltransferase, UDP-Sepharose or α-lactalbumin–Sepharose do not retard enzymatic activity when the resins are preequilibrated with either metal-free buffer or buffer containing EDTA. When 25 mM Mn^{2+} is added to the equilibration buffers, transferase activity is totally retarded. Addition of 20 mM Ca^{2+} in place of the Mn^{2+} did not retard activity on either column. With the α-lactalbumin–Sepharose column, transferase activity can be eluted from the column, in the presence of Mn^{2+}, by removal of *N*-acetylglucosamine from the buffer. When this elution is studied at different Mn^{2+} concentrations (Figure 3), it is found that the position of elution (from identical α-lactalbumin-Sepharose columns) is quite different at 0.1 mM Mn^{2+} than it is at 25 mM Mn^{2+}. At the low Mn^{2+} concentration, the transferase activity eluted at a significantly slower rate.

Preparation of S-Mercuric-N-dansylcysteine Derivative of Galactosyltransferase. The inactivation of galactosyltransferase by *S*-mercuric-*N*-dansylcysteine hydrochloride was studied as a function of time of reaction, concentration of reagent, and the presence of substrates and substrate analogues. When galactosyltransferase at a concentration of 0.05 mg/mL was incubated with varying concentrations of *S*-mercuric-*N*-dansylcysteine at 25 °C, it was found that maximum inactivation occurred after ~2 h (Figure 4A) with the highest *S*-mercuric-*N*-dansylcysteine concentration used, 0.1 mM. In these experiments modification was carried out in the presence of 5 mM *N*-acetylglucosamine, which was added to stabilize the galactosyltransferase. When *N*-acetylglucosamine was omitted from the reaction mixture and corrections were made for the instability of the transferase, identical results were obtained. Subsequently, *N*-acetylglucosamine was included in all the experiments described unless otherwise stated. Addition of 25 mM Mn^{2+} to the reaction mixture also had no effect on the rate or extent of inactivation. However, inclusion of 200 μM UDP-glucose afforded almost complete protection at the lower *S*-mercuric-*N*-dansylcysteine concentrations used and afforded considerable protection at 0.01 mM. In order

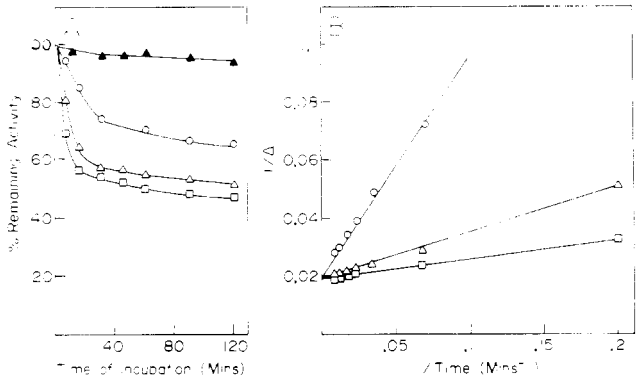

FIGURE 4: (A) Time course of *S*-mercuric-*N*-dansylcysteine modification of galactosyltransferase. Galactosyltransferase (0.18 mg/mL) was incubated with concentrations of *S*-mercuric-*N*-dansylcysteine of 0.2 mM (○), 0.05 mM (△), and 0.1 mM (□) in the absence (open symbols) and presence (closed symbols) of 200 μM UDP. Reaction was at 25 °C in 0.05 M cacodylate buffer, pH 7.4, containing 5 mM *N*-acetylglucosamine and 25 mM $MnCl_2$. (B) Data from (A) plotted as $1/\Delta$ (Δ = the extent of inactivation at any given time t) vs. $1/t$.

to determine the maximum extent of inactivation at each of the concentrations used, we plotted the data in Figure 4A in double-reciprocal form, $1/\Delta$ (Δ = the extent of inactivation at any given time t) vs. $1/t$. As can be seen in Figure 4B, upon extrapolation to infinite time, essentially the same extent of inactivation is found for each concentration used. On maximal inactivation with *S*-mercuric-*N*-dansylcysteine, it appears that the modified galactosyltransferase retains ~40% of its initial activity. Incubation of any of the modified samples of galactosyltransferase with 10 mM β-mercaptoethanol for 30 min at room temperature resulted in complete reactivation.

