Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.
Saitoh, T., & Changeux, J. P. (1980) *Eur. J. Biochem. 105*, 51–62.
Saitoh, T., Wennogle, L. P., & Changeux, J. P. (1979) *FEBS Lett. 108*, 489–494.
Saitoh, T., Oswald, R., Wennogle, L. P., & Changeux, J. P. (1980) *FEBS Lett. 116*, 30–36.
Sobel, A., Weber, M., & Changeux, J. P. (1977) *Eur. J. Biochem. 80*, 215–224.
Strader, C. D., & Raftery, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5807–5811.
Suarez-Isla, B. A., & Hucho, F. (1977) *FEBS Lett. 75*, 65–69.
Teichberg, V. I., Sobel, A., & Changeux, J. P. (1977) *Nature (London) 267*, 540–542.
Tzartos, S., & Lindstrøm, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.
Waksman, G., Oswald, R., Changeux, J. P., & Roques, B. (1980) *FEBS Lett. 111*, 23–28.
Wennogle, L. P., & Changeux, J. P. (1980) *Eur. J. Biochem. 106*, 381–393.
Witzemann, V., & Raftery, M. A. (1978a) *Biochemistry 17*, 3598–3604.
Witzemann, V., & Raftery, M. A. (1978b) *Biochem. Biophys. Res. Commun. 85*, 623–631.

Steady-State Kinetics and Inhibition Studies of the Aldol Condensation Reaction Catalyzed by Bovine Liver and *Escherichia coli* 2-Keto-4-hydroxyglutarate Aldolase†

Sharon R. Grady,‡ Janet K. Wang,§ and Eugene E. Dekker*

ABSTRACT: Two sensitive assays, one which fluorometrically measures only the L isomer of 2-keto-4-hydroxyglutarate after decarboxylation to L-malate and the other which spectrophotometrically determines both enantiomers by reductive amination with glutamate dehydrogenase, are described. By use of these assays, the steady-state kinetics of the aldol condensation of pyruvate with glyoxylate, as catalyzed by 2-keto-4-hydroxyglutarate aldolase from either bovine liver or *Escherichia coli*, were studied as was the inhibition of this reaction by glyoxylate and other anions. For the *E. coli* aldolase, double-reciprocal plots are linear except at high (above 5 mM) glyoxylate concentrations; apparent K_m values increase with increasing concentrations of the fixed substrate. The data are consistent with an ordered reaction sequence. Inhibition by halides follows the lyotropic or Hofmeister series. Esters are not good inhibitors; mono-, di-, and tricarboxylic acids are increasingly inhibitory. Of the substrate analogues tested, hydroxypyruvate is the most potent inhibitor. Inhibition studies with citrate, acetaldehyde, and glyoxylate (all competitive inhibitors) suggest there are two domains at the active site—the Schiff base forming lysyl residue which interacts with carbonyl analogues (like acetaldehyde) and a center of positive charge which binds anions (like citrate). In contrast to the bacterial enzyme, liver 2-keto-4-hydroxyglutarate aldolase is inhibited in a competitive manner by much lower concentrations (0.1 mM or even lower) of glyoxylate. Many salts and some carboxylic acids activate the liver enzyme. Similarly, substrate analogues like 2-ketobutyrate and fluoropyruvate are mild activators; no effect is seen with acetaldehyde. Besides glyoxylate, only glyoxal, 2-ketoglutarate, and hydroxypyruvate inhibit the aldol condensation reaction. A uniform value of 1 is found for the number of inhibitor molecules bound per active site of either liver or *E. coli* 2-keto-4-hydroxyglutarate aldolase.

2-Keto-4-hydroxyglutarate aldolase (2-keto-4-hydroxyglutarate glyoxylate-lyase) catalyzes the dealdolization of kHOGlt[1] (kHOGlt ⇌ pyruvate + glyoxylate), a terminal step in the degradation of L-hydroxyproline by mammals (Adams & Goldstone, 1960a,b; Maitra & Dekker, 1963). It catalyzes an analogous reaction (2-keto-4-hydroxybutyrate ⇌ pyruvate + formaldehyde), albeit less effectively, in L-homoserine catabolism (Lane et al., 1971). This enzyme, prepared in highly purified or homogeneous form from either rat liver (Maitra & Dekker, 1964; Adams, 1971b), bovine liver (Dekker et al., 1975a), or *Escherichia coli* (Dekker et al., 1975b), is a Schiff-base mechanism (class I type) aldolase. When compared with other class I aldolases, kHOGlt-aldolase is uniquely atypical in that it (1) forms a Schiff-base intermediate not only between the ε-amino group of an active-site lysyl residue and kHOGlt or pyruvate (as required mechanistically) but *also* with glyoxylate (thereby forming a dead-end or "abortive" Schiff base) (Kobes & Dekker, 1971a; Nishihara & Dekker, 1972), (2) is completely and irreversibly inactivated by cyanide *only* in the presence of glyoxylate (or other low molecular weight aldehydes) with stable formation of an aminonitrile (Hansen et al., 1974), and (3) is bifunctional, effectively catalyzing the β-decarboxylation of oxaloacetate as well as the aldol cleavage or formation of kHOGlt (Kobes & Dekker, 1971b).

