

Reversible Effect of Bicarbonate on the Inhibition of Mycobacterial and Yeast Transglucosylases by Mycoribnin¹

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ABSTRACT

LORNITZO, FRANK A. (Veterans Administration Hospital, Madison, Wis.), AND DEXTER S. GOLDMAN. Reversible effect of bicarbonate on the inhibition of mycobacterial and yeast transglucosylases by mycoribnin. *J. Bacteriol.* **89**:1086-1091. 1965.—The transglucosylase which catalyzes the formation of trehalose-6-phosphate from uridine diphosphate (UDP)-glucose and glucose-6-phosphate was purified from cell-free extracts of *Mycobacterium tuberculosis* H37Ra. During the purification procedure, the transglucosylase loses its sensitivity to mycoribnin, an inhibitor also found in these extracts. Sensitivity of the transglucosylase to mycoribnin is regained when the bicarbonate concentration of either enzyme or mycoribnin preparations is reduced to about 0.01 mM; sensitivity to mycoribnin is lost when low concentrations (< 1 mM) of bicarbonate are present in the reaction mixture. Transglucosylase preparations retain their bicarbonate-induced insensitivity to mycoribnin after dilution to a bicarbonate concentration which is ineffective for the initial conversion to insensitivity. The transglucosylases of brewer's yeast and of physiologically young (9-day) H37Ra cells, previously reported as insensitive to mycoribnin, have been partially purified. If bicarbonate is excluded from these preparations, the transglucosylases become sensitive to mycoribnin; bicarbonate abolishes this sensitivity. The H37Ra transglucosylase is specific for UDP-glucose and glucose-6-phosphate as substrates. UDP-galactose does not serve as a glycosyl donor; galactose-6-phosphate, ribose-5-phosphate, and glucose-1-phosphate do not act as glycosyl acceptors. Oligoribonucleotide analogues of mycoribnin do not inhibit substantially the H37Ra transglucosylase.

Cell-free extracts of the H37Ra strain of *Mycobacterium tuberculosis* contain a transglucosylase which catalyzes the formation of trehalose-6-phosphate from glucose-6-phosphate and uridine diphosphate (UDP)-glucose (Cabib and Leloir, 1958; Goldman and Lornitzo, 1962). A noncompetitive inhibitor of this transglucosylase is also found in these cell-free extracts (Goldman and Lornitzo, 1962). This inhibitor has been purified and shown to be an oligoribonucleotide containing adenine and guanine (Lornitzo and Goldman, 1964). The trivial name mycoribnin has been assigned to this inhibitor.

Several observations made it apparent that we were dealing with a system more complex than at first suspected. Goldman and Lornitzo (1962) reported that the transglucosylase present in extracts of physiologically young cultures of the

H37Ra strain and the transglucosylase of brewer's yeast were not sensitive to mycoribnin. In addition, as the transglucosylase of H37Ra was purified, it progressively lost its sensitivity to mycoribnin, although we observed that inhibitory activity could be enhanced by the addition of Co^{++} .

This report deals with the purification and properties of the transglucosylase of H37Ra, and also presents evidence that bicarbonate is responsible for the variable sensitivity to mycoribnin of the H37Ra and yeast transglucosylases. The mycoribnin-sensitive (or -insensitive) enzyme was reversibly converted to the insensitive (or sensitive) form; a possible mechanism for this conversion is presented.

MATERIALS AND METHODS

Reagents and assays. 1,2,3,4,6-Pentaacetyl- β -D-galactose (glucose-free) was obtained from Sigma Chemical Co., St. Louis, Mo. UDP-galac-

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tose was prepared through galactose-1-phosphate (Hansen, Rutter, and Krichevsky, 1955) by the method of Roseman et al. (1961). The product isolated as the dilithium salt was free from UDP-glucose. Ribonuclease (five times crystallized, pancreatic) was obtained from Sigma Chemical Co. The source of other reagents was given previously (Goldman and Lornitzo, 1962).

The one-step (directly coupled) transglucosylase assay procedure was carried out as described previously (Goldman and Lornitzo, 1962), except that in experiments with either purified mycoribin or other oligoribonucleotides the final magnesium content was reduced from 16 to 3.3 mM. The determination of trehalose-6-phosphate in the presence of other disaccharides and monosaccharides was carried out essentially as described by Goldman and Lornitzo (1962), with the following exceptions. Trehalose-6-phosphate was dephosphorylated with a phosphomonoesterase from *Escherichia coli* (the gift of Robert M. Bock of the University of Wisconsin). Also, the glucose analysis was based upon ADP formation in a glucose-adenosine triphosphate (ATP)-hexokinase (Type III hexokinase, Sigma Chemical Co.) system. (Control reactions with glucose and trehalose-6-phosphate confirmed the adequacy of this method.)