Characterization of S-Mercuric-N-dansylcysteine Derivative of Galactosyltransferase. Extent of Modification. Direct determination of the number of *S*-mercuric-*N*-dansyl groups introduced during inactivation using absorbance measurements is complicated by uncertainty in the absorption properties of the protein-bound chromophore and the undesirability of using high protein concentrations for accurate absorbance measurements. These problems were circumvented by making use of the reversibility of the modification by β-mercaptoethanol to introduce *N*-[^{14}C]ethylmaleimide into the original sites of modification by *S*-mercuric-*N*-dansylcysteine. The galactosyltransferase (1.02 mg) in a 20-mL final volume was modified by using 0.1 mM *S*-mercuric-*N*-dansylcysteine for 2 h, conditions where maximal inactivation occurs. The reaction was terminated by chromatography on Sephadex G-50 (40 × 2 cm, eluted with 50 mM cacodylate, pH 7.4, containing 5 mM cacodylate, pH 7.4, containing 5 mM *N*-acetylglucosamine and 25 mM Mn^{2+}) to remove unreacted reagent. To ensure complete removal of free reagent, we dialyzed the modified enzyme overnight against cacodylate buffer, pH 7.4, containing 25 mM Mn^{2+} and 5 mM *N*-acetylglucosamine. The resulting modified galactosyltransferase was then treated with 12 mM *N*-ethylmaleimide to block all unmodified sites of reaction with *N*-ethylmaleimide. The fluorescence of the introduced dansyl group was monitored before and after reaction with *N*-ethylmaleimide to check that the fluorescent label was not displaced by *N*-ethylmaleimide. Following reaction for 3 h, free *N*-ethylmaleimide was removed by Sephadex G-50 chromatography (as above) followed by dialysis. The dansyl–maleimide-labeled transferase was then dialyzed against cacodylate buffer, pH 7.4, containing 25 mM Mn^{2+}, 5 mM *N*-acetylglucosamine, and 10 mM β-mercaptoethanol. Following reaction with β-mercaptoethanol, the sample was extensively dialyzed against buffer containing *no* β-mercaptoethanol. Fluorescence studies (see later) showed that this treatment removed all the *S*-mercuric-*N*-dansyl label. Control experiments using native galactosyltransferase and *N*-[^{14}C]-ethylmaleimide showed (i) that a total of 3 sulfhydryl groups/mol of galactosyltransferase could be modified by extensive treatment with *N*-ethylmaleimide and (ii) that incubation of the modified transferase so obtained with β-mercaptoethanol neither restored any of the activity lost nor removed any of the ^{14}C label.

Table IV: Kinetic Parameters for Modified and Native Galactosyltransferase Determined at Saturating Mn^{2+} Concentrations

	modified enzyme	native enzyme[a]
Φ_0 (min)	0.0175 ± 0.0031	0.008 ± 0.0019
Φ_2 (μM·min)	0.453 ± 0.007	0.207 ± 0.002
Φ_3 (μM·min)	276 ± 5.7	99 ± 29
Φ_{23} (μM²·min)	1317 ± 59	1355 ± 42
V_{max} (min⁻¹)	57	125
K_m(UDPGal) (μM)	25.9	23
K_d(UDPGal) (μM)	4.8	13.7
K_m(GlcNAc) (mM)	15.7	11
K_d(GlcNAc) (mM)	2.9	6.6

[a] Taken from Bell et al. (1976).

The sample was now treated with *N*-[^{14}C]ethylmaleimide of specific activity 8 mCi/nmol and incubated at 25 °C for 3 h. Reaction was terminated by chromatography on Sephadex G-50, followed by extensive dialysis against cacodylate buffer to ensure complete removal of free *N*-ethylmaleimide. From the incorporation of ^{14}C it was calculated that 1.04 mol of ^{14}C had been incorporated per mol of galactosyltransferase, indicating that the original *S*-mercuric-*N*-dansyl derivative of galactosyltransferase contained 1 mol of fluorescent label/mol of protein.

