Substrate Specificity and Kinetic Studies on Thiogalactoside Transacetylase†

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ABSTRACT: Studies on the enzymology of thiogalactoside transacetylase (acetyl-CoA:galactoside 6-*O*-acetyltransferase, EC 2.3.1.18) from the *Lactose* operon of *Escherichia coli* are reported. Various divalent metal ions and chelators have negligible effects on the reaction. Several β-galactosides and related compounds have been tested as acetyl-acceptor substrates or inhibitors but none will saturate the enzyme at low concentrations. *p*-Nitrophenyl β-D-galactoside was found to be about twice as effective as isopropyl β-D-thiogalactopyranoside. Substrate kinetics and product inhibition studies are reported which are consistent with a reaction mechanism of the ordered bi-bi type with acetyl-CoA and CoA as the first substrate and final product, respectively. Inhibition constants for acetyl-CoA ($K_i = 5.7 \times 10^{-5}$ M) and CoA ($K_i = 6.8 \times 10^{-5}$ M) have been determined kinetically. These parameters should be equivalent to the equilibrium dissociation constants for the corresponding enzyme–reactant complexes. Other kinetic parameters have also been determined including the Michaelis constants for acetyl-CoA (1.8×10^{-4} M) and isopropyl β-D-thiogalactoside (0.77 M).

The *Lactose* operon of *Escherichia coli* contains three cistrons which code for the proteins β-galactosidase, β-galactoside permease, and thiogalactoside transacetylase (Jacob and Monod, 1961). Although the physiological roles of β-galactosidase and β-galactoside permease in the cell are clear, the function of thiogalactoside transacetylase is presently unknown. Clarification of certain aspects of the enzymology of thiogalactoside transacetylase could aid the formulation and evaluation of hypotheses concerning the role of this enzyme in lactose metabolism.

Thiogalactoside transacetylase catalyzes the transfer of the acetyl group from acetyl-CoA to the 6-hydroxyl of certain galactosides including iPrSGal[1] (Zabin *et al.*, 1959). The requirement for acetyl-CoA as the acyl-donor substrate has been shown to be quite specific (Zabin *et al.*, 1962; Alpers *et al.*, 1965). When the acetyl-CoA concentration is varied, simple Michaelis–Menten kinetics are observed with an apparent K_m of $<4 \times 10^{-5}$ M at 50 mM iPrSGal (Zabin *et al.*, 1962).

A number of β-galactosides and certain glucosides will serve as the acetyl-acceptor substrate (Zabin *et al.*, 1962; Alpers *et al.*, 1965; Fox and Kennedy, 1967). In contrast to the reasonable K_m for acetyl-CoA, no acceptor substrate tested will saturate the enzyme unless very high concentrations are used. Alpers *et al.* (1965) reported an apparent K_m for iPrSGal of 0.77 M. This value was obtained by extrapolation of rates at relatively low iPrSGal concentrations to minimize the apparent substrate inhibition which becomes severe at iPrSGal concentrations above about 0.5 M. Such a high K_m for the synthetic substrate iPrSGal may indicate that a "natural" acetyl-acceptor substrate has rather different structural features and that these features are important for proper binding to enzyme. Alternately, the optimal reaction conditions, including requirements for possible effectors, may not have been found.

However, in a reaction with two substrates, the apparent K_m for one may depend on the concentration of the other substrate. Even the K_m determined by extrapolation to infinite concentration of the alternate substrate will be a complex function of several rate constants and may not reflect the equilibrium dissociation constant for the enzyme–substrate complex in question.

In order to examine these uncertainties in more detail, a variety of reaction conditions and possible substrates have been tested. In addition, we have conducted substrate kinetics and product inhibition experiments to discriminate between possible kinetic mechanisms and to determine more definitive kinetic constants for the reactants.

