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Ultraviolet Photoinactivation of Galactosyltransferase. Protection by Substrates†

David J. Clymer, Collis R. Geren, and Kurt E. Ebner*

ABSTRACT: Galactosyltransferase was irreversibly inactivated upon exposure to ultraviolet light and the rate of inactivation followed apparent first-order kinetics. Significant protection against inactivation was observed in the presence of various combinations of substrates. UDPgalactose and Mn^{2+} together gave the most protection. Amino acid analyses revealed the loss of 1 mol of tryptophan per mol of galactosyltransferase upon ultraviolet photoinactivation. Further evidence for an essential tryptophan was provided by difference spectra and by inactivation with 2-hydroxy-5-nitrobenzyl bromide and protection against this reagent by Mn^{2+} and UDPgalactose. The protection by UDPgalactose and Mn^{2+} was greater than that provided by UDPgalactose alone. Since Mn^{2+} provided no protection by itself, this suggested that the formation of the galactosyltransferase–Mn^{2+}–UDPgalactose complex caused a conformational change which was responsible for the observed protection of the essential tryptophanyl residue.

Bovine skim milk galactosyltransferase (UDPgalactose: D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the transfer of galactose from UDPgalactose, forming β-1,4 linkages with glucose, GlcNAc,[1] or terminal GlcNAc groups of protein-bound β-glycosides (Brew et al., 1968; Schanbacher and Ebner, 1970; Fitzgerald et al., 1970a; Morrison and Ebner, 1971a). α-Lactalbumin is required to obtain significant rates in the reaction when glucose is the galactosyl acceptor, but inhibits the reaction when GlcNAc is the galactosyl acceptor (Schanbacher and Ebner, 1970; Morrison and Ebner, 1971a,b). In lactating mammary tissue, where α-lactalbumin is present in a significant concentration, the principal physiological role of galactosyltransferase is the production of lactose (Watkins and Hassid, 1962). Elsewhere, it is involved in glycoprotein biosynthesis (Schachter et al., 1970).

Based on the results of initial velocity and dead-end inhibition studies with GlcNAc as the galactosyl acceptor, Morrison and Ebner (1971c) have proposed an ordered mechanism for bovine milk galactosyltransferase with reactants adding in the order: Mn^{2+}, UDPgalactose, and GlcNAc. A further conclusion was that Mn^{2+} reacted with the free enzyme under conditions of thermodynamic equilibrium and did not dissociate after each turn of the catalytic cycle.

Magee and Ebner (1974) have demonstrated the existence of a critical sulfhydryl residue in galactosyltransferase by the use of sulfhydryl reagents which caused a distinct

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[1] Abbreviations used: GlcNAc, *N*-acetylglucosamine.

but incomplete loss of enzymatic activity. Further, they showed that inactivation of the enzyme by sulfhydryl reagents or proteolytic enzymes was prevented by the presence of substrates and suggested that this protection was due to conformational changes in the protein. The existence of such conformational changes upon substrate binding was demonstrated by circular dichroism measurements (Geren et al., 1975a).

The partial or total inactivation of enzymes and other proteins by irradiation with ultraviolet light has been documented in a number of reports (Dunlop, 1966; Katzenellenbogen et al., 1975; Vladimirov et al., 1970; Burridge and Churchich, 1970; Imahori, 1955; Forbes and Savige, 1962; Kenyon and Blois, 1965) and the possibility of the photodegradation of several amino acid residues such as tryptophan and tyrosine has been indicated. The present paper describes the inactivation of bovine milk galactosyltransferase by ultraviolet and visible light and protection against inactivation by substrates. A possible essential role for a tryptophan in galactosyltransferase was indicated by amino acid analyses, difference spectroscopy, fluorometry before and after photoinactivation, and inactivation by 2-hydroxy-5-nitrobenzyl bromide.