Rosso & Adams (1967), in describing kHOGlt-aldolase purified from rat liver, noted that kHOGlt formation is inhibited by glyoxylate. For examination of this specific observation together with the general kinetics of the reaction

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109. *Received April 15, 1980; revised manuscript received December 4, 1980.* This research was supported by the U.S. Public Health Service, National Institutes of Health Grant AM-03718.

‡ Predoctoral Trainee of the U.S. Public Health Service, Grant GM-00187. Present address: National Jewish Hospital and Research Center, Denver, CO 80206.

§ Present address: Department of Biochemistry and Chemistry, Southern Illinois University, Carbondale, IL 62901.

[1] Abbreviations used: kHOGlt, 2-keto-4-hydroxyglutarate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/81/0420-2497$01.25/0

more fully, sensitive and reliable procedures for following kHOGlt formation had to be established. Using homogeneous preparations of this novel aldolase that are now available to us from either bovine liver or *E. coli* (Dekker et al., 1975a,b) and newly developed assay procedures, we studied the steady-state kinetics of the aldol condensation of pyruvate with glyoxylate as well as inhibition of this reaction by glyoxylate and numerous other anions.

Materials and Methods

DL-kHOGlt was synthesized from *threo*-γ-hydroxy-DL-glutamate by nonenzymic transamination (Maitra & Dekker, 1963). Ultrol grade Hepes from Calbiochem Corp. was used; all other compounds including salts, substrate analogues, carboxylic acids, etc., were either reagent grade or the highest quality compounds commercially available from such companies as Sigma, Calbiochem, Eastman, Aldrich, Mallinckrodt, and Baker Chemical Co. Cells of *Escherichia coli* K-12 were grown and harvested as described (Dekker et al., 1975b). kHOGlt-aldolase was purified from extracts of bovine liver or *E. coli* according to the procedures of Dekker et al. (1975a,b); final preparations had specific activities of approximately 200 units/mg and 50 units/mg, respectively, where one unit is defined as the amount of protein that catalyzes the formation of 1 μmol of glyoxylate from DL-kHOGlt in 20 min under standard conditions (Maitra & Dekker, 1964). Homogeneity of enzyme preparations was determined by polyacrylamide gel electrophoresis. Beef liver catalase, beef liver glutamate dehydrogenase, and pig heart malate dehydrogenase were products of Worthington Biochemical Corp., Boehringer Mannheim, and Calbiochem, respectively. Absorbance measurements at a set wavelength were made with a Gilford Model 2000 spectrophotometer. Absorbance readings were either displayed on a digital readout meter or automatically plotted with the aid of a Honeywell recorder. A ratio fluorometer (Farrand Optical Co., Inc.) was used for fluorometric measurements.

Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard. In one assay of enzymatic activity, aldolase-catalyzed synthesis of kHOGlt was measured by peroxide decarboxylation of kHOGlt to malate. After enzyme reaction mixtures (0.5 mL) were incubated, 0.2 mL of 0.75 M acetate buffer, pH 5.0, containing 3% H_2O_2 was added to stop the reaction as well as to decarboxylate the product (kHOGlt) and substrates (pyruvate and glyoxylate). The resulting mixtures were incubated at 37 °C for 7 min to ensure complete decarboxylation. The pH of the solutions was then adjusted to approximately 8–9 with alkali and excess H_2O_2 destroyed by subsequently adding 1 μL (160 units) of catalase and incubating the mixtures for 5 min at 37 °C. L-Malate was measured fluorometrically in aliquots of the final solutions by method I of Lowry & Passonneau (1972). Malate dehydrogenase was added last; linearity of the reaction was determined as a function of both time and enzyme concentration. This assay is specific for L-malate (formed by decarboxylation of L-kHOGlt) and is not affected by the presence of D-malate (derived from D-kHOGlt). All rate data were determined from the slopes of plots (i.e., micromoles of malate formed vs. time); at least three time points were used. Rates are expressed as the number of micromoles of kHOGlt synthesized in 10 min/unit of enzyme. Assays with the *E. coli* aldolase, done over the course of 8 months with five separate enzyme preparations using 10 mM pyruvate and 1 mM glyoxylate, gave an average rate of 0.448 μmol of kHOGlt synthesized (10 min)$^{-1}$ (unit of enzyme)$^{-1}$ [±0.070 (SD) (n = 13)].