Materials and Methods

Thiogalactoside transacetylase was purified from extracts of *Escherichia coli* A324-5 (Fowler, 1972) according to the procedure of Zabin (1963) except that gel filtration was substituted for crystallization as the final step. The enzyme was homogeneous by the criteria of sodium dodecyl sulfate and pH 8.4 disc gel electrophoresis. Final specific activities were 1100 ± 150 units/mg (Zabin *et al.*, 1962). For kinetic experiments the enzyme concentrations were determined spectrophotometrically at 280 nm using an extinction of 0.95 for 1.0 mg/ml at pH 7.8 (Zabin, 1963).

Dr. Winfried Boos generously provided the following compounds: thioallolactose, 1-*S*-β-D-galactosyl-D-thioglycerol, 1-*O*-β-D-galactosyl-D-glycerol, 2-*O*-β-D-galactosyl-D-glycerol, neolactose, and thioallolactit. The various chelators and derivatives were kindly supplied by Dr. David Sigman.

The isopropyl 6-*O*-acetyl-β-D-thiogalactopyranoside was prepared enzymatically in a large-scale incubation of 476 mg (2 mmol) of iPrSGal with 11 mg of a 50% pure prepara-

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[1] Abbreviations used are: iPrSGal, isopropyl β-D-thiogalactopyranoside; NphGal, *o*-nitrophenyl β-galactopyranoside; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

tion of thiogalactoside transacetylase. Acetyl phosphate, coenzyme A, phosphotransacetylase, cysteine, and buffer were also added as indicated previously in a final volume of 10 ml (Zabin *et al.*, 1962). After incubation for 4 hr, the reaction was found to be complete by hydroxamate assay. The proteins were removed by addition of 30 ml of ethanol, and salts were removed by passage of the aqueous solution through a 1.9 × 20 cm column of Amberlite MB mixed-bed resins. The salt-free solution was dried *in vacuo*, and the residue was crystallized from hot ethyl acetate. The first crop of crystals, 114 mg, had a melting point of 130–131° (lit. (Herzenberg, 1961) mp 129–130°). The preparation contained less than 5% contamination by iPrSGal since no inhibition of NphGal hydrolysis by purified β-galactosidase was observed at 20 times the concentration of iPrSGal required for significant inhibition.

The following three assays for galactoside transacetylase activity were used according to the sensitivity desired and the properties of the substrate involved. (1) Alkaline hydroxamate assays for the acetyl ester product were performed as described by Zabin *et al.* (1962). (2) The radiometric assay developed by Fox and Kennedy (1967) was carried out with [^{14}C]acetyl-CoA. This assay depends on the removal of [^{14}C]AcCoA by passing the reaction mixture through a small Dowex 1 column. The amount of acetylated product is determined by the ^{14}C in the eluate. Hydrophobic compounds such as nitrophenyl glycosides were strongly retarded on the ion-exchange columns but were more readily recovered by equilibrating and eluting the columns with 5% methanol. Successive fractions were collected and counted for ^{14}C to be certain that elution was complete. All counting was done in Bray's solution (Bray, 1960) using a Packard Model 3003 Tri-Carb scintillation spectrometer. (3) The Nbs_2 assay for the CoA product was used as previously described (Alpers *et al.*, 1965). All assays were run at 30° in 0.5 M Tris-HCl (pH 7.8). EDTA (2 mM) was added in the assay system except for the studies on metal ions and chelators.

For substrate kinetics and product inhibition by isopropyl acetyl-β-D-thiogalactopyranoside the reaction was carried out in the presence of Nbs_2 and monitored continuously at 412 nm on a Beckman Kintrac VII recording spectrophotometer using an expanded scale of 0.1 ODU. The cuvet chamber was thermostated at 30° and the assay mixture lacking iPrSGal was preequilibrated for 5 min before starting the reaction by addition of iPrSGal. All reported rates have been corrected for a low rate of thionitrobenzoic acid formation observed prior to the addition of iPrSGal.

This assay system was also used without continous monitoring. In these cases Nbs_2 was omitted during the incubation at 30° but was added in two volumes of an ice-cold solution in Tris-HCl (pH 7.8). After 10 min in an ice bath the absorbance at 412 nm was determined on a Beckman DB spectrophotometer. Product inhibition by CoA, as well as studies of various potential substrates and effectors, were performed in this manner.