Experimental Procedure

Materials. Pyruvate kinase (type I containing 30 units of lactate dehydrogenase per mg of protein), NADH, phosphoenolpyruvate, N-acetylglucosamine, UDP, and 2-mercaptoethanol were obtained from Sigma Chemical Company. UDPgalactose was purchased from Calibiochem, 2-hydroxy-5-nitrobenzyl bromide from Nutritional Biochemicals Corp., methylene blue from Wilson Diagnostic, Inc., reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis from Bio-Rad, and guanidine hydrochloride from Schwarz/Mann. All other reagents were of analytical reagent quality. Galactosyltransferase was isolated from bovine skim milk by the procedure of Geren et al. (1975b). Stock solutions (1 mg/ml; specific activity 5–6 units/mg) of galactosyltransferase in 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5, were stored at −20 °C. Enzymic solutions were thawed and diluted with 20 mM Tris-HCl, 1 mM mercaptoethanol as required for each series of experiments immediately before use. Repeated freezing and thawing of enzymic solutions were avoided.

Methods. Galactosyltransferase activity was determined by the spectrophotometric assay of Fitzgerald et al. (1970b) using a Gilford Model 2400-2 automatic recording spectrophotometer. GlcNAc was used as the galactosyl acceptor and a unit was defined as that amount of enzyme which catalyzes the production of 1 μmol of *N*-acetyllactosamine per min at 24 °C.

Samples to be irradiated with ultraviolet light were placed in quartz cuvettes (2-ml volume, 1-cm light path) within a Rayonette Photochemical Reactor, catalog No. RPR-100, with 2537-Å lamps. Care was taken to ensure the reproducibility of cuvette placement within the reactor by scribing the outline of the cuvette base on the sample platform within the reactor. The desired light intensity was obtained by regulating the number of lamps actively discharging within the reactor chamber. Lamps were arranged around the sample cuvette in a symmetrical manner. Unless otherwise noted, all samples were irradiated by two actively discharging lamps. Samples to be irradiated with visible light were placed in 5-ml glass tubes (1-cm light path) within a cylinder containing either water or a 40% $CuSO_4$ solution 25 cm from a Sylvania flood lamp (150 W, 120 V). The solution around the sample tubes was circulated using a magnetic stirrer which helped maintain constant temperature during irradiation. The 40% $CuSO_4$ solution was used as an ultraviolet filter, absorbing all radiation having wavelengths shorter than 312 nm (Singh et al., 1962). In several experiments 0.001% w/v methylene blue was employed as a photosensitizer. With all irradiations the temperature of the exposed solutions was monitored, and appropriate controls were included to determine activity loss due to temperature alone.

Difference spectra were determined with a Cary 14 spectrophotometer. Fluorescence spectra were obtained with an Aminco-Bowman spectrofluorometer. An inactivation profile due to wavelength of incident light was determined by use of the Xenon Aminco-Bowman light source and monochromator (5-mm slits) with temperature maintained at 32 °C. Aliquots of galactosyltransferase were irradiated for 10 min at various wavelengths and enzymatic activity of the exposed samples was determined. Corrections were made for changes in intensity of the lamp at different wavelengths using the specifications supplied by the lamp manufacturer.

Samples for amino acid analysis were dialyzed against excess deionized distilled water, lyophilized and dissolved in constant boiling HCl (1 mg/ml). Hydrolysis was accomplished in evacuated, sealed glass tubes at 106 °C for 24 h. Duplicate assays of each sample were determined on a Beckman Model 121 automatic amino acid analyzer according to the method of Moore and Stein (1957). Tryptophan was determined by the spectrophotometric assay of Edelhoch (1967) as revised by Bredderman (1974). Tryptophan was also determined by the method of Liu and Chang (1971) which uses *p*-toluenesulfonic acid hydrolysis followed by analysis on the automatic analyzer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done as described by Magee et al. (1973), and gels were scanned with a Gilford 2400-2 spectrophotometer with a Model 2410 gel scanning attachment.