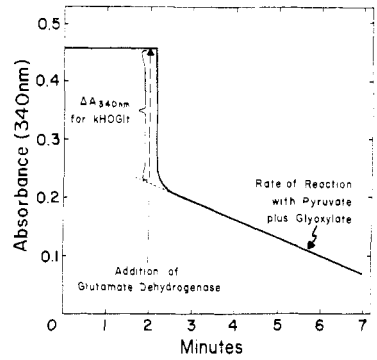

FIGURE 1: Measurement of DL-kHOGlt by glutamate dehydrogenase in the presence of pyruvate and/or glyoxylate. Besides the components listed in the text, the assay mixture contained 0.043 mM DL-kHOGlt, 0.5 mM sodium pyruvate, and 0.5 mM sodium glyoxylate. Glutamate dehydrogenase was added at the time shown on this recorder tracing. The absorbance scale is offset approximately 0.7 unit.

Since kHOGlt-aldolase from bovine liver or *E. coli* is known to catalyze the synthesis of a mixture of the two isomers of kHOGlt (Kobes & Dekker, 1971b; Nishihara & Dekker, 1972), another assay was necessary to determine total (D + L)kHOGlt formed. A modification of the method of Adams (1971a) was used. Glutamate dehydrogenase catalyzes the reduction of both kHOGlt isomers in the presence of NADH and NH_4^+, yielding *erythro*- and *threo*-γ-hydroxy-L-glutamate; the reaction is quantitated from the observed change in absorbance at 340 nm and the known extinction coefficient for NADH (Rafter & Colowick, 1957). Reaction with kHOGlt is extremely rapid but quite slow with pyruvate and/or glyoxylate; hence, the procedure is applicable in the presence of reasonably high levels of the latter two compounds. The assay mixture contained the sample of DL-kHOGlt to be measured, 100 mM potassium phosphate buffer, pH 7.4, 50 mM $(NH_4)_2HPO_4$, pH 8.0, and 0.25 mM NADH in a final volume of 1 mL. The initial absorbance at 340 nm is read, bovine liver glutamate dehydrogenase [18 units or 0.2 mg of a suspension in 2 M $(NH_4)_2SO_4$] is then added at a set time, and the reaction is allowed to proceed until only a slow rate of absorbance change is observed. Figure 1 shows a typical recording obtained in this assay procedure. Extrapolation of the slow rate of reaction with pyruvate and glyoxylate to the time of glutamate dehydrogenase addition gives the absorbance change due to reductive amination of kHOGlt. Working with known amounts of DL-kHOGlt, we found good agreement with the observed absorbance change at 340 nm. The same values were obtained in the presence or absence of pyruvate and/or glyoxylate.

For *E. coli* kHOGlt-aldolase, almost identical results were obtained with the glutamate dehydrogenase and malate dehydrogenase assays (for 12 samples, the average ratio of the former to the latter assay was 1.07). In the malate assay, therefore, no correction was applied to data obtained with the bacterial enzyme. A 50% correction, however, was found to be necessary in working with kHOGlt-aldolase from bovine liver. These results are entirely consistent with earlier experiments which showed that the liver enzyme is completely nonstereospecific (Kobes & Dekker, 1971b) while the *E. coli* aldolase markedly favors cleavage or synthesis of L-kHOGlt (Nishihara & Dekker, 1972).

Results

The steady-state kinetics of the aldol condensation of pyruvate with glyoxylate, catalyzed by *E. coli* kHOGlt-aldolase, are presented as reciprocal and secondary plots in Figure 2. Double-reciprocal plots are linear except at high (above 5 mM)