Enzymatic Properties of Modified Enzyme. Galactosyltransferase modified to the extent of 1 mol of dansyl/mol of protein was used in kinetic experiments to study the effects of modification on kinetic parameters. Table IV lists the ϕ parameters, determined as described under Experimental Procedures by using modified enzyme, a saturating Mn^{2+} concentration of 40 mM, UDP-galactose concentrations ranging from 0.1 to 20 μM, and *N*-acetylglucosamine concentrations from 1 to 20 mM. Also listed in Table IV are the K_m, K_d, and V_{max} values for native and modified enzymes.

In addition, the effects of addition of Mn^{2+} or Co^{2+} to apoenzyme were studied at saturating concentrations of UDP-galactose and *N*-acetylglucosamine. In the low concentration range, both metals had apparent K_m values identical with those reported above for the native enzyme.

Spectral Properties of Modified Enzyme. Excitation and emission spectra of the modified enzyme were recorded at a protein concentration of 0.13 mg/mL. With emission monitored at 505 nm and excitation scanned from 260 to 400 nm, two distinct excitation peaks were observed (Figure 5A), the first at 282 nm representing energy transfer from protein tryptophan residues and the second at 355 nm representing direct excitation of the dansyl moiety. With excitation at 355 nm, an emission maximum of 492 nm is observed (Figure 5B). When the excitation wavelength is varied from 320 to 400 nm and emission spectra are scanned, no shift in the emission maximum is observed. However, as expected, the maximum intensity of fluorescence does change. The *shape* of the emission spectrum observed at excitation wavelengths of 320, 355, and 400 nm is constant, as judged by emission spectral ratios, calculated by taking the peak intensity and comparing it to the intensity at a number of wavelengths on either side

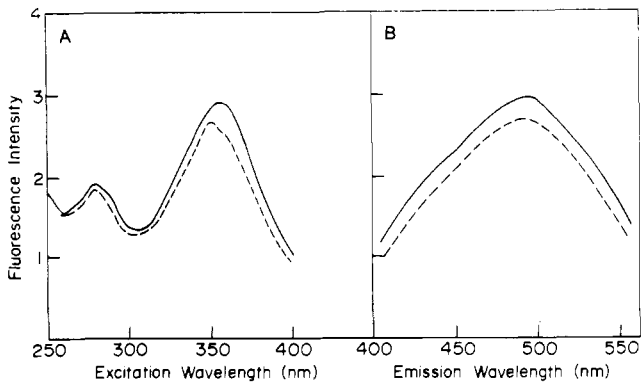

FIGURE 5: Fluorescence spectra of *S*-mercuric-*N*-dansylcysteine-modified galactosyltransferase. Spectra recorded at 25 °C in 0.5 M cacodylate buffer, pH 7.4, containing 5 mM *N*-acetylglucosamine and 25 mM $MnCl_2$. (A) Excitation spectra with emission monitored at 505 nm and (B) emission spectra with excitation at 355 nm, recorded in the absence (—) and presence (- - -) of 200 μM UDP.

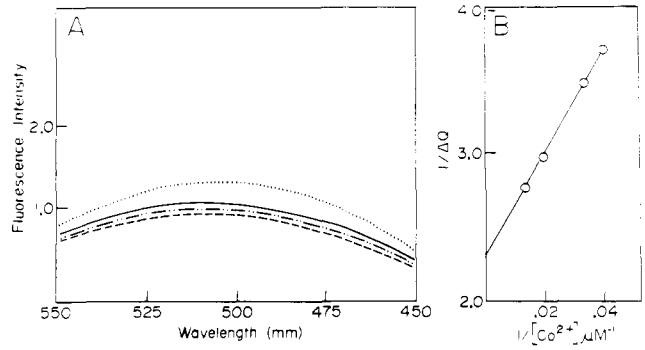

FIGURE 6: Dansyl → cobalt energy transfer measurements. (A) Emission spectra of *S*-mercuric-*N*-dansylcysteine-labeled galactosyltransferase with excitation at 355 nm in the absence (···) and the presence of cobalt concentrations of 25 (—), 30 (–··–), and 100 μM (- - -). Other conditions were as described in Figure 2 with the exception that $MnCl_2$ was omitted in these experiments. (B) Double-reciprocal plot of the cobalt ($1/[Co^{2+}]$).

of the emission maximum. Treatment of modified protein with β-mercaptoethanol followed by dialysis as described earlier resulted in the complete loss of dansyl fluorescence.