All rates have been corrected for an appropriate blank and, when necessary, by a standard curve to adjust for depletion of the limiting substrate.

Results

Metal Ion Effects. The effects of divalent metal ions on enzyme activity were tested in two ways. First, the appropriate salt (1 mM) was added directly to the Nbs_2 assay mixture lacking EDTA. $BaCl_2$, $CaCl_2$, $MgSO_4$, and $SrCl_2$ had negligible effect whereas $MnSO_4$ caused a 40% inhibition. The second type of experiment involved preincubating transacetylase (2 mg/ml) in 0.05 M Tris (pH 7.8) with the desired metal ion for 1 hr at 30° and subsequently diluting the enzyme 1000-fold into a Nbs_2 assay system lacking both EDTA and the metal ion. Such preincubations with 10^{-4} to 10^{-3} M $MnSO_4$ or $Cd(OAc)_2$ stimulated the enzyme activity up to two-fold; $ZnSO_4$ had no effect. The results in both types of experiments are comparable to those reported previously where assays were conducted in phosphate buffer (Zabin *et al.*, 1962; Zabin, 1963). A study of the rate dependence on iPrSGal concentration after preincubations with 5×10^{-4} M $MnSO_4$ or $Cd(OAc)_2$ indicated that the apparent K_m for iPrSGal was decreased about 40% while little or no change in the maximum velocity occurred.

Chelator Effects. Chelator effects were studied to examine further the possibility of a metal ion involvement in the transacetylase-catalyzed reaction. In these experiments transacetylase was preincubated with the desired chelator for 30 min in the Nbs_2 assay system which lacked acceptor substrate. iPrSGal was then added to initiate the reaction. Neither EDTA (2 or 10 mM) nor 8-hydroxyquinoline (4 mM) significantly affect the activity of transacetylase; 2–8 mM 1,10-phenanthroline inhibits slightly (30% at 8 mM). The inhibition by 1,10-phenanthroline is not enhanced by the presence of EDTA (2 mM). 1,10-Phenanthroline N^{10}-oxide, a poor chelator, also inhibits nearly as well. Furthermore, 2,2′-bipyridine, which chelates similarly to 1,10-phenanthroline, causes only a negligible inhibition even at 8 mM concentration. These results suggest that 1,10-phenanthroline does not inhibit transacetylase by chelation of an enzyme-bound metal ion.

Acyl-Donor Substrates. Oleoyl-CoA and crotonyl-CoA (10^{-4} M) have no significant activity when tested in the Nbs_2 assay system as acyl-donor substrates or as inhibitors of the reaction with acetyl-CoA. This is in agreement with previous reports (Zabin *et al.*, 1972; Alpers *et al.*, 1965) that only acetyl-CoA will serve as an effective donor substrate for transacetylase.

Acetyl-Acceptor Substrates. We have examined a series of potential substrates at different concentrations to compare their relative activities and to see if any will saturate transacetylase at concentrations lower than that required for iPrSGal. The first series of compounds in Table I are related to lactose, allolactose, and 1-*O*-β-D-galactosyl-D-glycerol. The latter two have been considered as candidates for the physiological inducer of the *Lactose* operon (Müller-Hill *et al.*, 1964). None of these were acetylated at appreciable rates nor did they saturate at low concentrations.

Since both galactose and glucose can be activated as nucleoside diphospho sugars, the possibility was considered that transacetylase might acetylate such compounds. However, three such compounds were inactive as acetyl acceptors (Table I).

A series of nitrophenyl glycosides were also tested (Table I). Although none of these saturate the enzyme at low concentrations, it is of interest that *p*-nitrophenyl β-D-galactoside is more active than iPrSGal. Epimerization at C-4 of the galactoside to give the glucoside decreases the relative rate but does not abolish activity. The mannoside is almost completely inactive. Although lactose itself is not acetylated, *p*-nitrophenyl β-D-lactoside is a reasonably good substrate. This is consistent with previous results (Zabin *et al.*, 1962; Alpers *et al.*, 1965) indicating that either a thioglycoside or a hydrophobic aglycon moiety is required for activity. The α anomer, *p*-nitrophenyl α-D-galactoside, shows a low but significant rate of acetylation. This compound is not hydrolyzed

TABLE I: Acetyl-Acceptor Specificity.