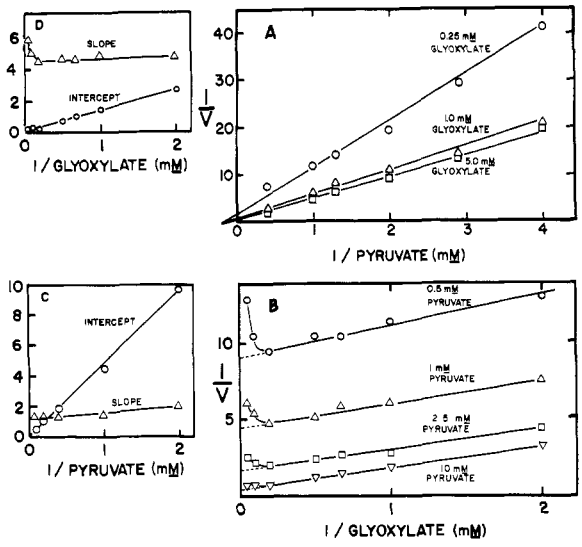

FIGURE 2: Reciprocal and secondary plots for the aldol condensation of pyruvate with glyoxylate (i.e., kHOGlt synthesis) catalyzed by *E. coli* kHOGlt-aldolase. Incubation mixtures (0.5 mL) contained 50 mM sodium Hepes buffer, pH 8.2, 0.2–0.3 unit of enzyme, and levels of substrates as shown. Concentration units are millimolar. kHOGlt was measured as malate; see Materials and Methods. Linear plots were determined by the least-squares method.

glyoxylate concentrations. The data are consistent with an ordered reaction sequence.

For both pyruvate and glyoxylate, apparent K_m values increase with increasing concentrations of the fixed substrate. Although this is typical of Ping-Pong mechanisms, it also occurs in sequential mechanisms when K_{ia} is smaller than K_a (Plowman, 1972). The equation applicable to sequential reactions (eq 1) approaches that for Ping-Pong mechanisms (eq 2) when $K_{ia} \ll K_a$ (Plowman, 1972).

$$K_a^{app} = \frac{K_a(1 + K_{ia}K_b/K_aB)}{1 + K_b/B} \quad (1)$$

$$K_a^{app} = \frac{K_a}{1 + K_b/B} \quad (2)$$

The observed convergence points in negative coordinates (Figure 2A,B) are compatible with this explanation. Convergence point coordinates for sequential bireactant mechanisms are $-1/K_{ia}$ and $1/V_{max}(1 - K_a/K_{ia})$ (Plowman, 1972). To have an intersection point below the x axis, K_{ia} must be smaller than K_a. The K_i values for pyruvate and glyoxylate are 0.28 mM (Figure 2A) and 0.08 mM (Figure 2B), respectively. K_m values for pyruvate and glyoxylate can be calculated from the secondary plots (Figure 2C,D). When Hofstee plots and least-squares analysis of the data are used, a K_m value of 14.3 ± 7.1 mM for glyoxylate is obtained and V_{max} = 10.5 ± 4.4 μmol of kHOGlt synthesized (10 min)$^{-1}$ (unit of enzyme)$^{-1}$. On the other hand, only an approximate K_m value for pyruvate can be projected from the linear portion of the lines plotted in Figure 2B; we estimate a value of ~45 mM.

Inhibition by glyoxylate appears to be competitive since the slope but not the intercept replot is nonlinear (Figure 2D). Futhermore, Figure 3 shows that the inhibition becomes linear at high glyoxylate concentrations, a result that indicates glyoxylate combines in a "dead-end" manner with the form of kHOGlt-aldolase that normally interacts with pyruvate (Cleland, 1970). Previously, in studies wherein ketimine– and aldimine–aldolase complexes were reduced with $NaBH_4$ (Nishihara & Dekker, 1972), it was found that glyoxylate competes for the same site on the enzyme; kinetic results and

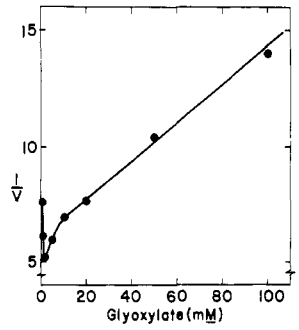

FIGURE 3: Glyoxylate inhibition of kHOGlt synthesis catalyzed by *E. coli* aldolase. Incubation mixtures (0.5 mL) contained 50 mM sodium Hepes buffer, pH 8.2, 1 mM sodium pyruvate, 0.2–0.3 unit of enzyme, and levels of sodium glyoxylate as shown. kHOGlt was determined as malate; see Materials and Methods.

Table I: Inhibition of kHOGlt Synthesis (*E. coli* Aldolase) by Halides[a]

halide added	concn (mM)	inhibition (%)
NaF	20	19
	50	28
NaCl	20	38
	50	59
NaBr	20	48
	50	65
NaI	20	59
	50	81

[a] Incubation mixtures (0.5 mL) contained 50 mM sodium Hepes buffer, pH 8.2, 0.2–0.3 unit of enzyme, 1 mM sodium glyoxylate, 10 mM sodium pyruvate, and the halide salt indicated. kHOGlt was determined as malate; see Materials and Methods.

substrate binding studies, therefore, are in agreement.