The emission spectrum of the dansyl derivative of galactosyltransferase was totally insensitive to the addition of Mn^{2+} or *N*-acetylglucosamine; however, addition of 100 μM UDP in the presence of 25 mM Mn^{2+} caused a small but significant shift in the emission maximum as well as small changes in intensity (Figure 5).

Energy Transfer Experiments Using S-Mercuric-N-dansylcysteine-Modified and Native Galactosyltransferase. (1) Dansyl → Cobalt Energy Transfer. As described earlier, addition of Mn^{2+}, up to a concentration of 25 mM, to the dansylated transferase had no effect on the fluorescence of the dansyl label. However, when cobalt, in the range of 0–100 μM, was added to labeled enzyme (Figure 6), the fluorescence of the dansyl group progressively decreased, appearing to approach a plateau value. The maximum quenching produced by the addition of cobalt to the dansyl-labeled transferase was obtained from a double-reciprocal plot of $1/\Delta Q$, where ΔQ is the change in fluorescence quantum yield obtained by graphical analysis, vs. $1/[Co^{2+}]$ (Figure 6B). This extrapolation indicates that at saturating Co^{2+} concentrations, the dansyl label exhibits 71% of its initial fluorescence.

(2) Europium → Cobalt Energy Transfer. Europium, on excitation at 395 nm, shows a complex emission spectrum with a number of bands occurring in the 550–700 nm region and two distinct maxima at 610 and 650 nm. When 10 μM

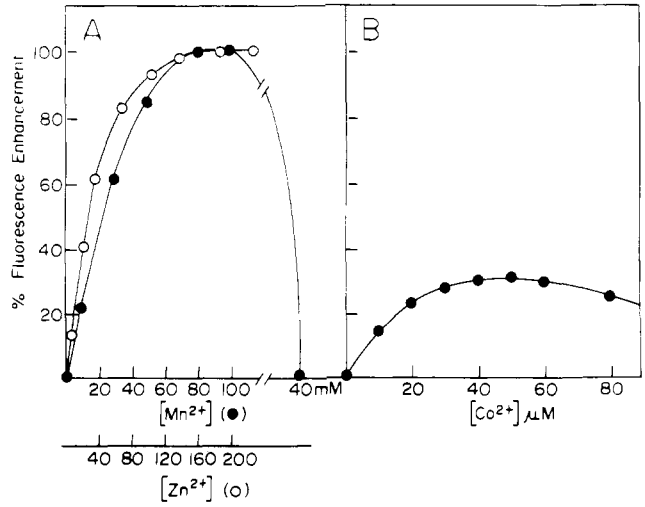

FIGURE 7: Europium → cobalt energy transfer measurements. (A) Titration of europium (0.1 mM) in the presence of galactosyltransferase (2.3 μM) with Mn^{2+} (●) or Zn^{2+} (○). Europium fluorescence was monitored with excitation at 395 nm and emission at 610 nm in 0.05 M cacodylate buffer, pH 7.4, containing 5 mM *N*-acetylglucosamine. (B) Titrations of europium in the presence of galactosyltransferase with Co^{2+}. Conditions were as described in Figure 5A.

apogalatosyltransferase is added to a 1 mM europium solution, no change in the intensity or position of these two peaks is seen. When, however, manganese is also added, a considerable enhancement of the europium fluorescence occurs. Figure 7A shows the titration of a fixed concentration (0.1 mM) of Eu^{3+} in the presence of 2.3 μM apogalactosyltransferase, with increasing concentrations of Mn^{2+} or Zn^{2+}. Also shown is the effect of addition of sufficient Mn^{2+} to completely saturate both sites I and II with Mn^{2+}. As can be seen, with both Mn^{2+} and Zn^{2+}, the europium fluorescence increases to reach a similar enhancement. Saturating Mn^{2+} concentrations, however, completely reverse the europium fluorescence enhancement. When a similar titration is performed with cobalt instead, the europium fluorescence is again enhanced but reaches a much lower plateau than that observed with Mn^{2+} or Zn^{2+} (Figure 7B). Comparison of the plateau levels reached in the presence of Mn^{2+} or Zn^{2+} with that reached in the presence of Co^{2+} indicated that the Co^{2+}–Eu^{3+} enzyme exhibited only 30% of the europium fluorescence of the Mn^{2+}–Eu^{3+} or the Zn^{2+}–Eu^{3+} enzyme.