The effect on galactosyltransferase activity of 2-hydroxy-5-nitrobenzyl bromide as a fairly tryptophan specific inactivating agent (Koshland et al., 1964; Horton and Koshland, 1965) was investigated. A stock solution of 10 mg/ml of 2-hydroxy-5-nitrobenzyl bromide in absolute methanol was freshly prepared before each experiment. An aliquot of such a solution was added to galactosyltransferase in 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5, shaken vigorously, and allowed to incubate for 5 min to effectively hydrolyze the unreacted reagent. The activity of the modified galactosyltransferase was compared with that of a control which had been treated in the same manner with only absolute methanol.

Results

Galactosyltransferase exhibited a rapid, time-dependent loss of enzymatic activity upon exposure to ultraviolet light. The rate of inactivation was intensity dependent, and the process followed apparent first-order kinetics throughout more than 99% inactivation. The apparent first-order rate constants of inactivation with two, three, and five lamps actively discharging in the Rayonette reactor were 0.092 ± 0.004, 0.171 ± 0.001, and 0.244 ± 0.006 min^{-1}, respectively. The rate constants were determined by a least-square fit and the errors are expressed as the standard deviation. The rate of inactivation was not appreciably affected by varying the mercaptoethanol concentration of the enzymic solution

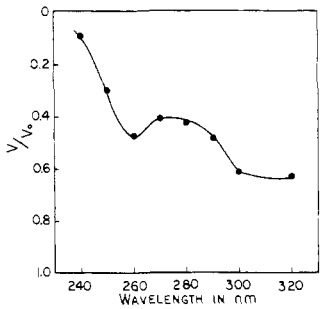

FIGURE 1: The effect of wavelength of incident light on rate of photoinactivation of galactosyltransferase. Separate samples of 0.89 unit/ml galactosyltransferase were exposed to light of the indicated wavelength for 10 min (see Experimental Procedure for detail) and residual enzymatic activity was determined. V_0 was the activity of enzyme incubated at 32 °C for 10 min without irradiation, while V was the activity after irradiation with light of the indicated wavelength.

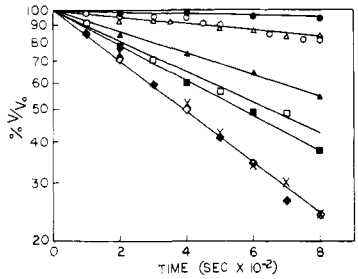

FIGURE 2: Ultraviolet photoinactivation of galactosyltransferase in the presence of substrates. Galactosyltransferase, 0.35 unit/ml in 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5, containing various combinations of substrates was exposed to ultraviolet light in the Rayonette reactor with two functional lamps. The symbols represent galactosyltransferase irradiated in the presence of: no substrates (◊); 2 mM Mn^{2+} (X); 20 mM GlcNAc (♦); 2 mM Mn^{2+} and 20 mM GlcNAc (■); 1 mM UDPgalactose (□); 1 mM UDPgalactose and 20 mM GlcNAc (▲); 1 mM UDPgalactose, 2 mM Mn^{2+}, and 20 mM GlcNAc (△); and 1 mM UDPgalactose and 2 mM Mn^{2+} (○). A 32 °C nonirradiated control is represented by ●. All substrate concentrations are those used in routine galactosyltransferase assay.

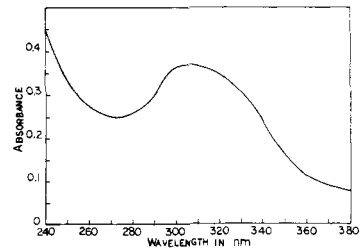

FIGURE 3: Difference spectrum between irradiated and nonirradiated galactosyltransferase. Half of a 0.86 mg/ml galactosyltransferase solution in 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5, was exposed to ultraviolet light for 1 h in the Rayonette reactor with two operating lamps (7% residual activity), while the other half was incubated at 32 °C for 1 h (97% residual activity). The difference spectrum was recorded with a Cary 14 spectrophotometer with the temperature control in the reference cell and the irradiated enzyme in the sample cell.

