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Competition by 4-Chloronitrosobenzene for the Active Glycolaldehyde Intermediate of Transketolase

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The incubation of 4-chloronitrosobenzene with yeast transketolase, Mg^{2+}, and thiamine pyrophosphate in the presence of excess xylulose-5-phosphate resulted in the formation of *N*-(4-chlorophenyl)glycolhydroxamic acid. This enzyme-catalyzed C_2 transfer displayed a K_m of 0.92 m*M* and a V_{max} of 5.2×10^{-2} μmol min^{-1} unit $enzyme^{-1}$. Conversion was inhibited by the normal acceptor sugar, ribose-5-phosphate, with a K_i of 0.35 m*M*. Kinetic analysis showed inhibition was competitive in nature, reinforcing the proposed theory for similarity in catalytic formation of both the hydroxamic acid and sedoheptulose-7-phosphate. Most interesting about the conversion of this alternative substrate is that even at high concentrations of ribose-5-phosphate, a significant amount of the nitroso compound was converted to the hydroxamic acid, implying that 4-chloronitrosobenzene can successfully compete for active glycoaldehyde. Using the yeast enzyme as a model for transketolase in higher organisms, the adventitious conversion of such xenobiotics *in vivo* is proposed.

INTRODUCTION

Transketolase (sedoheptulose-7-phosphate D-glyceraldehyde 3-phosphate glycolaldehyde-transferase, EC 2.2.1.1.) reactions are characterized by the production of an "active glycolaldehyde" intermediate which results from a C_2 transfer from a donating sugar phosphate to enzyme-bound thiamine. Its subsequent condensation with the acceptor sugar, ribose-5-phosphate, produces sedoheptulose-7-phosphate and regenerates the transketolase-bound thiamine (*1*). Previous reports from this laboratory have shown that low concentrations of nitrosobenzene were converted in high yield to *N*-phenylglycolhydroxamic acid by action of this enzyme (*2*). The conversion, like the usual transketolase reactions, requires Mg^{2+}, a donating sugar phosphate (in this case, xylulose-5-phosphate) and thiamine pyrophosphate. Conversion was postulated to proceed by nucleophilic attack of enzyme-bound "active glycolaldehyde" (I) (Fig. 1) (*3*) on the nitroso nitrogen, yielding a transient, enzyme-bound intermediate. Cleavage of the intermediate produces *N*-phenylglycolhydroxamic acid and regenerates transketolase-bound thiamine (*2*). An analogous mechanism has been proposed to account for the production of *N*-phenylacetohydroxamic acid by thiamine-dependent pyruvate decarboxylases (*4*).

The conversion of aromatic nitroso xenobiotics to hydroxamic acids *in vivo* is expected to be significant if *in vitro* conversions of this type could be demon-

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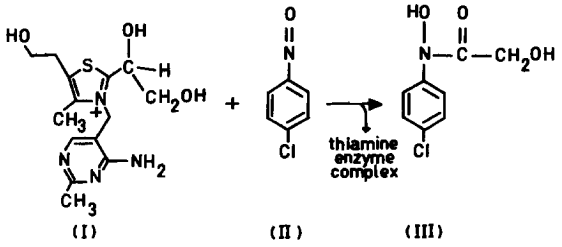

FIG. 1. Proposed mechanism for the production of glycollic acid-derived hydroxamic acids by transketolase. (I) Active glycoaldehyde; (II) 4-chloronitrosobenzene; (III) *N*-(4-chlorophenyl)glycolhydroxamic acid.

strated at high concentrations of the usual acceptor sugar, ribose-5-phosphate. The earlier cited work by Corbett and Chipko employing the yeast enzyme (*2*) suggests that such conversions do occur at relatively high concentrations of the acceptor sugar although at greatly reduced rates.

