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Comparative Kinetics of Mg^{2+}-, Mn^{2+}-, Co^{2+}-, and Ni^{2+}-Activated Glyoxalase I. Evaluation of the Role of the Metal Ion†

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ABSTRACT: The disproportionation of the hemimercaptals of glutathione and α-ketoaldehydes into the corresponding thiol esters of glutathione and α-D-hydroxycarboxylic acids, catalyzed by rat erythrocyte glyoxalase I, requires a divalent metal ion. Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} were compared for their effects on a number of parameters: (1) V_{max} values using methylglyoxal as the α-ketoaldehyde agree within a factor of two for all four M^{2+}–glyoxalase I; (2) activation volumes, $\Delta V^{\ddagger}$, are similar (14 ± 2 cm^3/mol) regardless of which metal ion is present; (3) the stereospecific transfer of hydrogen from the hemimercaptal carbon to the α-ketone is insensitive to changes in the metal ion, giving 95 ± 2% D-lactate after hydrolysis of the thiol ester obtained from the disproportionation of the glutathione hemimercaptal of methylglyoxal; (4) deuterium isotope effects using phenylglyoxal and α-deuteriophenylglyoxal were observed on V_{max} for all four M^{2+}–glyoxalase I indicating that the breaking of the C–H bond of the hemimercaptal is rate determining. These results lead to the conclusion that the metal ion participates in the glyoxalase I reaction as a superacid which is able to polarize the α-ketone group to facilitate the disproportionation reaction. To support the proposal for a role for the metal ion at the active site of the enzyme, the approximate location of the metal ion was determined. The kinetics of inactivation of glyoxalase I by dansyl chloride indicate that the dansyl group modifies a residue at or near the active site. Dansylated apoglyoxalase I when titrated with Mn^{2+} shows extensive fluorescence quenching, indicating that the metal ion is binding close to the dansyl group.

The disproportionation of α-ketoaldehydes, such as methylglyoxal, into the corresponding α-hydroxycarboxylic acids proceeds by two enzyme-catalyzed reactions. The first reaction, catalyzed by glyoxalase I (*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5), requires glutathione as cofactor and involves the conversion of an α-ketoaldehyde into a thiol ester of glutathione and an α-hydroxycarboxylic acid; the second reaction, catalyzed by glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), involves the hydrolysis of the thiol ester to regenerate glutathione and liberate a free α-hydroxycarboxylic acid. There has been considerable interest in the mechanism of glyoxalase I especially regarding the involvement of a one- or a two-substrate pathway (Cliffe and Waley, 1961; Kermack and Matheson, 1957). Because glutathione adds to the α-ketoaldehyde rapidly and reversibly in a nonenzymic reaction, the determination of whether the hemimercaptal is the substrate for glyoxalase I or whether the α-ketoaldehyde and glutathione are the substrates has not been a simple problem. Our earlier studies on yeast glyoxalase I suggested that the one-substrate mechanism is the major pathway (Vander Jagt et al., 1975). In addition, yeast glyoxalase I shows very broad specificity for both aliphatic and aromatic α-ketoaldehydes with little sensitivity in its kinetic parameters V_{max} and K_m to variations in the substrate (Vander Jagt et al., 1972a). This is in contrast to the disproportionation of α-ketoaldehydes in alkaline solution, a model for glyoxalase I, where the polarity of the α-ketone group is a major factor in the rate of reaction (Vander Jagt et al., 1972b). For both the yeast glyoxalase I

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reaction and the model reaction, intramolecular hydride migration appears to be the rate-determining step (Vander Jagt and Han, 1973).

The present study involves an evaluation of the role of the divalent metal ion in glyoxalase I. All sources of glyoxalase I thus far examined contain Mg^{2+}. The participation of Mg^{2+} in the glyoxalase I reaction was suggested previously, although no experimental data are available to support the direct participation of Mg^{2+} (Davis and Williams, 1966). One problem with using yeast glyoxalase I for studies on the role of the divalent metal ion is that metal ion exchange has not yet been accomplished. Glyoxalase I from mammalian sources, however, is amenable to replacement of the metal ion (Davis and Williams, 1966; Mannervik et al., 1972; Uotila and Koivusalo, 1975). We report here results from a study of the comparative kinetics of glyoxalase I from rat erythrocytes where the Mg^{2+} was replaced by various divalent cations.