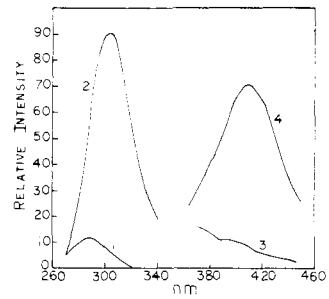

FIGURE 4: Fluorescence spectra of irradiated and nonirradiated galactosyltransferase. A galactosyltransferase solution, 0.14 mg/ml in 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5, was divided into two equal aliquots. One aliquot was inactivated with ultraviolet light, while the other was incubated at the same temperature as that of the sample being irradiated for the duration of exposure. Uncorrected spectra obtained with an Aminco-Bowman spectrofluorometer include control excitation (1), irradiated excitation (2), control emission (3), and irradiated emission (4). The excitation and emission λ_{max} for the irradiated enzyme were 305 and 410 nm respectively. The temperature control enzyme spectra were obtained using these same wavelengths.

from 0 to 10 mM. The effect of wavelength of incident light on rate of photoinactivation is illustrated by Figure 1. No recovery of activity after photoinactivation was observed with prolonged incubation in the absence or presence of substrates. No further time-dependent loss of activity occurred in partially ultraviolet photoinactivated galactosyltransferase samples after removal from the Rayonette reactor.

Significant protection of the enzyme against ultraviolet inactivation was observed in the presence of certain combinations of substrates. As Figure 2 illustrates, the presence of 2 mM Mn^{2+} and 1 mM UDPgalactose gave excellent protection. A similar rate of inactivation was obtained with 2 mM Mn^{2+}, 1 mM UDPgalactose and 20 mM GlcNAc. The combinations of 1 mM UDPgalactose and 20 mM GlcNAc, 2 mM Mn^{2+} and 20 mM GlcNAc, and 1 mM UDPgalactose alone gave some degree of protection. Galactosyltransferase was inactivated at the same apparent rate as enzyme and 2 mM Mn^{2+}, and enzyme and 20 mM GlcNAc. Protection of the galactosyltransferase-Mn^{2+} complex by UDPgalactose was a function of UDPgalactose concentration. A dissociation constant of 0.101 ± 0.005 mM for UDPgalactose from the enzyme-Mn^{2+}-UDPgalactose complex was determined as described by Magee and Ebner (1974). The combination of 1 mM UDP and 2 mM Mn^{2+} also gave excellent protection against ultraviolet photoinactivation of galactosyltransferase. A K_d of 0.299 ± 0.005 mM was determined for dissociation of UDP from the enzyme-Mn^{2+}-UDP complex.

Photoinactivation was observed with visible light irradiation, and the rate of inactivation was enhanced by the presence of 0.001% methylene blue, a photosensitizer. The apparent first-order rate constant for inactivation of galactosyltransferase in the absence of methylene blue was 0.16 ± 0.01 min^{-1} while in the presence of this reagent a value of 0.61 ± 0.05 min^{-1} was obtained. Use of the 40% $CuSO_4$ solution as a uv filter reduced the rate of inactivation, but the process continued at a significant rate. The reduction in rate was probably due to decreased intensity of incident light. Inclusion of 5 mM 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, an anti-oxidant, had no effect upon the visible light inactivation of galactosyltransferase. The halftime of inactivation was increased approximately threefold by the presence of 1 mM UDPgalactose and 2 mM Mn^{2+}

Comparison of the sodium dodecyl sulfate gel electrophoresis patterns of ultraviolet photoinactivated (7% residual activity) and control galactosyltransferase revealed no changes in apparent molecular weights. As shown by the difference spectra in Figure 3, a significant change in the absorbance of galactosyltransferase with a maximum at 300–310 nm occurred upon ultraviolet photoinactivation. The inactivated enzyme also exhibited a changed fluores-