We felt it necessary to determine quantitatively the effect of ribose-5-phosphate concentration on the enzyme-catalyzed rate of nitrosoaromatic conversion to the corresponding phenylglycolhydroxamic acid. Nitrosobenzene is rather volatile and can result in an overestimation of reaction velocities; thus, in order to obtain a true quantitative measure for this conversion we chose the less volatile halogenated derivative, 4-chloronitrosobenzene (II), as the substrate. In addition, we employed yeast transketolase for these model studies, as transketolase purified from other sources (including mammalian tissues) contains pentose phosphate isomerase activity (*5*); thus, for kinetic studies, ribose-5-phosphate concentrations would only be approximate. Classical competition studies (*6*) utilizing ribose-5-phosphate as the competitor and 4-chloronitrosobenzene as substrate showed that this nitrosoaromatic was converted to *N*-(4-chlorophenyl)glycolhydroxamic acid (III), and that the conversion was competitively inhibited by ribose-5-phosphate.

EXPERIMENTAL

Materials and methods. Yeast transketolase, D-xylulose-5-phosphate, D-ribose-5-phosphate, and thiamine pyrophosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). 4-Chloronitrosobenzene was synthesized according to the method of Lutz and Lytton (*7*) and purified by repeated recrystallization from ethanol. *N*-(4-Chlorophenyl)glycolhydroxamic acid was prepared by the reaction of 4-chlorophenylhydroxylamine with glycollic acid in the presence of dicyclohexylcarbodiimide (*8*).

Assay procedure. Stock solutions of 4-chloronitrosobenzene were prepared in 0.05 *M* Trizma · HCl buffer, pH 7.6, at concentrations of 0.19, 0.28, 0.38, 0.54, and 0.74 m*M*. Essential cofactors were prepared together in 50 ml of the buffer to contain 0.4 m*M* thiamine pyrophosphate, 6.0 m*M* $MgCl_2$, and 2.8 m*M* D-xylulose-5-phosphate (84% purity). Ribose-5-phosphate solution (20 m*M*) was prepared

separately in the buffer containing the cofactors and xylulose-5-phosphate. Ribose-5-phosphate solutions of varying concentrations were then prepared by mixing the Trizma-cofactor solution with the ribose-5-phosphate solution as follows:

Ribose-5-phosphate soln. (ml)	Trizma-cofactor soln. (ml)	Resulting Ribose-5-PO_4 conc. (m*M*)
0.3	0.2	12.0
0.2	0.3	8.0
0.1	0.4	4.0
1.05	0.45	2.0
0.025	0.475	1.0

To carry out a rate determination, 0.5 ml of 4-chloronitrosobenzene solution and 0.5 ml of ribose-5-phosphate solution were mixed in a quartz curvette (1-cm, light path). To this mixture was added 0.2 units of transketolase in 1 ml of Trizma buffer. One unit of transketolase produces 1 μmol of glyceraldehyde-3-phosphate per min from xylulose-5-phosphate (in the presence of ribose-5-phosphate) at pH 7.6. Enzyme activity was determined as described by Saitou *et al.* (*9*). Disappearance of the nitroso compound was monitored spectrophotometrically at 320 nm on a Beckman model 24 uv spectrophotometer. All rates were determined at 22°C. A control was prepared using heat-denatured transketolase and was incubated with all concentrations of 4-chloronitrosobenzene in the absence of ribose-5-phosphate. Decreases in optical density were observed for 3 min in each case, using the difference between the 1 min reading and 2 min reading as the rate of conversion. The rate of A_{320} change was linear over this time span. Authenticity of the product was determined as described previously for the *N*-phenylglycolhydroxamic acid (*2*).

RESULTS AND DISCUSSION

Incubation of 4-chloronitrosobenzene with transketolase resulted in the production of *N*-(4-chlorophenyl)glycolhydroxamic acid, as demonstrated by cochromatography with the authentic compound, the characteristic violet color that formed when sprayed with $FeCl_3$ reagent, and its uv spectrum. Each incubate revealed the presence of this single hydroxamic acid, analogous to results obtained previously with nitrosobenzene (*2*). Heat-inactivated enzyme failed to produce the hydroxamic acid, establishing that enzyme catalysis was necessary for hydroxamate production. In all cases, the sugar, xylulose-5-phosphate, was in excess as the donating substrate at 2.8 m*M* and its concentration remained essentially unchanged throughout the reaction. In the presence of 2 m*M* xylulose-5-phosphate and in the absence of nitrosobenzene derivatives, the Michaelis constant for the acceptor substrate ribose-5-phosphate is reported to be 0.4 m*M* (*10*).