Substrate	Concn (mM)	% iPrSGal Reaction[a]	Assay[b]
Lactose	28	0	H
Neolactose	10	2	H
Thioallolactose	50	2	H
	10	2	D
Thioallolactit	50	0	H
	10	4	D
1-*O*-β-D-Galactosyl-D-glycerol	10	9	D
2-*O*-β-D-Galactosyl-D-glycerol	10	10	D
1-*S*-β-D-Galactosyl-D-thioglycerol	50	18	H
	10	16	D
UDP-galactose	3	0	D
GDP-glucose	3	0	D
ADP-glucose	3	0	D
p-Nitrophenyl β-D-galactoside	10	210	D, H
	5	230	D, H
	2	210	D
p-Nitrophenyl β-D-glucoside	5	51	H
	2	50	D, R
p-Nitrophenyl β-D-mannoside	5	1	H
	2	3	D, R
p-Nitrophenyl β-D-lactoside	5	45	H
	2	60	R
p-Nitrophenyl α-D-galactoside	10	32	H, D
	5	33	H, D
	2	27	R
p-Nitrophenyl β-D-thiogalactoside	5	27	H
	2	25	R
o-Nitrophenyl β-D-galactoside	5	105	D
	2	85	R
o-Nitrophenyl β-D-thiogalactoside	10	90	H
	5	100	D

[a] All assays compared to a control with an equal concentration of iPrSGal. [b] Assays are designated as H (hydroxamate), R (radiometric), and D (Nbs_2). Values from different assays agreed within experimental error. All D and R assays were done with 0.2 mM acetyl-CoA and corrected for blanks lacking acetyl-CoA. H assays were corrected for blanks in which the acceptor substrate was added after arsenolysis of the acetyl phosphate and acetyl-CoA.

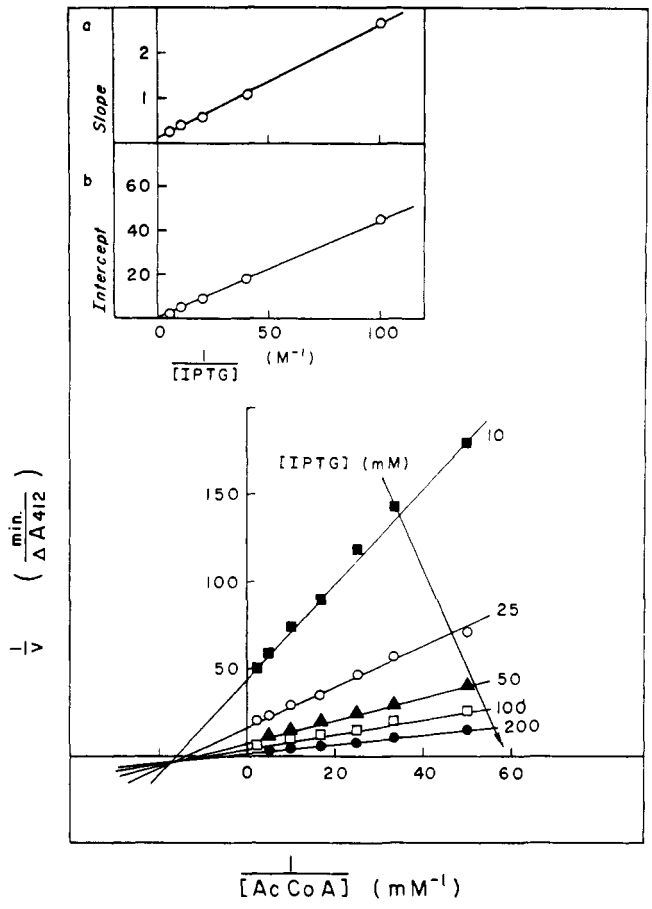

FIGURE 1: Initial velocity pattern with acetyl-CoA as the variable substrate at different fixed concentrations of iPrSGal. The slopes and *y* intercepts are replotted against the reciprocal iPrSGal concentration in the insets a and b, respectively.

by purified β-galactosidase; but since some α-galactosides act as competitive inhibitors of β-galactosidase (Kuby and Lardy, 1953), this does not exclude the possibility of contamination by the β anomer. Although *p*-nitrophenyl β-D-thiogalactoside is less active than the oxygen analog, the corresponding thio- and oxygalactosides of *o*-nitrophenol show equivalent activities.