Table I: Ultraviolet Photoinactivation of Galactosyltransferase.[a]

Irradiation Time (h)	Activity (%)	Residues per 51 000 Molecular Weight[b] Methionine	Half-Cystine	Tryptophan	Tyrosine	Phenylalanine	Histidine
0	100	6.9	4.6	4.1	15.4	14.4	8.4
1	7	6.9	4.6	3.1	15.4	14.1	8.4

[a] Three milliliters of 1.18 mg/ml of galactosyltransferase in 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5, was separated into 2- and 1-ml aliquots. The 2-ml sample was irradiated with ultraviolet light in the Rayonette reactor as described in Experimental Procedure. The 1-ml sample was incubated in room light for 1 h at the same temperature (32 °C) as the sample being irradiated and is represented by the 0 irradiation time above. Amino acid analyses were performed in duplicate as described in Experimental Procedure. The total amino acid composition of active galactosyltransferase was similar to that reported by Trayer and Hill (1971). [b] Based on sodium dodecyl sulfate gel electrophoresis, the galactosyltransferase used in the present experiments contained approximately equal amounts of the high (58 000) and low (44 000) molecular weight forms reported by Magee et al. (1973). Lehman et al. (1975) reported that such a mixture of galactosyltransferase contained 13.8% carbohydrate. The values in the table were based on an average molecular weight of 51 000 of which 13.8% was assumed to be carbohydrate.

Table II: Inactivation of Galactosyltransferase by 2-Hydroxy-5-nitrobenzyl Bromide. Protection by Substrates.

Substrates Added[a]	Inhibition[b] (%)
None	49.4
$MnCl_2$	46.0
GlcNAc	46.1
UDPgalactose	27.3
$MnCl_2$ + GlcNAc	35.2
$MnCl_2$ + UDPgalactose	24.7
$MnCl_2$ + UDPgalactose + GlcNAc	24.7

[a] The concentration of $MnCl_2$ was 2 mM, GlcNAc was 20 mM, and UDPgalactose was 1 mM. [b] The percentage inhibition was measured after a 5-min incubation at 24 °C with 0.85 mM 2-hydroxy-5-nitrobenzyl bromide. The buffer was 20 mM Tris-HCl, 1 mM mercaptoethanol, pH 7.5. The control consisted of 100 μl of the same enzyme solution (0.14 mg/ml) treated with a volume of anhydrous methanol equal to that used to add the 2-hydroxy-5-nitrobenzyl bromide (2 μl). This control retained 93% of the activity of untreated enzyme.

cence spectrum with a new excitation λ_{max} of 305 nm and an emission λ_{max} of 410 nm (Figure 4). The excitation λ_{max} corresponds well with the area of maximum change in the difference spectrum. Comparison of the amino acid compositions of ultraviolet photoinactivated and control galactosyltransferase demonstrated a decrease of approximately 1 mol of tryptophan per mol of enzyme. No change was observed in the content of any other residue (Table I).

Galactosyltransferase was also inactivated by 2-hydroxy-5-nitrobenzyl bromide, and the amount of inactivation was proportional to the amount of reagent used. The enzyme (0.14 mg/ml) was totally inactivated when the 2-hydroxy-5-nitrobenzyl bromide concentration was greater than 2.4 mM. As shown by Table II, inactivation by this compound could be hindered by the presence of Mn^{2+} and UDPgalactose.

Discussion

Bovine milk galactosyltransferase is rapidly and irreversibly inactivated upon exposure to ultraviolet light and the inactivation reaction approximates first-order kinetics. The rate of inactivation is dependent upon intensity and wavelength of incident light. The presence of Mn^{2+} alone gives no protection against photoinactivation while the presence of Mn^{2+} and UDPgalactose provides maximal protection. The addition of GlcNAc to the enzyme-Mn^{2+}-UDPgalactose complex gives no increase in protection. These observations are consistent with the substrate protection against inactivation of galactosyltransferase by sulfhydryl reagents and trypsin observed by Magee and Ebner (1974).