TABLE 1

RATES OF 4-CHLORONITROSOBENZENE CONVERSION TO HYDROXAMIC ACID AT SELECTED CONCENTRATIONS—INHIBITION BY RIBOSE-5-PHOSPHATE[a]

4-Chloronitrosobenzene concentration (m*M*)	Rate of conversion (μmol/min/unit enzyme)	Percentage of uninhibited rate at each ribose 5-phosphate concentration (m*M*)				
		0.25	0.50	1.0	2.0	3.0
0.185	0.03	75	60	50	33	27
0.135	0.024	75	50	46	29	17
0.095	0.018	61	55	39	22	11
0.070	0.014	60	46	39	ND[b]	ND
0.047	0.012	58	42	33	17	04

[a] All rates were determined spectrophotometrically (320 nm) at 22°C in a Beckman Model 24 Spectrophotometer. Conditions for the transketolase-catalyzed conversion, as well as for the ribose-5-phosphate inhibition of the conversion, are described in the Experimental section under "Assay Procedure."

[b] ND, not determined.

As can be seen from Table 1, the rate of conversion of nitrosoaromatic to glycolhydroxamate was concentration dependent. With xylulose-5-phosphate as the donating substrate, the highest rate of 4-chloronitrosobenzene conversion was seen at the highest substrate concentration (0.185 m*M*) in the absence of ribose-5-phosphate (Table 1). The lowest conversion rate (4% of the uninhibited rate at that concentration) was observed at the lowest substrate concentration (0.047 m*M*) in the presence of 3.0 m*M* ribose-5-phosphate. At each concentration, the rate of nitroso conversion was progressively inhibited by increasing concentrations of ribose-5-phosphate. Figure 2 shows the double reciprocal plot of the kinetic data obtained with 4-chloronitrosobenzene as substrate and ribose-5-phosphate as inhibitor. As is evidenced from the figure, ribose-5-phosphate inhibition of the 4-chloronitrosobenzene to hydroxamic acid conversion was competitive in nature up to 1.0 m*M* ribose-5-phosphate. Kinetic constants derived for the 4-chloronitrosobenzene to hydroxamic acid conversion were K_m = 0.92 m*M* and V_{max} = 5.2 × 10^{-2} μmol min^{-1} unit $enzyme^{-1}$; and for the ribose-5-phosphate inhibition of the conversion, the K_i was 0.35 m*M*. At the two higher ribose-5-phosphate concentrations employed (2.0 and 3.0 m*M*), the inhibition patterns became noncompetitive in nature and were not used for the calculation of K_i.

With these data, we have generated a model to predict the relative conversion rate of 4-chloronitrosobenzene to *N*-(4-chlorophenyl)glycolhydroxamic acid in tissues. Although the model was derived from data generated from yeast transketolase, we feel that such a model should at least approximate the situation within mammalian tissues. Normal *in vivo* equilibrium concentrations of total pentose phosphate in rat tissues have been reported to range from a low in muscle of 220 μmol/kg, to highs in liver (480 μmol/kg) and kidney (720 μmol/kg) (*13*). Transketolase activities in these tissues roughly parallel pentose phosphate

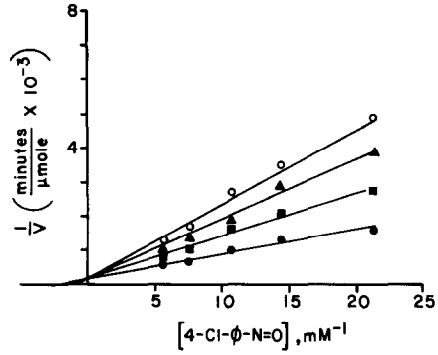

FIG. 2. Double reciprocal plot of kinetic data obtained on the ribose-5-phosphate-inhibited enzymatic conversion of 4-chloronitrosobenzene to 4-(chlorophenyl)glycolhydroxamic acid: (●) in the absence of D-ribose 5-phosphate; (■) in the presence of 0.25 m*M* D-ribose 5-phosphate; (▲) in the presence of 0.5 m*M* D-ribose 5-phosphate; (○) in the presence of 1.0 m*M* D-ribose 5-phosphate. The condition of incubation are described in the Experimental section under Assay Procedure.