Discussion

In our previous analysis of the kinetic mechanism of galactosyltransferase (Bell et al., 1976), it was established that, at saturating manganese concentrations, either donor or acceptor substrates could bind to an enzyme–Mn^{2+} complex.

The kinetic parameters obtained at low Mn^{2+} concentrations are characterized by the fact that both ϕ_{12} and ϕ_{13} are zero. The simplest kinetic mechanism consistent with this observation is one where Mn^{2+} binds to apoenzyme as an obligatory first substrate, followed by a random equilibrium addition of either UDP-galactose or *N*-acetylglucosamine (Dalziel, 1969). However, at high Mn^{2+} concentrations the rate equation contains all eight ϕ parameters of eq 1, consistent with a random addition of UDP-galactose, *N*-acetylglucosamine, and a second Mn^{2+} ion to an enzyme–Mn^{2+} complex (Dalziel, 1969).

The equilibrium nature of the addition of the second Mn^{2+} ion and the other two substrates to the enzyme–Mn^{2+} complex suggests that Mn^{2+} and UDP-galactose can either bind separately to the enzyme or add as a UDP-galactose–Mn^{2+}

complex. Since normal Michaelis–Menton behavior is observed over a wide range of Mn^{2+} concentrations (0.1–40 mM) and estimates for the dissociation constant for Mn^{2+}–UDP-galactose range from 10 to 20 mM (Berliner & Wong, 1975; Dwek, 1973), it would appear that either route for the combination of Mn^{2+} and UDP-galactose to any given enzyme form is equivalent (Macfarlane & Ainsworth, 1972).

As has been discussed previously (Bell et al., 1976; Dalziel, 1969), a number of the dissociation constants for this mechanism can be calculated from the initial rate parameters experimentally determined. Several of these dissociation constants were calculated previously from experiments at a fixed, saturating Mn^{2+} concentration of 40 mM, and there is good correlation between the values obtained in the present study and those reported earlier (Table IV).

From the data at low Mn^{2+} concentrations, it is apparent that the Michaelis constant for UDP-galactose ($\phi_2/\phi_0 = K_{132}$) is much higher than when the second metal ion is present (93 μM compared to 22 μM), consistent with the observation that the apparent K_m for the second Mn^{2+} ion decreases as the UDP-galactose concentration is increased. These observations, together with the suggestion that Mn^{2+}–UDP-galactose is probably as good a substrate as Mn^{2+} and UDP-galactose separately, suggest that this second metal ion and UDP-galactose may form a cyclic metal bridge type complex with the enzyme where both metal and UDP-galactose can interact separately with the enzyme as well as with each other. It should be noted that the Michaelis constant for the first Mn^{2+} ion is equal to $k_{cat}/(k + 1)$ (Dalziel, 1969) and is *not* a dissociation constant but is independent of the UDP-galactose concentration as in fact observed.

From studies using alternate metal ions, it is apparent that Zn^{2+} and Co^{2+} can both bind to site I in a manner similar to that of Mn^{2+}. The K_m values of all three metals binding to site I are independent of UDP-galactose concentration and all appear to support activity, though to differing extents in the absence of metal binding to site II. Once a metal has bound to site I, a variety of metals can bind to site II, causing either activation or inhibition.

Of the metals which bind to site I, Zn^{2+}, Co^{2+}, and Mn^{2+} also bind to site II. However, Mn^{2+} binding to site II results in activation, whereas Zn^{2+} or Co^{2+} binding results in an inhibition. In addition to Mn^{2+}, four other metals, Ca^{2+}, Al^{3+}, Eu^{3+}, and Tb^{3+}, all cause activation on binding to site II, though as shown in Table III Mn^{2+} activates to a much greater extent. Although these five metals are similar in causing activation on binding to site II, they can be subdivided into two groups, one containing Mn^{2+}, Ca^{2+}, and Al^{3+} which do not inhibit at high concentrations and a second containing the two lanthanides which cause inhibition at high concentrations (>1 mM). Of these metals, Mn^{2+} is the only one which can support activity by binding to site I alone. It may be that at high concentrations, the lanthanides can displace Mn^{2+} from site I without supporting activity themselves, giving the observed inhibition at high lanthanide concentrations.