The comparison of amino acid analyses of active and photoinactivated galactosyltransferase (Table I) indicates the loss of a single tryptophan residue per molecule of enzyme upon inactivation. The difference spectra (Figure 3) are also indicative of tryptophan destruction (Dunlop, 1966). The inactivation by 2-hydroxy-5-nitrobenzyl bromide and protection against this reagent by substrates provide further evidence for an essential tryptophan.

The protection of the enzyme against ultraviolet photoinactivation by UDPgalactose alone may be due to the absorption of light by UDPgalactose which has an absorption maximum at 262 nm. The Rayonette reactor was equipped with 254-nm output lamps. However, this does not explain the enhanced protection by Mn^{2+} and UDPgalactose since Mn^{2+} alone does not protect. An explanation for the observed results is that the formation of the enzyme-Mn^{2+}-UDPgalactose complex causes a conformation change, and that this conformation hinders the ultraviolet light induced destruction of the particular tryptophanyl residue. Geren et al. (1975a) have reported changes in the circular dichroism spectra of galactosyltransferase upon formation of the enzyme-Mn^{2+}-UDPgalactose complex which correspond to tyrosine and tryptophan perturbations. The present results indicate that a tryptophan could have an essential role in the assumption of the correct catalytic conformation. The partial protection of galactosyltransferase against ultraviolet photoinactivation by Mn^{2+} and GlcNAc, and UDPgalactose and GlcNAc could be due to an incomplete conformation change. Barker et al. (1972) have shown that Mn^{2+} greatly enhances the binding of galactosyltransferase to GlcNAc-Sepharose which would indicate the formation of an enzyme-Mn^{2+}-GlcNAc complex. The inactivation reaction induced by visible light in the presence of methylene blue appears similar to that induced by ultraviolet light, as protection by the various combinations of substrates closely approximates that obtained with ultraviolet light.

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Characterization of a Cytochrome P-450 Dependent Monoterpene Hydroxylase from the Higher Plant *Vinca rosea*†

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ABSTRACT: A monooxygenase isolated from 5-day old etiolated *Vinca rosea* seedlings was shown to catalyze the hydroxylation of the monoterpene alcohols, geraniol and nerol, to their corresponding 10-hydroxy derivatives. Hydroxylase activity was dependent upon NADPH (neither NADH nor combination of NADH, $NADP^+$, and ATP served as substitutes) and O_2. Geraniol hydroxylation was enhanced by dithiothreitol (monothiols were less effective) and inhibited by phospholipases, thiol reagents, metyrapone, and cytochrome *c*, as well as other inhibitors of cytochrome P-450 systems. Geraniol was hydroxylated at a faster rate than nerol, but the alcohols possessed similar apparent K_m values. The membrane-bound hydroxylase was solubilized by treatment with sodium cholate, Renex-30, or Lubrol-WX. Cholate-treated enzyme was resolved by DEAE-cellulose chromatography and reconstitution of the hydroxylase was effected utilizing different fractions containing cytochrome P-450, a NADPH-cytochrome *c* reductase, and lipid.

The biological conversion of the acyclic monoterpene alcohols, geraniol and its cis isomer, nerol, to cyclopentano derivatives involves allylic oxygenation, dehydrogenation, and cyclization (Scheme I) (Banthorpe et al., 1972). In the higher plant, *Vinca rosea,* this metabolic sequence represents the beginning of indole alkaloid biosynthesis, and one of the initial steps is hydroxylation at the C-10 methyl group to afford the diols, 10-hydroxygeraniol and 10-hydroxynerol (Escher et al., 1970; Battersby et al., 1970). A NADPH-dependent monooxygenase found in crude extracts of *V. rosea* catalyzes this hydroxylation (Meehan and Coscia, 1973), and this enzyme has been identified as one of a growing number of cytochrome P-450 dependent oxygenases which have been discovered in higher plants (Cotte-

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