Several inactive analogs of the acetyl-acceptor substrates have also been tested as possible dead end competitive inhibitors of iPrSGal. However, the reaction with iPrSGal (25 mM) was not inhibited by any of the following compounds: lactose (28 mM), galactose (56 mM), melibiose (28 mM), raffinose (25 mM), mannose (56 mM), dulcitol (30 mM), *p*-nitrophenyl β-D-fucoside (20 mM), or *p*-nitrophenyl β-D-thiofucoside.

Substrate Kinetics. The Nbs_2 assay and a recording spectrophotometer were used to study the substrate kinetics for transacetylase. Figure 1 is a double-reciprocal plot of initial velocity against acetyl-CoA concentration with various fixed concentrations of iPrSGal. The associated insets a and b are replots of the corresponding slopes and *y* intercepts, respectively, against the reciprocal iPrSGal concentration.

The intersecting pattern seen in Figure 1 indicates the mechanism is sequential and not a Ping-Pong type which would yield parallel lines. However, these initial velocity patterns do not discriminate between a rapid-equilibrium random and an ordered mechanism. In addition, one cannot determine the order of substrate participation in an ordered mechanism from this information. Product inhibition studies are required to discriminate between these possibilities.

Product Inhibition. Inhibition by isopropyl 6-*O*-acetyl-β-D-thiogalactopyranoside was studied by using the spectrophotometric assay as above. Isopropyl 6-*O*-acetyl-β-D-thiogalactopyranoside (75 mM) inhibits noncompetitively on both acetyl-CoA and iPrSGal, at subsaturating concentrations of the alternate substrate.

Inhibition by CoA was studied in a similar assay system by incubating in the absence of Nbs_2. CoA inhibits competitively on acetyl-CoA (Figure 2) and noncompetitively against iPrSGal at subsaturating concentrations of the alternate substrate.

The observed pattern of product inhibition is inconsistent with a rapid-equilibrium random mechanism since for the latter each product would be a competitive inhibitor against either substrate when the alternate substrate is subsaturating.