The two-step transglucosylase assay procedure (Goldman and Lornitzo, 1962) was replaced by the following procedure. Each reaction mixture contained, in a total volume of 0.10 ml, 0.96 μ mole of tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 6.8), 0.4 μ mole of phosphate buffer (pH 6.8), 1.2 mg of serum albumin, 0.16 μ mole of UDP-glucose, 0.4 μ mole of glucose-6-phosphate, 0.8 μ mole of magnesium chloride, 0.2 μ mole of ethylenediaminetetraacetate, and 0.4 to 2.0 units of transglucosylase. Control mixtures lacking either UDP-glucose or glucose-6-phosphate were processed along with the experimental mixtures. The mixtures were incubated at 23 C for 60 min. At the end of this time, water (and missing substrate, as required) was added to a final volume of 0.20 ml; 10 μ liters of 0.1 M acetic acid were added to each mixture, and deproteinization was completed by heating the acidified mixture at 100 C for 5 min. The turbid suspensions were clarified by centrifugation at 2,000 $\times g$, and samples (50 μ liters) were removed for UDP analyses. Another sample (25 μ liters) was removed for trehalose-6-phosphate analysis as described above.

Purification of the H37Ra transglucosylase. All steps were carried out at 2 C unless otherwise noted. Gel filtrations were carried out at room temperature. Ammonium sulfate fractionations were carried out at pH 7 to 7.5 with a saturated (2 C) solution of ammonium sulfate.

Cell-free extracts of H37Ra were prepared by grinding the cells in a colloid mill as described previously (Goldman and Lornitzo, 1962). The crude cell-free extract was fractionated with ammonium sulfate. The fraction precipitating

between 0.28 and 0.55 saturation (AS-1) was recovered by centrifugation for 10 min at 30,000 $\times g$, dissolved in water, and refractionated with ammonium sulfate. The fraction precipitating between 0.30 and 0.55 saturation (AS-2) was recovered, dissolved in water, and desalted by passage through Sephadex G-50 (equilibrated with 10 mM Tris-acetate-10 mM ammonium sulfate; final pH, 7.0) with 10 mM Tris-acetate-10 mM ammonium sulfate (final pH, 7.0) as the eluting buffer. The Sephadex G-50 eluate was brought to 2 mM with magnesium chloride and further fractionated on a column of Sephadex G-200 (equilibrated with 10 mM Tris-acetate-2 mM magnesium chloride; final pH, 7.0). The rate of filtration through Sephadex G-200 was increased by the addition of an equal volume of glass beads 130 μ in diameter (Superbrite) to the swollen gel before the column was prepared. The transglucosylase was eluted from the column with 10 mM Tris-acetate-2 mM magnesium chloride.

The presence of 2 mM magnesium chloride in both the equilibration and elution buffers was essential. If magnesium acetate was omitted, the transglucosylase was but partially excluded from the dextran gel and, accordingly, was recovered over a wide volume.

Alternatively, the Sephadex G-50 eluate could be further fractionated with dilute acetic acid. The pH of the Sephadex G-50 eluate was reduced to 5.8 with acetic acid. Any insoluble material was removed by centrifugation and discarded. Magnesium acetate was added to the supernatant solution to a final concentration of 20 mM. The pH of the resulting solution was reduced to 5.0 by the further addition of acetic acid. The precipitate was recovered by centrifugation and dissolved in 20 mM Tris-maleate buffer (pH 6.8).

Purification of the yeast transglucosylase. The transglucosylase of fresh, dried brewer's yeast was prepared according to the method of Cabib and Leloir (1958) through the dialyzed second ammonium sulfate precipitation step. The transglucosylase was then further purified by fractional precipitation with acetic acid. Any precipitate forming at pH 5.8 was removed by centrifugation and discarded. The supernatant solution was made 20 mM with respect to magnesium acetate; the pH of the resulting solution was brought to 5.0 with acetic acid. The precipitate which formed was recovered by centrifugation and dissolved in 20 mM Tris-maleate buffer of pH 6.8.