The observations of the behavior of galactosyltransferase on the two affinity resins UDP-Sepharose and α-lactalbumin–Sepharose are entirely consistent with the deductions based on the kinetic experiments reported here. In the absence of any metal ion, the transferase does not interact with either resin. Calcium alone does not allow an interaction to occur, consistent with the observation that calcium alone does not support activity. Significantly, quite different elution from α-lactalbumin–Sepharose occurs when *N*-acetylglucosamine is removed from the wash buffer, depending on the Mn^{2+} concentration. At low Mn^{2+} concentrations (0.1 mM) where only site I would be expected to be saturated with Mn^{2+}, the transferase is bound to the resin more tightly (i.e., elutes at a later point) than if both metal sites are saturated with Mn^{2+} (25 mM).

The results presented here and the forgoing discussion suggest that metal binding to site I alone is sufficient to support catalysis and that binding a second metal (to site II) causes either an activation or an inhibition. The variety of metals which can support activity on binding to site I, the lack of effect of UDP-galactose on site I binding, and the requirement for metal to bind to site I prior to other substrates or a second metal binding suggest that the site I–metal interaction may be involved in maintaining the structural integrity of either the catalytic site or the protein itself, rather than playing a direct role in the chemical mechanism of the reaction or in substrate binding. However, the second metal binding site appears to be associated intimately with the nucleotide sugar donor binding site.

While the results reported here concerning the activation effects of calcium on galactosyltransferase confirm those reported by Powell & Brew (1976), the kinetic studies with manganese alone differ substantially from those of Powell and Brew. As shown here, when apoenzyme is prepared without using EDTA, the enzyme responds to increasing manganese concentrations with a saturation curve indicating two classes of sites. Powell and Brew, on the other hand, found sigmoidal saturation with respect to manganese, which suggests either positive cooperativity between metal sites in an allosteric manner or an absolute dependence on more than one metal ion in the catalytic mechanism. As shown by the kinetic studies reported here, neither of these alternatives can apply. Rather, since Powell and Brew prepared their apoenzyme by using EDTA, which is shown in the work described here to form an enzyme–metal–EDTA complex under the conditions used by Powell and Brew, it is probable that the sigmoidal saturation curves reported by these workers are due to a small amount of EDTA remaining which would indeed generate a sigmoidal saturation curve.

The chemical modification studies reported here with the fluorescent sulfhydryl reagent *S*-mercuric-*N*-dansylcysteine are in general agreement with studies reported with other sulfhydryl reagents such as *N*-ethylmaleimide (Kitchen & Andrews, 1974) or *p*-(hydroxymercuri)benzoate (Magee & Ebner, 1974). With all of the sulfhydryl reagents previously studied and that reported here, only UDP-galactose or analogues of the sugar nucleotide substrate appear to offer any protection against inactivation. Unlike the studies reported earlier using *p*-(hydroxymercuri)benzoate or *N*-ethylmaleimide where 90–95% inactivation was observed, the maximal inactivation with *S*-mercuric-*N*-dansylcysteine is only ~60%. That this is indeed the maximal inactivation with this reagent is indicated by the observation that similar extents of inactivation, though not rates of inactivation, are obtained with three different concentrations of *S*-mercuric-*N*-dansylcysteine.

Characterization of the extent of modification of the maximally inactivated transferase was achieved by making use of the lability of the dansyl-enzyme to β-mercaptoethanol. The stability of the derivative obtained by inactivation with *N*-ethylmaleimide is not reversed by incubation with β-mercaptoethanol, whereas inactivation by *p*-(hydroxymercuri)benzoate is completely reversed by β-mercaptoethanol.