High salt concentrations were found to inhibit the synthesis of kHOGlt. The chloride salts of Na^+, K^+, Li^+, Cs^+, Rb^+, Mg^{2+}, and Ca^{2+} (all at an identical chloride ion concentration) were tested to determine if this was an effect of the cation or anion. The experimental conditions given in the legend to Table I were used. Essentially the same level of inhibition (i.e., 9% inhibition at 10 mM, final concentration, 41% inhibition at 40 mM, and 62% inhibition at 90 mM) was seen with each compound. The observed inhibition, therefore, is most likely due to the chloride ion. Table I shows that the degree of inhibition depends on the nature of the anion, progressively increasing through the series F^-, Cl^-, Br^-, I^-.

The possible inhibition caused by a number of substrate analogues, carboxylic acids, and esters was tested; the results are listed in Table II. In general, esters do not appear to be good inhibitors. Mono-, di-, and tricarboxylic acids are increasingly inhibitory, suggesting that the effect may be partially due to ionic strength. Of the substrate analogues tested, hydroxypyruvate is the most potent inhibitor. Actual data are not given, but when the inhibition patterns for selected compounds were determined (Dixon, 1953), it was found that sodium citrate, acetaldehyde, and hydroxypyruvate are competitive inhibitors with K_i values of 7.5 mM, 11 mM, and 12.5 μM, respectively. In contrast, noncompetitive inhibition is seen with sodium chloride; the convergence point of reciprocal plots at different chloride concentrations is not on the x axis.

The number of inhibitor molecules that bind can be determined by eq 3 (Bergmann & Segal, 1954; Nygaard, 1961;

$$\log [(V_0/V_i - 1)] = r \log I + C \quad (3)$$

Fridovich, 1963), where r is the number of moles of inhibitor bound per mole of active site, I is the inhibitor concentration, V_0 is the rate of the reaction in the absence of inhibitor, V_i

Table II: Effects of Substrate Analogues, Carboxylic Acids, and Salts on kHOGlt Synthesis Catalyzed by *E. coli* Aldolase[a]

compound (40 mM final concn)	inhibition (%)	compound (40 mM final concn)	inhibition (%)
formate	29	azelate	63
acetate	23	sebacate	62
propionate	22	citrate	63
valerate	28	tartrate	78
hexanoate	34	ethyl formate	11
heptanoate	34	ethyl acetate	0
oxalate	73	diethyl tartrate	11
malonate	53	Hepes	11[b]
succinate	57	chloride	34
glutarate	47	acetone	0
3,3-dimethyl-glutarate	49	acetaldehyde	67
adipate	53	2-ketobutyrate	44
pimelate	57	hydroxypyruvate	49[c]
suberate	62	hydroxypyruvate	100[d]

[a] Incubation mixtures (0.5 mL) contained 50 mM sodium Hepes buffer, pH 8.2, 0.2–0.3 unit of enzyme, 1 mM sodium glyoxylate, 10 mM sodium pyruvate, and the compound listed. kHOGlt was determined as malate; see Materials and Methods. [b] 40 mM Hepes in addition to the 50 mM normally present. [c] 0.02 mM final concentration. [d] 1 mM final concentration.

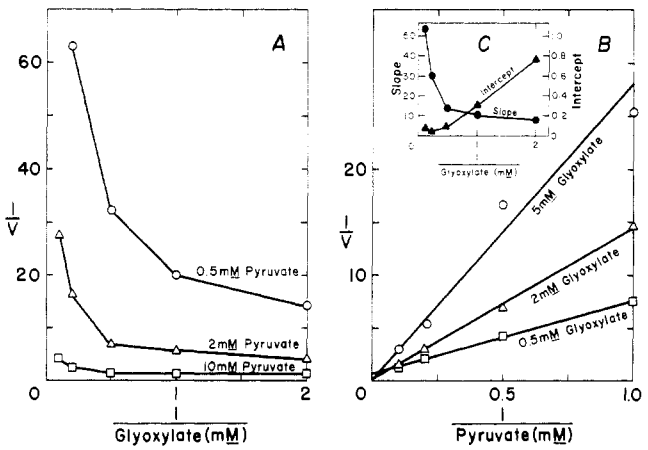

FIGURE 4: Reciprocal and secondary plots for the aldol condensation of pyruvate with glyoxylate (i.e., kHOGlt synthesis) catalyzed by bovine liver kHOGlt-aldolase. Incubation mixtures (0.5 mL) contained 50 mM sodium Hepes buffer, pH 8.2, 0.24 unit of enzyme, and levels of substrates as shown. Rates are expressed as μmol of kHOGlt synthesized in 10 min per unit of enzyme. kHOGlt was measured by the glutamate dehydrogenase assay; see Materials and Methods (Figure 1).