Purification of the transglucosylase of physiologically young cultures of H37Ra. Since only small amounts of these cells (9 days of incubation instead of the standard 28 days) were available, an alternate extraction procedure was introduced. The cells were harvested and washed as usual and then ground for 15 min in a chilled mortar with an equal weight of alumina (Alcoa, A-305). The thick paste was diluted with 10 mM Tris-0.25 M sucrose buffer (pH 7.0) and centrifuged at 30,000 $\times g$ for 15 min; the residue was discarded. The supernatant solution was centrifuged at 100,000 \times

g for 40 min; the residue was discarded. The supernatant solution was fractionated with ammonium sulfate in the same manner as the standard H37Ra crude extracts. The ammonium sulfate fraction containing transglucosylase activity was filtered twice through Sephadex G-50 as described above.

RESULTS

Purification of the H37Ra transglucosylase. The data of Table 1 show that sensitivity to mycoribnin is progressively lost during purification of the transglucosylase but is recovered after desalting by fractionation on Sephadex or on fractionation at a low pH.

Properties of the H37Ra transglucosylase. The requirement for magnesium in the dextran-gel purification of the transglucosylase may be due to the formation of stable high molecular weight aggregates of the enzyme. Magnesium also increased somewhat the stability of the partially purified enzyme to storage at both 2 and -17 C. The Sephadex G-200 eluate lost 90% of its activity in about 4 hr at 2 C. It was slightly more stable at -17 C; bovine serum albumin (5 mg/ml) reduced the rate of inactivation of these preparations at -17 C, permitting storage for 2 to 3 days.

The transglucosylase prepared by fractionation with acetic acid in the presence of 2 mM magnesium acetate lost most of its activity when stored at -17 C for 4 days.

Substrate specificity of the H37Ra transglucosylase. Galactose-6-phosphate, glucose-1-phosphate, and ribose-5-phosphate could not accept a glucosyl unit from UDP-glucose. UDP-galactose did not transfer a galactosyl unit to glucose-6-phosphate. Galactose-6-phosphate, glucose-1-phosphate, ribose-5-phosphate, and UDP-galactose did not inhibit the formation of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate.

Effect of analogues of mycoribnin on the H37Ra transglucosylase. Synthetic poly-A (13 units per molecule on the average) caused a slight inhibition of the H37Ra transglucosylase. [Poly-A denotes a series of oligoribonucleotides synthesized from adenosine diphosphate (ADP; ppA) in the presence of polynucleotide phosphorylase



where n is variable. These oligoribonucleotides can be fractionated on the basis of n .] The specific activity of poly-A was about 20% that of mycoribnin. A pancreatic ribonuclease digest of thymus ribonucleic acid (RNA), with the general composition of $(\text{Ap})_m(\text{Gp})_n\text{Pyp}$ ($14 > (m+n) > 7$), stimulated the H37Ra transglucosylase. A pancreatic ribonuclease digest of H37Ra RNA,

TABLE 1. Purification of the H37Ra transglucosylase

Fractionation step	Vol	Total protein	Specific activity*	Total units	Sensitivity to mycoribnin
	ml	mg			%
Crude extract....	100	1,900	2.2	3,100	100†
AS-1.....	25	500	6	3,000	20
AS-2.....	19	380	8	3,040	5
G-50 eluate.....	23	380	7	2,650	60
G-200 eluate.....	25	40	26	1,060	80
Mg ⁺⁺ -acetic acid residue.....	3.8	38	31	1,240	90

* Millimicromoles of trehalose-6-phosphate formed per minute per milligram of protein.

† Estimated.

TABLE 2. Effect of cobalt and bicarbonate on the inhibition of the H37Ra transglucosylase by mycoribnin*

Addition to basic system	Amt (μmoles) of UDP formed per min†		Decrease
	Without mycoribnin	With mycoribnin	
None.....	2.20	1.66	0.54
Co ⁺⁺	2.05	1.38	0.67
HCO ₃ ⁻	2.24	2.08	0.16
Co ⁺⁺ and HCO ₃ ⁻	2.24	1.82	0.42

* The standard one-step assay system for the transglucosylase was employed including 2.2 units of enzyme (Sephadex G-50 eluate stage) and 0.6 unit of partially purified mycoribnin. Cobalt, when added, was at a final concentration of 0.2 mM. The reaction was allowed to proceed for 8 min, at which time bicarbonate (0.2 mM, final concentration) was added as shown. The reaction was followed for an additional 8 min. Each reaction mixture was followed in parallel with a control mixture which lacked UDP-glucose.

† Corrected for a control mixture lacking UDP-glucose.

with the general composition of $(\text{Ap})_m(\text{Gp})_n\text{Pyp}$ ($6 > (m+n) > 3$), was slightly inhibitory to the H37Ra transglucosylase. The specific activity was about 20% that of mycoribnin.