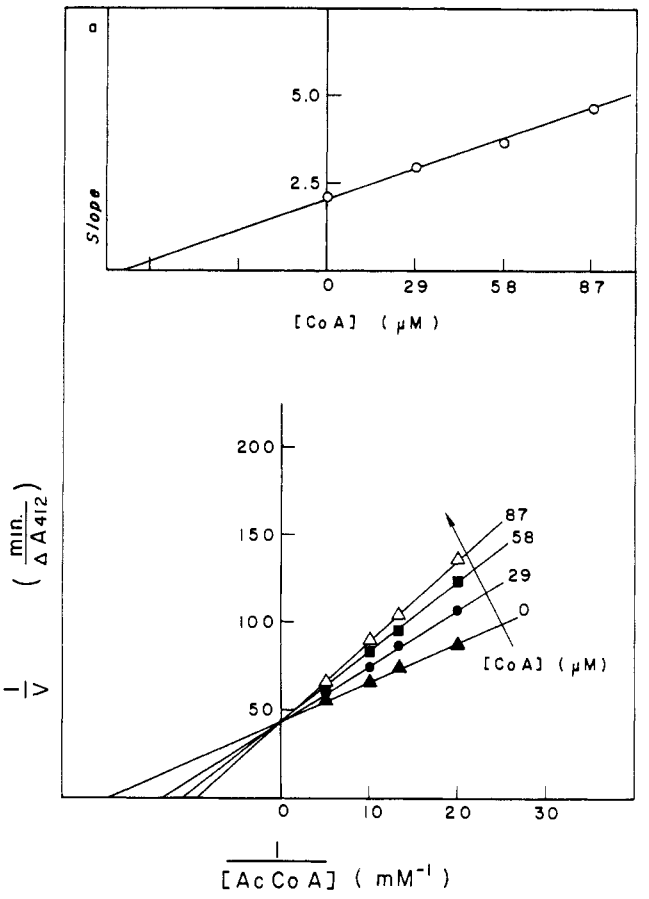

FIGURE 2: Product inhibition by CoA against acetyl-CoA as the variable substrate. iPrSGal concentration was fixed at 50 mM. Inset a is the replot of slopes against CoA concentration.

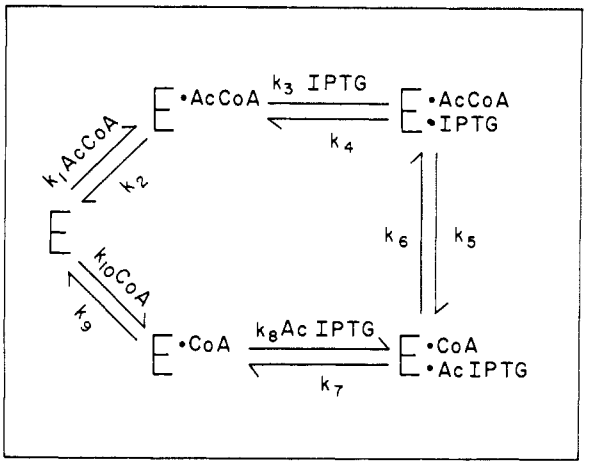

FIGURE 3: Ordered bi-bi kinetic mechanism proposed for thiogalactoside transacetylase.

However, the pattern of product inhibition is consistent with an ordered mechanism in which either acetyl-CoA or CoA can bind to the same enzyme form.

Proposed Mechanism and Kinetic Constants. The simplest kinetic mechanism consistent with the above results and involving (arbitrarily) a single step for chemical transformation of substrates to products is an ordered bi-bi mechanism as represented in Figure 3.

According to Cleland's notation (Cleland, 1963), the rate equation for this mechanism is represented in eq 1, where A, B, P, and Q represent acetyl-CoA, iPrSGal, isopropyl acetyl-β-D-thiogalactopyranoside, and CoA, respectively; V_1 and V_2 are the maximum velocities in the forward and reverse directions, respectively; and K_{eq} is the equilibrium constant for the forward direction. Each reactant is represented by two types of kinetic constants which are designated by the subscripts i (inhibition constant) and m (Michaelis constant).

$$v = \frac{V_1V_2\left(AB - \frac{PQ}{K_{eq}}\right)}{K_{iA}K_{mB}V_2 + K_{mB}V_2A + K_{mA}V_2B + V_2AB + \frac{K_{mQ}V_1P}{K_{eq}} + \frac{K_{mP}V_1Q}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{K_{mQ}V_1AP}{K_{iA}K_{eq}} + \frac{K_{mA}V_2BQ}{K_{iQ}} + \frac{V_2ABP}{K_{iP}} + \frac{V_1BPQ}{K_{iB}K_{eq}}} \quad (1)$$

Each of these parameters can be defined in terms of the rate constants in our proposed mechanism, but we list only four which are particularly relevant (eq 2).

$$K_{iA} = \frac{k_2}{k_1}$$

$$K_{iQ} = \frac{k_9}{k_{10}}$$

$$K_{mA} = \frac{k_5k_7k_9}{k_1(k_7k_9 + k_6k_9 + k_5k_9 + k_5k_7)} \quad (2)$$

$$K_{mB} = \frac{(k_3k_7 + k_4k_7 + k_4k_6)k_9}{k_3(k_7k_9 + k_6k_9 + k_5k_9 + k_5k_7)}$$

It is quite apparent that the Michaelis constants for acetyl-CoA and iPrSGal are not equivalent to the equilibrium dissociation constants for the corresponding enzyme–substrate complexes. However, K_{iA} and K_{iQ} should be equal to the actual dissociation constants for the enzyme complexes with acetyl-CoA or CoA, respectively.