Experimental Section

Materials

Glyoxalase I from rat erythrocytes was purified by the procedure reported recently (Han et al., 1976). The enzyme preparation used in this study was a 9000-fold purified preparation which was nearly homogeneous on polyacrylamide gel electrophoresis. Glutathione (Sigma) was >99% pure by sulfhydryl titration with *N*-ethylmaleimide. Commercial methylglyoxal was purified by distillation. The aromatic α-ketoaldehydes and the deuterated analogues were prepared as described previously (Vander Jagt et al., 1972b; Vander Jagt and Han, 1973). *N*,*N*-Diethylcysteamine (Aldrich) was standardized by sulfhydryl titration with *N*-ethylmaleimide. Chlorides of Mg^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} were reagent grade chemicals and were used directly. Dansyl chloride (Sigma) was used as purchased. Solutions of the various metal ions were prepared using distilled, deionized water which had been passed several times through a 2 × 30 cm column of Chelex 100 (Bio-Rad).

Methods

Kinetics. Kinetics studies were carried out using a Gilford 222 modified Beckman DU with temperature controlled by a circulating water bath. For first-order kinetics, rate constants were obtained from computer-calculated least-squares slopes of plots of log absorbance change vs. time. Correlation coefficients generally were better than 0.999. The dissociation constants of the hemimercaptals of glutathione and the α-ketoaldehydes are defined as: K_{diss} = [total α-ketoaldehyde]·[glutathione]/[hemimercaptal] where total α-ketoaldehyde is essentially equal to the concentration of the hydrated form, owing to the high degree of hydration of these very reactive aldehydes (Vander Jagt et al., 1972b). Initial rate studies for the glyoxalase I reaction were carried out by following thiol ester formation at 240 nm for methylglyoxal and by following loss of reactant at the apparent isosbestic point between α-ketoaldehyde and hemimercaptal for the aromatic α-ketoaldehydes. Pertinent spectral data and K_{diss} values have been reported (Vander Jagt et al., 1972a, 1975; Vander Jagt and Han, 1973). The kinetic constants for glyoxalase I reactions were determined under conditions where the initial rates are proportional to the enzyme concentrations. Initial rate studies at 5 °C (Table I) were measured as limiting rates in the presence of excess glyoxalase I.

Preparation of Apoenzyme. Two milliliters of a concentrated solution of glyoxalase I purified from rat erythrocytes, 0.5–1 mg/mL, was placed in a dialysis bag which previously was boiled for 5 min in 0.01 M Na_2EDTA solution and thereafter was washed with distilled, deionized water. The dialysis bag was sealed and placed in a plastic beaker containing buffer prepared by mixing 84 mL of Chelex-treated sodium phosphate buffer, μ = 0.01, pH 7; 3 mL of 1 M imidazole; 3 mL of 0.1 M Na_2EDTA; and 10 mL of glycerol. Dialysis was carried out with gentle stirring at 4 °C. The buffer was replaced every 6 h for the first 24 h. Then dialysis was continued in the same buffer without imidazole and Na_2EDTA for an additional 12 h with change of buffer every 3 h. Less than 1% activity remained. The apoenzyme was stored as a 50% glycerol solution at 4 °C and was stable for months. Reactivation with Mg^{2+} gave essentially quantitative recovery of activity.