In the experiments reported here, it is shown that reactivation of the *S*-mercuric-*N*-dansylcysteine-modified transferase by β-mercaptoethanol is accompanied by the complete removal

of the fluorescent label from the transferase and complete recovery of activity. The switch-labeling procedure used here, using cold *N*-ethylmaleimide to block any possible sites of reaction with *N*-ethylmaleimide prior to removal of the fluorescent label, followed by incubation with ^{14}C-labeled *N*-ethylmaleimide to introduce a radioactive label into the sites of *S*-mercuric-*N*-dansylcysteine modification clearly indicates that only 1 mol of *S*-mercuric-*N*-dansylcysteine is introduced per mol of transferase. The control experiments described with *N*-ethylmaleimide indicate that in the colostral enzyme there are three sulfhydryl groups that can be modified by extensive treatment with *N*-ethylmaleimide. Since amino acid analyses of the colostral enzyme indicate a total of 3 sulfhydryl groups/mol, it would appear that all are accessible to *N*-ethylmaleimide. In accord with previous work (Magee & Ebner, 1974; Kitchen & Andrews, 1974), only a very small residual activity (2–5%) was observed after extensive modification by *N*-ethylmaleimide. Since a maximum of only 1 mol of dansyl reagent could be incorporated per mol of transferase it appears that only one of the three sulfhydryl groups is susceptible to modification by *S*-mercuric-*N*-dansylcysteine. In view of the kinetics of modification observed, it is unlikely that the total of 1 dansyl incorporated per mol of enzyme is the result of partial modification of 2 or 3 sulfhydryl groups fortuitously giving a maximum of 1 mol modification.

The spectral properties of the modified enzyme also suggest that a single, unique sulfhydryl group has been fluorescently labeled by reaction with *S*-mercuric-*N*-dansylcysteine. If two or more sulfhydryl residues had been partially modified by reagent, the emission spectral ratios representing the shape of the emission spectrum for the dansyl fluorescence would most probably have changed as the excitation wavelength was changed. The fact that no such change is observed indicates that the introduced fluorophores are in identical environments, suggesting a unique modification of a single sulfhydryl residue. The likelihood of two different sulfhydryl residues each being partially modified giving derivatives with identical fluorescent properties is extremely small. The large blue shift compared to the free label suggests that the fluorophore is in a highly hydrophobic environment on the protein. The observation of an excitation maximum at 285 nm indicates that excitation energy absorbed by protein tryptophan residues is transferred by resonance energy transfer to the introduced dansyl label. The lack of spectral changes upon addition of Mn^{2+} or *N*-acetylglucosamine to the modified protein suggests that, while complexes such as enzyme–Mn^{2+} and enzyme–Mn^{2+}–*N*-acetylglucosamine are known to occur (Bell et al., 1976), the introduced dansyl group is not sensitive to either their formation or conformational changes that *may* occur in the protein as a result of their formation. However, the changes in emission maximum and intensity induced by addition of UDP suggest that the introduced fluorophore either is located near the nucleotide binding site or is sensitive to conformational changes induced by nucleotide binding.

From the quenching of either the dansyl group fluorescence or the europium fluorescence by cobalt, distances between the cobalt, in metal site I, and either the dansyl group or europium, in metal site II, can be calculated from eq 2. For these calculations, the critical distance R_0 for energy transfer of the appropriate donor–acceptor couple must be obtained from eq 3. In these studies, the overlap integral, J, has been obtained graphically from the emission spectrum of the donor fluorescence and the absorption spectrum of the acceptor. From the absorption spectrum of galactosyltransferase–cobalt and free cobalt (not shown), a molar extinction coefficient at 530 nm of enzyme-bound cobalt of ~ 140 cm^{-1} M^{-1} has been calculated. The quantum yield of the dansylated transferase was found to be 0.11, relative to a value of 0.06 for dansylglycine (Chen, 1967). By use of these data, a value of 23 Å is calculated for R_0 for the dansyl–cobalt couple if the refractive index is taken as 1.33 (Horrocks et al., 1975) and the orientation factor K^2 is assumed to be $^2/_3$. Because of the sixth power dependence of R_0 on these factors, small variations in them will have negligible effects on the value of R_0. Similar calculations for the europium–cobalt couple give a value of R_0 of 20 Å.