Since we measured initial velocities in the forward direction, the general rate equation can be simplified to describe the lines plotted in Figures 1 and 2 (Cleland, 1963). One can then extract values for these kinetic parameters from the data as follows: K_{iA} (5.7×10^{-5} M), K_{iQ} (6.8×10^{-5} M), K_{mA} (1.8×10^{-4} M), and K_{mB} (0.77 M). The V_1 from Figure 1 corresponds to a turnover number of $\sim 8 \times 10^3$ mol of CoA/min per mol of enzyme based on a molecular weight of 65,300 for the transacetylase dimer (Zabin, 1963).

Discussion

We have presented steady-state kinetic evidence consistent with an ordered bi-bi mechanism for this enzymatic reaction. The results presented here are also consistent with an iso-Theorell–Chance mechanism in which iPrSGal and isopropyl 6-*O*-acetyl-β-D-thiogalactopyranoside can bind to differ-

ent isomeric forms of the free enzyme. A distinction between this and the ordered bi-bi mechanism can only be made by binding studies (Cleland, 1963). The possibility of a stable covalent acetyl-enzyme form, however, appears untenable since the latter would likely result in a Ping-Pong type of mechanism. Kinetic evidence for this latter type of mechanism has been reported for human placental choline acetyltransferase (Schuberth, 1966). However, Chase and Tubbs (1966) reported kinetic studies on pigeon breast carnitine acetyltransferase indicating a rapid-equilibrium random mechanism for that enzyme. As noted in the latter studies, we cannot exclude the transitory formation of a covalent acetyl-enzyme during the transformation of the ternary complex with substrates to that with products. In addition, the reaction we have studied may be misleading due to the low apparent affinity for the acetyl-acceptor substrate. A natural acceptor substrate with a high affinity might yield a more random reaction mechanism. It is evident that acetyltransferases act by a variety of mechanisms.

Our proposed mechanism does not account for the reported substrate inhibition at high iPrSGal concentrations (Alpers *et al.*, 1965) for three reasons. First, the reported inhibition was seen at such high concentrations of iPrSGal that contamination of the latter may be responsible. Secondly, even if iPrSGal itself causes the inhibition, it may have no relevance to the physiological reaction which presumably involves lower reactant concentrations. Finally, all our reactions have been conducted at lower iPrSGal concentrations where this inhibition is not significant.

According to an ordered bi-bi mechanism the limiting K_m values for acetyl-CoA (1.8×10^{-4} M) and for iPrSGal (0.77 M) do not directly reflect the equilibrium dissociation constants for the corresponding substrate–enzyme complexes. In contrast, the values for K_{iAcCoA} (5.7×10^{-5} M) and K_{iCoA} (6.8×10^{-5} M) should be equivalent to the dissociation constants for the complexes of enzyme with acetyl-CoA or CoA, respectively.

The values of these kinetic parameters strongly suggest that the physiological reaction catalyzed by thiogalactoside transacetylase involves acetyl-CoA. Either an acetyl-acceptor substrate with structural features not present in the compounds tested or effectors which improve the binding of the acceptor substrate must be involved in a physiological reaction. We have examined the possibility that divalent metal ions might affect the binding of the acetyl-acceptor substrate. Although preincubation of the enzyme with Mn^{2+} or Cd^{2+} causes a slight decrease in the apparent K_m for iPrSGal the resulting value remains abnormally high. The studies with chelators do not indicate the presence of an enzyme-bound metal ion which would be necessary for activity. We have also conducted experiments (not reported here) indicating that neither energy charge (Atkinson and Walton, 1967) nor 3′,5′-cAMP have significant effects on transacetylase activity (Musso, 1972).

Certain tentative conclusions regarding structural features of the acceptor substrate were suggested by the data obtained with nitrophenyl glycosides. Galactosides and glucosides are acetyl acceptors, but the manoside is not, indicating an apparent importance of the configuration at C-2 of the glycosyl moiety. However, no substantial clue to the physiological acetyl-acceptor substrate was obtained.

Acknowledgments

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