TABLE I: Initial Rates of Hemimercaptal Formation and Thiol Ester Formation for the Glyoxalase I Reaction with Methylglyoxal, 5 °C.[a]

pH[b]	[M] (mM)	[G] (mM)	V_{AD} (M/min)[c]	V_p (M/min)
5.0	1.0	5.0	$0.89 \pm 0.14 \times 10^{-4}$	
	2.5	2.5	$1.7 \pm 0.3 \times 10^{-4}$	
	5.0	0.5	$2.0 \pm 0.3 \times 10^{-4}$	$1.7 \pm 0.2 \times 10^{-4}$
	5.0	1.0	$2.6 \pm 0.4 \times 10^{-4}$	$2.3 \pm 0.3 \times 10^{-4}$
	5.0	2.5	$3.5 \pm 0.5 \times 10^{-4}$	$3.3 \pm 0.2 \times 10^{-4}$
	5.0	5.0	$3.7 \pm 0.6 \times 10^{-4}$	$3.8 \pm 0.3 \times 10^{-4}$
7.0	1.0	5.0	$1.7 \pm 0.3 \times 10^{-4}$	$2.1 \pm 0.4 \times 10^{-4}$
	2.5	2.5	$4.8 \pm 0.7 \times 10^{-4}$	$4.7 \pm 0.2 \times 10^{-4}$
	5.0	0.5	$9.2 \pm 1.4 \times 10^{-4}$	$9.2 \pm 0.4 \times 10^{-4}$
	5.0	1.0	$9.0 \pm 1.3 \times 10^{-4}$	$9.6 \pm 0.8 \times 10^{-4}$
	5.0	2.5	$9.5 \pm 1.4 \times 10^{-4}$	$9.0 \pm 0.3 \times 10^{-4}$
	5.0	5.0	$9.4 \pm 1.4 \times 10^{-4}$	$9.7 \pm 1.3 \times 10^{-4}$

[a] Methylglyoxal (M) and glutathione (G) react to form hemimercaptal with initial rates V_{AD} in absence of glyoxalase I. The reaction in presence of excess glyoxalase I forms thiol ester product with initial rates V_p. [b] Sodium acetate and sodium phosphate buffers used at pH 5.0 and 7.0, μ = 0.2. [c] V_{AD} taken from Vander Jagt et al. (1975).

Fluorescence Studies. Fluorescence spectra were recorded on a Perkin-Elmer MPF44a with corrected spectra accessory. Apoglyoxalase I was treated at 25 °C, pH 8, sodium phosphate (μ = 0.1), 30% acetone, with 1 mM dansyl chloride until the amount of activity which could be restored with Mg^{2+} was less than 2%. Then glutathione was added to react with the excess dansyl chloride, and the resulting mixture was chromatographed on Sephadex G-25 (1 × 20 cm) to separate dansyl-apoenzyme from dansyl-glutathione. Spectra of dansyl-apoenzyme (pH 7), with and without added divalent metal ions, were recorded at 450–650 nm with excitation at 322 nm.

High Pressure Studies. High pressure kinetics measurements were made using a stainless steel cell with sapphire windows, similar to cells reported in the literature (Mustafa et al., 1971). Pressure was applied with a High Pressure Equipment Co. Model 50-6-15 pressure generator and was monitored with an Acragage 600 series high pressure gauge. The effects of pressure in the range 1–700 atm on the glyoxalase I reaction were measured under conditions where the concentration of the hemimercaptal of GSH and methylglyoxal was high enough to saturate the enzymes. Initial rate measurements could be recorded within 15 s after addition of all reactants.

Stereospecificity Studies. The effect of metal ion exchange on the stereospecificity of the glyoxalase I reaction was evaluated by determining the amounts of D- and L-lactate present after base hydrolysis of the thiol ester. The glyoxalase I reac-