is the rate with inhibitor, and $C = \log [K_m/[K_i (K_m + S)]]$. A plot of $\log [(V_0/V_i - 1)]$ vs. log I will have a slope of r. Values of r determined for citrate, acetaldehyde, hydroxypyruvate, and chloride are 0.84, 1.04, 1.12, and 0.91, respectively. When high concentrations of glyoxylate are used, 1.2–1.4 mol is bound as an inhibitor. When considered in the context that 1 mol of either substrate (pyruvate or glyoxylate) is bound by this enzyme (Nishihara & Dekker, 1972), these data indicate that only one molecule of each type of inhibitor can bind per active site of *E. coli* kHOGlt-aldolase.

The steady-state kinetics for the aldol condensation reaction catalyzed by bovine liver kHOGlt-aldolase are shown in Figure 4A–C. Reciprocal plots involving pyruvate are linear (Figure 4B), but those with glyoxylate show extensive substrate inhibition (Figure 4A). Secondary plots from slopes and intercepts of Figure 4B are given in Figure 4C; both the slope and the intercept are affected by glyoxylate. The intercept effect, however, is not apparent until the glyoxylate concentration is over 2 mM whereas the slope effect is seen at all

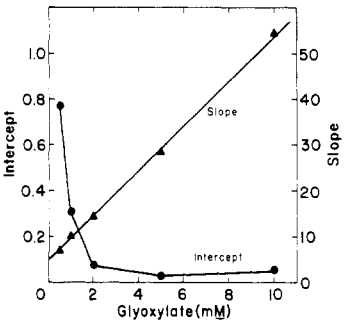

FIGURE 5: Glyoxylate inhibition of kHOGlt synthesis catalyzed by bovine aldolase. Experimental conditions are the same as in the legend to Figure 4.

Table III: Effects of Substrate Analogues, Carboxylic Acids, and Salts on kHOGlt Synthesis Catalyzed by the Bovine Liver Aldolase

addition to standard assay[a]	final concn (mM)	percent initial activity
sodium glyoxylate[b]	1	63
	4	30
	9	18
glyoxal	20	64
	50	44
acetaldehyde	20	99
	50	99
sodium fluoropyruvate	20	125
lithium hydroxypyruvate	0.05	80
	1.0	44
	20	9
sodium 2-ketobutyrate	20	123
	50	181
sodium 2-ketoglutarate	20	88
	50	32
sodium acetate	20	184
sodium oxalate	20	165
sodium citrate	20	108
sodium chloride	20	224
potassium chloride	20	243
lithium chloride	20	133

[a] The standard assay mixture (0.5 mL) contained 5 mM sodium Hepes buffer, pH 8.2, 1 mM sodium pyruvate, 1 mM sodium glyoxylate, and 0.2–0.3 unit of liver enzyme. kHOGlt was determined by the glutamate dehydrogenase assay; see Materials and Methods (Figure 1). [b] Amount in addition to 1 mM present in the assay mixture.

glyoxylate concentrations tested (0.5 mM and higher). Further assays indicated that inhibition by glyoxylate occurs at concentrations as low as 0.1 mM (possibly even lower). Glyoxylate inhibition is linear with respect to the slope effects (Figure 5), indicating inhibition is of the linear competitive type at low levels of glyoxylate with some small intercept effect superimposed at higher concentrations (Figure 4C). Hyperbolic activation by glyoxylate is suggested by the intercept plot (Figure 5). This activation might be expected since glyoxylate is a substrate and implies that its K_m value is in the millimolar range. Since the secondary plots are nonlinear, K_m values cannot be calculated.

How specific is glyoxylate inhibition of bovine liver kHOGlt-aldolase activity? We found that pyruvate does not inhibit the aldol condensation reaction even at high concentrations (i.e., 200 mM). Many salts (like KCl) and some carboxylic acids (i.e., sodium acetate or sodium oxalate) activate rather than inhibit whereas sodium citrate has little effect (see Table III). Similarly, substrate analogues like 2-ketobutyrate and fluoropyruvate are mild activators, but no effect is seen with acetaldehyde. Besides glyoxylate, only

glyoxal, 2-ketoglutarate, and hydroxypyruvate inhibit the aldol condensation reaction, with hydroxypyruvate being slightly more effective than glyoxylate. Hydroxypyruvate and 2-ketoglutarate were determined to be competitive inhibitors, with K_i values of 0.7 mM and 31 mM, respectively. Glyoxal is also a competitive inhibitor at concentrations up to 20 mM (K_i = 28 mM) but is noncompetitive at 50 mM. The pattern of glyoxal inhibition may be similar to that with glyoxylate where slope (competitive inhibition) and not intercept effects are noticeable at low concentrations.