concentrations (*14*). The total pentose phosphate concentration has been shown to consist of equimolar concentrations of xylulose-5-phosphate, ribose-5-phosphate, and ribulose-5-phosphate (*10*); thus, tissue concentrations of ribose-5-phosphate are about 70–240 μmol/kg tissue. Knowledge of the ribose-5-phosphate concentration in a specific tissue, coupled with K_i data, lends itself to extrapolations of nitrosoaromatic conversion by that tissue. Since the value for the K_m of an enzyme is unaffected by substrate concentration, the exact enzyme activity in that tissue is not critical. The rate of nitrosoaromatic conversion can simply be determined by plotting percentage maximum rate of conversion against the concentration of the nitrosoaromatic (at a specific ribose-5-phosphate concentration). Half-maximal inhibition of the nitrosoaromatic to hydroxamate conversion were found to occur at 0.35 m*M* ribose-5-phosphate, a concentration which ostensibly never occurs *in vivo*. Therefore, from a kinetic viewpoint, this enzymatic production of hydroxamic acid should occur in the presence of normal concentrations of the sugar phosphate acceptor, ribose-5-phosphate. Thus, 4-chloronitrosobenzene can actively compete for "active glycoaldehyde" generated by transketolase *in vitro*.

As can be seen from Fig. 3, at 0.25 m*M* ribose-5-phosphate, a concentration which approximates liver and kidney concentrations, the percentage of maximum conversion rate of 4-chloronitrosobenzene to hydroxamic acid was a linear function of its concentration. As lower concentrations of the nitrosoaromatic were utilized, the rate of conversion decreased to a minimum value of about 50%. Therefore, one can postulate that at very low concentrations of nitrosoaromatic, as expected for a xenobiotic, such compounds are converted *in vivo* at significant rates approximating 50% of their conversion rate in the absence of ribose-5-phosphate.

A correlation of yeast transketolase activity with transketolase activity in higher organisms may seem speculative. However, considering that the action of transketolase—that of ketol group transfer to an aldehyde acceptor—is the same

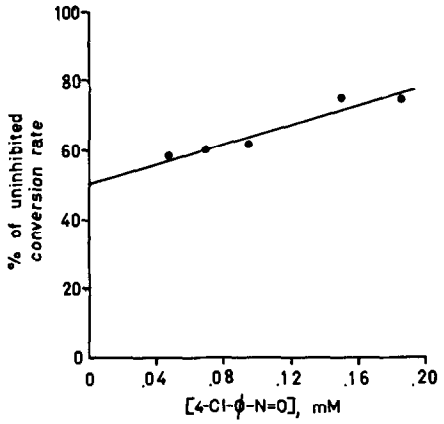

FIG. 3. Rates of 4-chloronitrosobenzene conversion to 4-(chlorophenyl)glycolhydroxamic acid in the presence of 0.25 m*M* ribose-5-phosphate. All rates were determined spectrophotometrically (320 nm) at 22°C in a Beckman Model 24 spectrophotometer. The conversion rate was determined at each 4-chloronitrosobenzene concentration in the presence (inhibited) and absence (uninhibited) at 0.25 m*M* ribose-5-phosphate. The data are expressed as the "percentage of the uninhibited conversion rate" at each selected concentration. The conditions of incubation are further described in the Experimental section under "Assay Procedure."

regardless of the utilized substrates (*10*) such a comparison is intuitively justified. A number of compounds serve as ketol group donors, or as aldehyde acceptors (*10*). Our data establish that yeast transketolase will also utilize 4-chloronitrosobenzene as an aldehyde acceptor *in vitro*. It is reasonable to expect a similar reaction for mammalian transketolases. Recent *in vitro* studies in our laboratory have shown that rat liver homogenates produce *N*-(4-chlorophenyl)glycolhydroxamic acid in appreciable quantities when incubated in the presence of thiamine pyrophosphate, Mg^{2+}, and donor sugar phosphate (*15*). Previously we found that intact algal cells also convert nitrosoaromatics to hydroxamic acids (*16*). With this evidence, it is our opinion that the production of glycolhydroxamic acids from aromatic nitroso compounds in mammalian systems is the result of transketolase action. The results of the present study clearly demonstrate the probability that glycolhydroxamate production by complex homogenates and by intact cells is the result of transketolase activity.

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