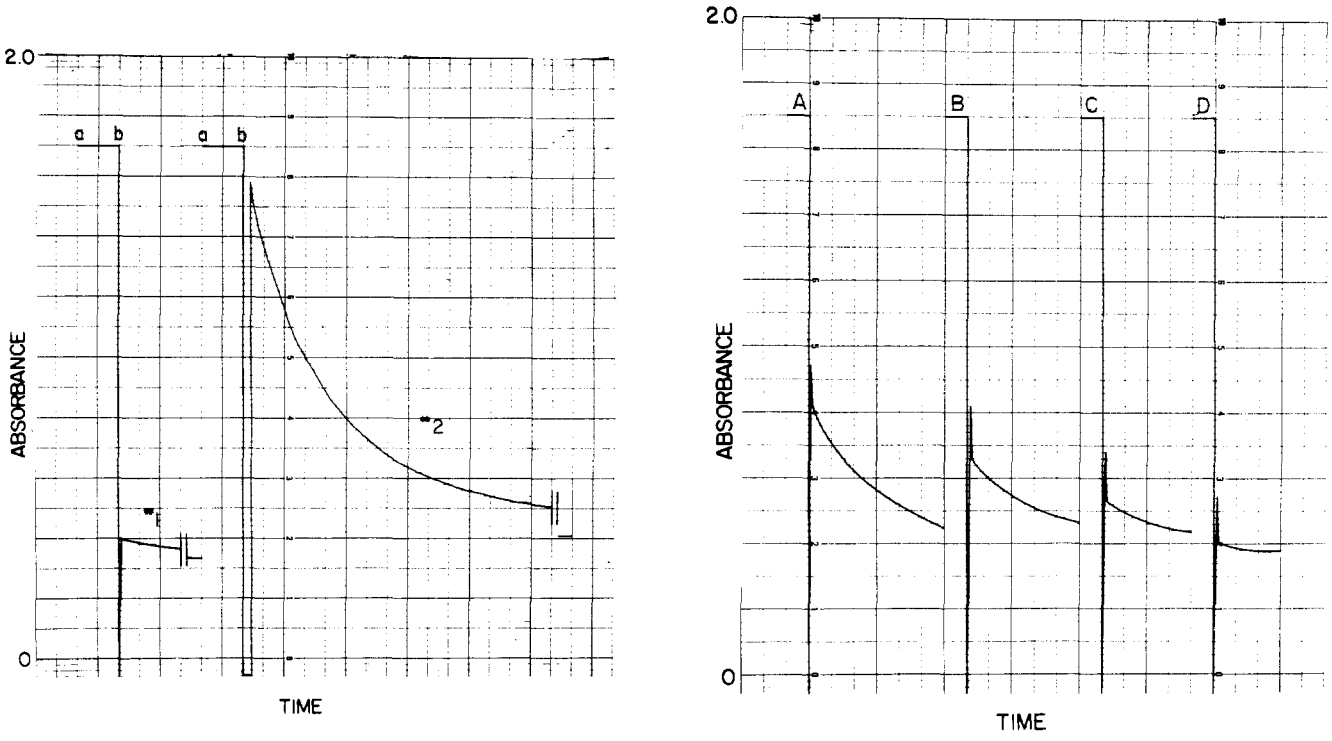

FIGURE 1: (Left) Disproportionation of phenylglyoxal with rat erythrocyte glyoxalase I. (1) The preenzymic reaction to form hemimercaptal (a) was carried out before enzyme was added (b); (2) first enzyme and phenylglyoxal were added (a) before GSH was added (b). (Right) Glyoxalase I catalyzed disproportionation of phenylglyoxal in the presence of various concentrations of GSH. In each reaction, glyoxalase I is added after equilibration of phenylglyoxal and GSH. (PG) = 0.3 mM; (GSH) is (A) 1 mM; (B) 2 mM; (C) 4 mM; (D) 25 mM. All data at 5 °C, pH 7, sodium phosphate, μ = 0.1, 20 s/div, 2-OD scale, 263 nm.

tion was run at pH 7 using various metal ion activated enzymes. After the reactions had gone to completion, the thiol esters were hydrolyzed at pH 10. Glutathione was measured by *N*-ethylmaleimide titration. The D- and L-lactate were measured with the specific lactate dehydrogenases from *L. leichmannii* and beef heart, respectively. Assays for lactate were carried out in pH 9.5 glycine containing 0.4 M hydrazine and 4 mM NAD^+.

Results

One- vs. Two-Substrates Mechanism. Previous studies on the effects of pH, temperature, and added thiols on the kinetics of yeast glyoxalase I suggested that the main reaction pathway for this enzyme involves the disproportionation of the hemimercaptal of glutathione and an α-ketoaldehyde in a one-substrate mechanism, rather than involving glutathione and an α-ketoaldehyde in a two-substrate pathway (Scheme I). This same type of analysis was carried out with glyoxalase I from rat erythrocytes in order to establish whether the one-substrate pathway also predominates for this enzyme.