Plots of log $[(V_0/V_i - 1)]$ vs. log [I] for 2-ketoglutarate, glyoxal, and hydroxypyruvate gave r values of 0.95, 0.99, and 0.90, respectively.

Discussion

The kinetic properties of kHOGlt-aldolase from *E. coli* and bovine liver are quite different. Glyoxylate, which is physiologically a product of the reaction and is chemically similar to pyruvate, strongly inhibits the liver enzyme. A priori, this effect might be expected since the degree of specificity required to eliminate such product inhibition would be high and only a low evolutionary advantage gained (Cleland, 1970). Any complications that might potentially occur as a consequence of glyoxylate inhibition of this reaction are essentially removed, of course, if low in vivo glyoxylate levels are maintained. The *E. coli* aldolase, in contrast, is only mildly inhibited in a linear competitive fashion by glyoxylate as well as by many other compounds. In bacteria, therefore, there is a somewhat better basis to suggest this enzyme-catalyzed aldol-type reaction may accomplish either C–C bond cleavage or formation in vivo.

While several classes of compounds (including salts and mono-, di-, and tricarboxylic acids as well as many substrate analogues) inhibit *E. coli* kHOGlt-aldolase, only certain substrate analogues inhibit the liver aldolase. Somewhat surprisingly, analogues found to be inhibitory in the studies reported here do not necessarily cause inactivation when incubated with the enzyme in the presence of sodium borohydride (Kobes & Dekker, 1971b; Nishihara & Dekker, 1972). For example, treatment of either liver or *E. coli* kHOGlt-aldolase with borohydride in the presence of hydroxypyruvate causes no inactivation, yet it is a good inhibitor of the enzyme from either biological source. On the other hand, treatment of the liver aldolase with hydroxypyruvate in the presence of cyanide does cause irreversible inactivation (Hansen, 1971). The substrate analogues, 2-ketobutyrate and acetaldehyde, do not inhibit catalysis of C–C bond formation by the liver enzyme, but both compounds do inactivate the enzyme (appreciably or mildly, respectively) in the presence of borohydride (Kobes & Dekker, 1971b) or cyanide (Hansen, 1971; Hansen et al., 1974). The basis for these differing effects is not immediately apparent. One difference to be noted is in the design of the experiments; glyoxylate, an extremely good inhibitor of liver kHOGlt-aldolase, is present in the inhibition experiments but not in the activation studies. Conceivably, competition by glyoxylate can mask weak inhibitory effects.

A uniform value of 1 is found for the number of inhibitor molecules bound per active site of liver or *E. coli* kHOGlt-aldolase. These results are in line with earlier data obtained by different procedures; when ketimine or aldimine intermediates are stabilized by reduction with borohydride (Kobes & Dekker, 1971a; Nishihara & Dekker, 1972) or cyanide addition (Hansen et al., 1974), 1 mol of either kHOGlt, pyruvate, or glyoxylate is bound per mol of enzyme.

Inhibition of the *E. coli* aldolase by halides appears to follow the lyotropic or Hofmeister series (West et al., 1966; von Hippel & Schleich, 1969). These effects, however, may be partially electrostatic in nature (Warren et al., 1966; von Hippel & Schleich, 1969) since the concentrations required to cause inhibition are much lower than usual. Similar results have been reported for acetoacetate decarboxylase (Fridovich, 1963) and fumarylacetoacetate fumarylhydrolase (Braun & Schmidt, 1973), both of which also have negatively charged substrates.

Inhibition studies with *E. coli* kHOGlt-aldolase indicate that there are two distinct domains at the active site. One would be the Schiff base forming lysyl residue which interacts with substrate analogues having a carbonyl group (like acetaldehyde). The other is most likely a center of positive charge which binds anions, including inhibitors like citrate. The active site includes both domains since both types of inhibitors are competitive with kHOGlt synthesis. Glyoxylate, having both functionalities, appears to manifest its inhibitory effect primarily by interaction with the carbonyl binding site (i.e., the normal pyruvate site). It is quite possible, of course, that the two domains are in juxtaposition; this seems reasonable in that a bulky anionic compound like citrate can prevent binding of the carbonyl substrate (i.e., is competitive) whereas chloride ion only masks the anion-binding domain (i.e., is noncompetitive). Wang & Himoe (1974) envisaged a similar situation for enolase inhibition by fluoride. Inhibition in this instance, although not competitive, was considered to be the consequence of fluoride being able to block a cationic site (which normally accepts hydroxide ion generated from the β-hydroxyl group of 2-phosphoglycerate) but not being large enough to prevent phosphoglycerate binding. Somewhat analogous considerations may apply for chloride interaction with the glyoxylate binding site of *E. coli* kHOGlt-aldolase.