Effect of bicarbonate on the action of mycoribnin. Several procedures were tried to prevent the loss of sensitivity or to alter the sensitivity to mycoribnin of a given transglucosylase preparation; only procedures which involved metal ions had any restorative effect. For example, in the presence of 0.1 mM Co⁺⁺ the sensitivity to mycoribnin

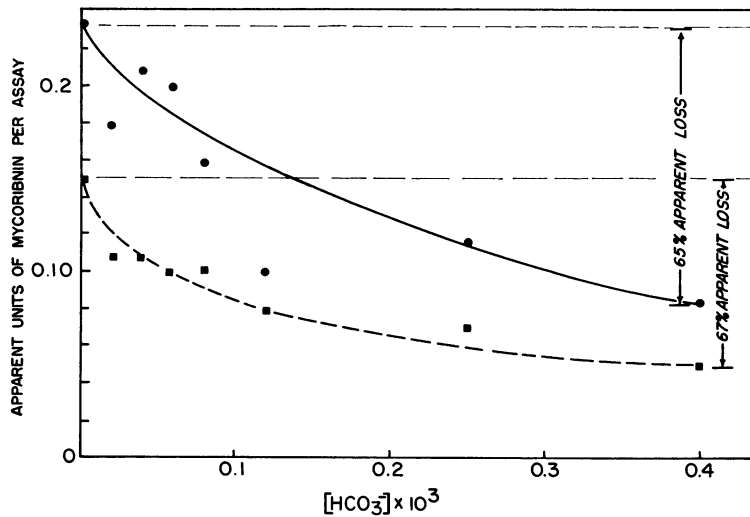


FIG. 1. Effect of bicarbonate concentration on the inhibition of the H37Ra transglucosylase by mycoribnin. The standard one-step assay system for the transglucosylase was employed; each reaction mixture contained 1.0 unit of transglucosylase (Sephadex G-50 eluate stage), bicarbonate as shown, and partially purified mycoribnin as follows: ●, 0.25 units; ■, 0.13 units. Each complete reaction mixture was corrected for a control mixture lacking UDP-glucose.

of a partially insensitive transglucosylase preparation could be almost completely restored. Because mycoribnin is treated with bicarbonate during its purification (Lornitzo and Goldman, 1964), these observations, together with others, suggested that the formation of the enzyme-inhibitor complex might be sensitive to bicarbonate. Divalent metal ions could reverse this effect by reducing the concentration of bicarbonate. As shown in Table 2, bicarbonate blocks the inhibition of the transglucosylase by mycoribnin; Co^{++} restores this sensitivity.

Preincubation of the transglucosylase with bicarbonate (1 mM) at 37 C, followed by dilution with fresh buffer and assay for transglucosylase activity, yielded essentially the same results. Approximately 50% of the transglucosylase's sensitivity to mycoribnin was lost, even though the concentration of bicarbonate after dilution was 25 μM . Bicarbonate (25 μM) had no effect when added directly to the transglucosylase-mycoribnin assay system (Fig. 1).

The residual bicarbonate concentration in the enzyme preparations could be reduced to about 10 μM by bubbling CO_2 -free nitrogen through the solution. Mycoribnin-insensitive transglucosylase preparations so treated were converted uniformly to the mycoribnin-sensitive form with no loss of the transglucosylase activity.

Conversion of the yeast transglucosylase to a mycoribnin-sensitive form. The high concentrations of glycolytic enzymes and intermediates in

TABLE 3. Purification of the yeast transglucosylase

Fractionation step	Vol	Total protein	Specific activity*	Total units
	ml	mg		
Crude extract†	75	1,650	7	11,550
AS-1†	12	660	20	13,200
AS-2†	10	600	16	9,600
AS-2 (dialyzed)†	12	600	11	6,600
Mg ⁺⁺ -acetic acid residue	3.0	160	20	3,200

* Millimicromoles of trehalose-6-phosphate formed per minute per milligram of protein.

† According to the procedure of Cabib and Leloir (1958).

cell-free extracts of brewer's yeast suggested the possibility that sufficient bicarbonate was being produced by side reactions to prevent the inhibition of the transglucosylase by mycoribnin. This could account for our reported inability to inhibit the yeast transglucosylase with mycoribnin (Goldman and Lornitzo, 1962). The following experiments were undertaken to clarify these results.