Scheme I

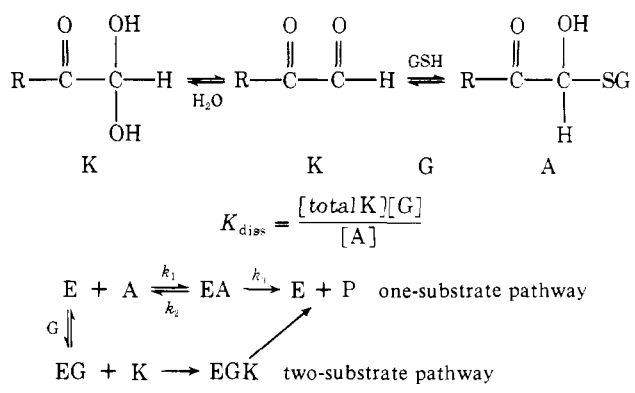

Table I shows the initial rates of hemimercaptal formation, measured in the pH range 3–7 at 5 °C, for the reaction of methylglyoxal with glutathione and compares these rates with the initial rates of thiol ester product formation measured under conditions where the rates are independent of the enzyme concentration. At pH 7, the initial rates of hemimercaptal formation and thiol ester formation are identical and independent of the concentration of glutathione. This leads to the conclusion that hemimercaptal formation proceeds by rate-determining dehydration of hydrated methylglyoxal; furthermore, to the extent that any two-substrate pathway is involved in the glyoxalase I reaction, the enzyme must utilize the unhydrated form of the α-ketoaldehyde since product formation is not faster than dehydration of methylglyoxal.

At lower pH, the initial rates of hemimercaptal formation are no longer independent of the glutathione concentration. At pH 3, the initial rates are almost directly proportional to the amount of glutathione. At pH 5, where the enzyme is still active, the dependence of rate on the concentration of glutathione is less than at pH 3. Nevertheless, the fact that at pH 5 there is some dependence of rate on the glutathione concentration suggests that dehydration of methylglyoxal is not entirely rate determining. This provides a situation where the two-substrate pathway can be evaluated. Since dehydration of methylglyoxal is somewhat faster than formation of hemimercaptal, one would expect that the initial rates of product formation in the enzyme reaction would be faster than the initial rates of hemimercaptal formation if a two-substrate pathway is important. Within experimental error, the initial rates of hemimercaptal formation and thiol ester product formation at pH 5 are identical, supporting the one-substrate pathway.

Studies on the effects of order of addition of reactants on the limiting enzyme catalyzed reaction at pH 7, 5 °C, are consistent with a one-substrate pathway. Figure 1 illustrates the

TABLE II: Substrate Specificity of Mg^{2+}-, Mn^{2+}-, Co^{2+}-, and Ni^{2+}-Activated Glyoxalase I.

	Mg^{2+}		Mn^{2+}		Co^{2+}		Ni^{2+}	
Ketoaldehyde	Rel V_{max}[a]	K_m (M)[b]	Rel V_{max}	K_m (M)	Rel V_{max}	K_m (M)	Rel V_{max}	K_m (M)
Methylglyoxal	1.0	9×10^{-5}	1.0	5×10^{-5}	1.0	4×10^{-5}	1.0	4×10^{-5}
Phenylglyoxal	0.5	4×10^{-5}	0.07	2×10^{-5}	0.8	5×10^{-5}	3.1	11×10^{-5}
p-Chlorophenylglyoxal	0.7	2×10^{-5}	0.4	2×10^{-5}	3.2	5×10^{-5}	6.9	6×10^{-5}
p-Methoxyphenylglyoxal	0.3	1×10^{-5}	0.03	2×10^{-5}	0.4	2×10^{-5}	0.9	2×10^{-5}