The results reported here place kHOGlt-aldolase within a growing group of enzymes that appear to have a nonspecific anion-binding locus in the active site; quite frequently, this is true for enzymes whose substrates themselves are anions under physiological conditions. Aspartate transaminase (Cheng & Martinez-Carrion, 1972), ascorbate oxidase (Gerwin et al., 1974), fumarylacetoacetate fumarylhydrolase (Braun & Schmidt, 1973), and serine transhydroxymethylase (Schrich & Diller, 1971), to mention a few, are all nonspecifically but competitively inhibited by anions. Acetoacetate decarboxylase is also inhibited by many anions, but the inhibition is not competitive with respect to substrate (Fridovich, 1963).

Acknowledgments

We thank Dr. Fedor Medzihradsky for guidance in developing the assays and use of his fluorometer.

References

Adams, E. (1971a) *Methods Enzymol. 17B*, 275–276.
Adams, E. (1971b) *Methods Enzymol. 17B*, 280–284.
Adams, E., & Goldstone, A. (1960a) *J. Biol. Chem. 236*, 3492–3498.
Adams, E., & Goldstone, A. (1960b) *J. Biol. Chem. 235*, 3504–3512.
Bergmann, F., & Segal, R. (1954) *Biochem. J. 58*, 692–698.
Braun, C. V., & Schmidt, D. E., Jr. (1973) *Biochemistry 12*, 4878–4881.
Cheng, S., & Martinez-Carrion, M. (1972) *J. Biol. Chem. 247*, 6597–6602.
Cleland, W. W. (1970) *Enzymes 2*, 1–65.
Dekker, E. E., Kobes, R. D., & Grady, S. R. (1975a) *Methods Enzymol. 42*, 280–285.
Dekker, E. E., Nishihara, H., & Grady, S. R. (1975b) *Methods Enzymol. 42*, 285–290.
Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Fridovich, I. (1963) *J. Biol. Chem. 238*, 592–598.
Gerwin, B., Burstein, S. R., & Westley, J. (1974) *J. Biol. Chem. 249*, 2005–2008.
Hansen, B. A. (1971) Ph.D. Thesis, The University of Michigan, Ann Arbor, MI.
Hansen, B. A., Lane, R. S., & Dekker, E. E. (1974) *J. Biol. Chem. 249*, 4891–4896.
Kobes, R. D., & Dekker, E. E. (1971a) *Biochemistry 10*, 388–395.
Kobes, R. D., & Dekker, E. E. (1971b) *Biochim. Biophys. Acta 250*, 238–250.
Lane, R. S., Shapley, A., & Dekker, E. E. (1971) *Biochemistry 10*, 1353–1363.
Lowry, O. H., & Passonneau, J. V. (1972) in *A Flexible System of Enzymatic Analysis*, p 201, Academic Press, New York.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maitra, U., & Dekker, E. E. (1963) *J. Biol. Chem. 238*, 3660–3669.
Maitra, U., & Dekker, E. E. (1964) *J. Biol. Chem. 239*, 1485–1491.
Nishihara, H., & Dekker, E. E. (1972) *J. Biol. Chem. 247*, 5079–5087.
Nygaard, A. P. (1961) *J. Biol. Chem. 236*, 2779–2782.
Plowman, K. M. (1972) in *Enzyme Kinetics*, pp 46–53, McGraw-Hill, New York.
Rafter, G. W., & Colowick, S. P. (1957) *Methods Enzymol. 3*, 887–890.
Rosso, R. G., & Adams, E. (1967) *J. Biol. Chem. 242*, 5524–5534.
Schirch, L., & Diller, A. (1971) *J. Biol. Chem. 246*, 3961–3966.
von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 418–574, Marcel Dekker, New York.
Wang, T., & Himoe, A. (1974) *J. Biol. Chem. 249*, 3895–3902.
Warren, J. G., Stowring, L., & Morales, M. F. (1966) *J. Biol. Chem. 241*, 309–316.
West, E. S., Todd, W. R., Mason, H. S., & van Bruggen, J. T. (1966) in *Textbook of Biochemistry*, 4th ed., pp 93–95, Macmillan, New York.