Preparations of yeast transglucosylase at the dialyzed second ammonium sulfate stage (Table 3) were not affected by mycoribnin. Desalting of this transglucosylase preparation with Sephadex G-50 did not affect this lack of sensitivity to mycoribnin. Sensitivity to mycoribnin was

TABLE 4. *Effect of bicarbonate on the inhibition of yeast transglucosylase by mycoribnin**

Reaction mixture	Product formed (μ moles)	
	UDP	Trehalose 6-phosphate
Complete.....	48	42
Less UDP-glucose.....	0	0
Glucose-6-phosphate.....	0	0
Plus		
Mycoribnin (0.3 unit)†.....	29	26
Bicarbonate (0.2 μ mole)†.....	44	34
Mycoribnin and bicarbonate†.....	44	38

* Two-step transglucosylase assay procedure using 0.8 unit of yeast enzyme (Mg^{++} -acetic acid residue stage).

† Control mixtures lacking UDP-glucose or glucose-6-phosphate were carried through the procedure in parallel with each experimental mixture.

TABLE 5. *Effect of bicarbonate on the inhibition of the transglucosylase of physiologically young cultures of H37Ra by mycoribnin**

Reaction mixture	UDP formed	Decrease
	μ moles/min	
Complete.....	0.54	
Complete plus mycoribnin.....	0.32	0.22
Complete plus bicarbonate.....	0.48	
Complete plus mycoribnin and bicarbonate.....	0.36	0.12

* The standard one-step assay system for the transglucosylase was employed including 0.5 unit of transglucosylase (second gel-filtration stage). Additions to the system included mycoribnin (0.8 units) and bicarbonate (0.2 mM, final concentration), as shown. Each reaction mixture was followed in parallel with a control mixture lacking UDP-glucose.

shown, however, when the yeast transglucosylase was fractionated with acetic acid-magnesium acetate as described above. The sensitivity of the yeast transglucosylase to mycoribnin was lost in the presence of 2 mM bicarbonate (Table 4).

Conversion of the transglucosylase of physiologically young H37Ra to a mycoribnin-sensitive form. The transglucosylase present in the crude cell-

free extract of physiologically young cultures of H37Ra had a specific activity of 3.5 to 4.0 ($m\mu$ -moles per minute per mg of protein) and was not sensitive to mycoribnin. Purification of the transglucosylase by the ammonium sulfate procedure raised the specific activity to about 8; the enzyme was not inhibited by mycoribnin. After two gel-filtration steps, the transglucosylase for the first time showed definite sensitivity to mycoribnin (about 40%). These data as well as the action of bicarbonate on this reaction (Table 5) suggest that the extracts of young cultures of H37Ra are saturated with respect to bicarbonate, leading to the insensitivity of the transglucosylase to mycoribnin.

DISCUSSION

We noted previously that the transglucosylase(s) of both brewer's yeast and young cultures of H37Ra was insensitive to mycoribnin. At least two possible explanations could be advanced for this insensitivity. (i) The two insensitive transglucosylases are different one from another, and both differ from the enzyme of old cultures of H37Ra. (ii) Some additional, as yet unknown, factor is required for the inhibition of the transglucosylase by mycoribnin.

The data presented above suggest that neither explanation is valid. When prepared free from bicarbonate, the transglucosylases of brewer's yeast, young cells of H37Ra, and old cells of H37Ra were all sensitive to mycoribnin. Sensitivity was lost when the transglucosylases were treated with millimolar concentrations of bicarbonate. This bicarbonate-induced change in the enzyme was not reversed by dilution of the enzyme preparation to a bicarbonate concentration ordinarily ineffective in altering the mycoribnin sensitivity of the transglucosylase.

The mycoribnin-insensitive transglucosylases of young cells of H37Ra and of yeast could be made mycoribnin-sensitive. The demonstrations of these sensitivity changes were reproducible; typical experimental results are given. We made no attempt to prepare these enzymes in a form completely sensitive to mycoribnin as was done with the H37Ra enzyme (Table 1). For the purposes of this report, it was of importance only to demonstrate (i) that the enzymes could be converted to a mycoribnin-sensitive form and (ii) that this conversion could be reversed by bicarbonate. The physiological changes responsible for the changes in intracellular bicarbonate concentration as the H37Ra culture reached full growth are as yet unknown.

The data presented above on the effect of bicarbonate on the mycoribnin-transglucosylase system make possible several conclusions. (i)

Since either loss or recovery of sensitivity to mycoribnin is unaccompanied by a change in transglucosylase activity, mycoribnin and substrate(s) are probably not bound at the same site. (ii) The binding of mycoribnin to the enzyme either effects a configurational change in the protein or otherwise physically blocks the substrate binding site. (iii) Bicarbonate probably is bound to the same site on the protein as is mycoribnin; the dissociation constant of bicarbonate is significantly smaller than that of mycoribnin, because displacement by mycoribnin is not observed whereas displacement of mycoribnin by bicarbonate is observed.

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