[a] For each series, the V_{max} for methylglyoxal is set at 1.0 and the other values are relative to methylglyoxal, 25 °C, pH 7, phosphate buffer, $\mu = 0.01$. [b] K_m are the Michaelis constants for the hemimercaptals of GSH and the α-ketoaldehydes; dissociation constants of the hemimercaptals and appropriate spectral data have been reported (Vander Jagt et al., 1972, 1973, 1975).

effects of order of addition of reactants using phenylglyoxal as the α-ketoaldehyde; the same results were obtained with methylglyoxal. Initial rates of hemimercaptal formation and product formation (Table I) are identical only if glyoxalase I is *not* added last; reversal of the order of addition gives a "burst" of product. The size of the "burst" depends upon the amount of hemimercaptal present as shown in Figure 1. These results by themselves do not rule out a two-substrate pathway. The "burst" could simply indicate that unhydrated α-ketoaldehyde is produced more rapidly from hemimercaptal breakdown than from dehydration of hydrated α-ketoaldehyde. However, these results along with the pH 5 data in Table I strongly support a one-substrate pathway as the main pathway. The rate of the slow reaction following the burst agrees with the rate of hemimercaptal formation, suggesting that the burst represents rapid consumption of the hemimercaptal present when enzyme is added. In addition, the size of the burst agrees with the calculated amount of hemimercaptal present in the preequilibrium reaction.

An additional test of the possible contribution of a two-substrate pathway was made by examining the effects of nonglutathione thiols on the "burst" kinetics. The effects of various concentrations of *N*,*N*-diethylcysteamine on the glyoxalase I reaction were measured where in each case glutathione is added last to initiate the reaction. There is indeed a partial "burst" at low concentrations of *N*,*N*-diethylcysteamine which supports the idea that hemimercaptal breakdown can provide unhydrated phenylglyoxal more rapidly than dehydration of hydrated phenylglyoxal. However, at higher concentrations of this thiol, the extent of the "burst" is greatly diminished. This is most easily interpreted as indicating that this thiol is competing with the fixed concentration of glutathione for unhydrated phenylglyoxal. At higher concentrations of this thiol, glutathione competes less effectively and, consequently, the rate of formation of glutathione hemimercaptal needed for the enzyme reaction is reduced. Thus, all of the results from studies on the effects of pH and added thiols on the kinetics of rat erythrocyte glyoxalase I are similar to previous results with yeast glyoxalase I (Vander Jagt et al., 1975) and support a one-substrate pathway, at least under the experimental conditions used in this study. There are data which suggest that, at low substrate concentrations, both the one- and two-substrate pathway may participate (Mannervik et al., 1974).

Activation of Apoglyoxalase I with Divalent Metal Ions. The main question of interest in this study concerns the role of the divalent metal ion in the glyoxalase I reaction. Reactivation of apoglyoxalase-I by Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} was carried out at pH 7 in dilute sodium phosphate buffer. Plots of $100/\%V_{max}$ vs. $1/M^{2+}$ gave linear reactivation plots, in contrast to results reported recently for reactivation of apoglyoxalase I from sheep liver (Uotila and Koivusalo, 1975). The relative V_{max} values for these metal ion activated enzymes are 1.0, 0.46, 0.85, and 0.40 for the Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}-activated enzymes, respectively. The dissociation constants for metal ions, determined from the reactivation plots, are 66, 7.6, 3.5, and 4.5 μM for these four metal ion activated enzymes, respectively. These are upper estimates based upon use of total metal ion concentrations in the reactivation plots. However, available data on metal ion complexes (Christensen et al., 1975) suggest these dissociation constants are within a factor of two of the real dissociation constants. Interestingly, Mn^{2+}, Co^{2+}, and Ni^{2+} appear to be an order of magnitude better than Mg^{2+} in terms of tightness of binding.