From these values of R_0 and the quenching of donor fluorescence (either dansyl or europium) by cobalt, a distance of 19 Å can be calculated between the cobalt binding site (metal site I) and the dansyl group attached to the sulfhydryl residue. Similarly, a distance of 18 Å is calculated between the two metal binding sites. The major uncertainty in these estimates is the precise value of the orientation factor K^2. Theoretical limits for K^2 are 0 and 4.0. The value usually used is $^2/_3$ which assumes a random orientation of chromophores. Changing the value of K^2 to 0.3 or 1.5 gives values of R_0 of 21.4 and 28 Å, respectively. Where little is known about the orientation of the chromophores, it would seem reasonable to put limits of ±3 Å on distances estimated by using $^2/_3$ as K^2.

The energy transfer experiments reported here thus give distances of 18 Å between metal sites I and II and 19 Å between site I and a fluorophore attached to a sulfhydryl group in or near the UDP-galactose binding site. The distance between the two metal sites is certainly consistent with the suggestion discussed earlier that only one of the metal sites (site II) is located in or near the substrate binding sites, while the other (site I) is a structural site. The fluorescence studies with europium have further significance to this suggestion. It was suggested on the basis of kinetic studies that metal must bind to site I prior to substrates binding and prior to metal binding to site II. The observation that the enhancement of Eu^{3+} fluorescence occurs only in the presence of sufficient Mn^{2+} (or other site I metals) to significantly saturate site I confirms this suggestion.

In both sets of experiments with cobalt reported here, similar "dissociation constants" for cobalt can be estimated from Figures 3 and 5B of ~ 15 μM. These values are somewhat smaller than the K_m for cobalt of 68 μM. However, the K_m for the first metal bound is $K_{cat}/(R + 1)$ and is not a dissociation constant. In fact, since K_{cat} is quite probably larger than K_{-1} for metal binding, K_m should be greater than K_D.

The chemical modification and fluorescence studies with the *S*-mercuric-*N*-dansylcysteine-modified enzyme strongly suggest that the modified sulfhydryl is located near the nucleotide substrate binding site and that this sulfhydryl is located some 19 Å from metal site I. This observation is also consistent with the previous suggestion, and the above calculations for the distance between the two metal sites, that metal site II is associated with the nucleotide substrate binding site.

References

Barker, R., Olsen, K. W., Shaper, H. H., & Hill, R. L. (1972) *J. Biol. Chem. 247*, 7135–7147.

Bell, J. E., Beyer, T. A., & Hill, R. L. (1976) *J. Biol. Chem. 251*, 3003–3014.

Berliner, L. J., & Wong, S. S. (1975) *Biochemistry 14*, 4977–4982.

Brew, K., & Hill, R. L. (1975) *Rev. Physiol., Biochem. Pharmacol. 72*, 105–158.

Chen, R. F. (1967) *Arch. Biochem. Biophys. 120*, 609.

Dalziel, K. (1969) *Biochem. J. 114*, 547–556.
Draper, N. R., & Smith, H. (1968) *Applied Regression Analysis*, Wiley, New York, London, and Sydney.
Dwek, R. A. (1973) *Nuclear Magnetic Resonance (NMR) in Biochemistry*, Clarendon Press, Oxford, England.
Eisinger, J. (1974) *J. Mol. Biol. 84*, 643–647.
Hill, R. L., & Brew, K. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 411–490.
Horrocks, W. D., Holmquist, B., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4764–4768.
Kitchen, B. J., & Andrews, P. (1974) *Biochem. J. 143*, 587–590.
Leavis, P. C., & Lehrer, S. S. (1974) *Biochemistry 13*, 3042–3048.
Macfarlane, N., & Ainsworth, S. (1972) *Biochem. J. 129*, 1035–1047.
Magee, S. C., & Ebner, K. E. (1974) *J. Biol. Chem. 249*, 6992–6998.
Powell, J. T., & Brew, K. (1976) *J. Biol. Chem. 251*, 3654–3652.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Trayer, I. P., & Hill, R. L. (1971) *J. Biol. Chem. 246*, 6666–6675.
Weber, G. (1960) *Biochem. J. 75*, 335–345.

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).

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

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