Substrate Specificity of M^{2+}-Activated Glyoxalase I. The effects of change in metal ion on the substrate specificity of glyoxalase I were measured for Mg^{2+}-, Mn^{2+}-, Co^{2+}-, and Ni^{2+}-activated enzyme using methylglyoxal and several substituted phenylglyoxals as substrates (Table II). Previous studies on yeast glyoxalase I revealed broad substrate specificity for aliphatic and aromatic α-ketoaldehydes with V_{max} and K_m values very insensitive to variations in the α-ketoaldehyde (Vander Jagt et al., 1972a). This same insensitivity was observed with rat erythrocytes glyoxalase I (Han et al., 1976). Both of these enzymes, as isolated, contain Mg^{2+}. Table II shows that the replacement of Mg^{2+} by Mn^{2+}, Co^{2+}, or Ni^{2+} causes a modest change in substrate specificity. Specifically, V_{max} values appear to be more sensitive to the nature of the α-ketoaldehyde when Mg^{2+} is replaced by another divalent cation. For all of the M^{2+}-glyoxalase I reactions (Table II), the use of substituted phenylglyoxals reveals that electron-withdrawing groups speed up the reaction. This is consistent with intramolecular hydride migration being rate determining and being dependent upon the polarity of the α-ketone. The major difference between the M^{2+}-glyoxalase I reactions is the sensitivity of V_{max} to variations in substituents.

Isotope Effects in the M^{2+}-Glyoxalase I Reactions. Yeast glyoxalase I shows a deuterium isotope effect on both V_{max} and K_m when methylglyoxal and perdeuteriomethylglyoxal are compared. These pairs of α-ketoaldehydes also show an isotope effect when they are disproportionated to the corresponding α-hydroxycarboxylic acids in alkaline solution (Vander Jagt and Han, 1973). These results suggested that rate-determining intramolecular hydride migration occurs both in the enzyme reaction and in the model reaction. Table III shows results from a study of the isotope effects on V_{max} and K_m for various M^{2+}-glyoxalase I from rat erythrocytes. There is an isotope effect both on V_{max} and on K_m, and the magnitude of this effect on these parameters is similar. However, this isotope effect on V_{max} and K_m is sensitive to the particular divalent metal ion present in the M^{2+}-glyoxalase I, being smallest with Ni^{2+}-glyoxalase I. If the metal ion functions in the enzyme reaction as a superacid which is able to polarize the α-ketone group and thereby facilitate rate-determining intramolecular hydride

TABLE III: Isotope Effects in the Glyoxalase I Reaction: Comparison of Mg^{2+}-, Mn^{2+}-, Co^{2+}-, and Ni^{2+}-Activated Enzyme.

Ketoaldehyde	Metal ion	$\frac{V_{max}(H)}{V_{max}(D)}$[a]	$\frac{K_m(H)}{K_m(D)}$
Methylglyoxal and perdeuteriomethylglyoxal	Mg^{2+}	1.7 ± 0.1	2.8 ± 0.3
	Mn^{2+}	3.5 ± 0.2	1.6 ± 0.1
	Co^{2+}	1.4 ± 0.1	1.1 ± 0.1
	Ni^{2+}	1.0 ± 0.1	1.0 ± 0.1
Phenylglyoxal and α-deuteriophenylglyoxal	Mg^{2+}	4.8 ± 0.3	3.9 ± 0.8
	Mn^{2+}	3.8 ± 0.3	1.8 ± 0.2
	Co^{2+}	4.5 ± 0.6	4.2 ± 0.8
	Ni^{2+}	2.1 ± 0.1	3.0 ± 0.4

[a] All data are at 25 °C, pH 7, phosphate buffer, μ = 0.01, with 0.2 mM of the appropriate metal ion present.

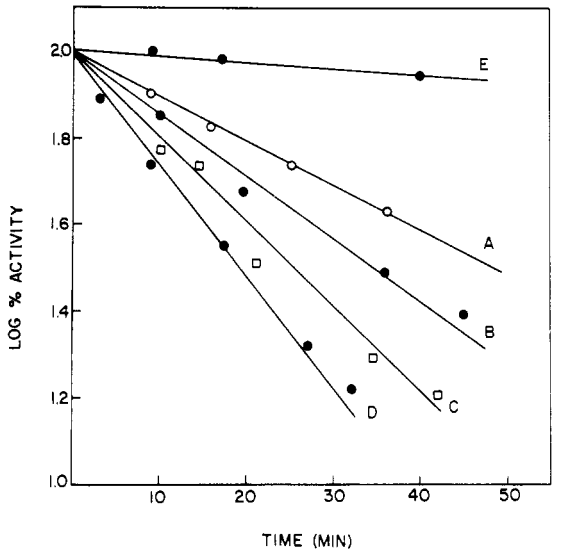

FIGURE 2: Inactivation of Mg^{2+}-glyoxalase I by various concentrations of dansyl chloride. Reactions were run in pH 8 sodium phosphate buffer containing 30% acetone, 25 °C. Dansyl chloride concentrations were (A) 0.6 mM; (B) 0.9 mM; (C) 1.5 mM; and (D) 2.2 mM. Plot E shows the effects of 30 mM *N*-acetyl-(*S*-*p*-chlorobenzyl)glutathione in protecting against inactivation by 1 mM dansyl chloride.

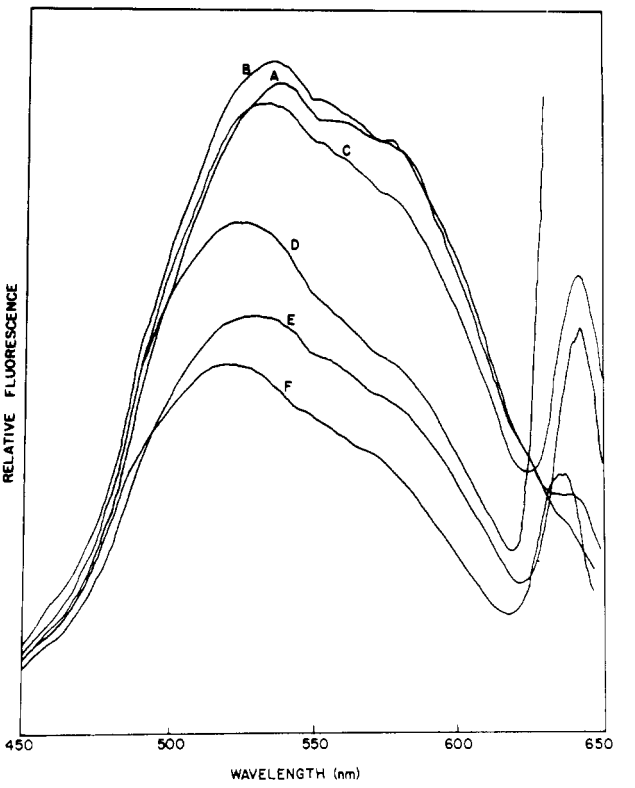

FIGURE 3: (A) Fluorescence spectrum of dansylated apoglyoxalase I; (B) fluorescence spectrum after the addition of 2 mM Mn^{2+} to A. The spectrum was recorded at various times after the addition of the Mn^{2+}; (C) 15 min; (D) 30 min; (E) 60 min; (F) 120 min. All spectra ca. 25 °C, pH 7, sodium phosphate, μ = 0.01.

migration, then the variation in the size of the isotope effect with different metal ions in the enzyme is presumably a measure of the extent of hydride migration in the transition state. The fact that the isotope effect is seen in K_m suggests that $k_3 > k_2$ and that $K_m \simeq k_3/k_1$ regardless of which divalent metal ion is present (Scheme I).

Location of the Metal Ion in Glyoxalase I. The above results from studies of the effects of metal ion replacement on the substrate specificity and on the deuterium isotope effects argue for a direct role for the metal ion. However, no data are available which establish experimentally that the divalent metal ion resides near the substrate binding site. A recent study of the inactivation of glyoxalase I from porcine erythrocytes and yeast by amino-group reagents suggests that these reagents form a reversible inactivator–enzyme complex prior to the actual inactivation (Mannervik et al., 1975). Since this inactivation could be prevented by competitive inhibitors of glyoxalase I, the results also suggest that the inactivation reaction takes place at the active site. This suggests a method for introducing a fluorescent label at the active site of apoglyoxalase I and then titrating the modified apoenzyme with Mg^{2+} and Mn^{2+} in order to test for fluorescence quenching by the paramagnetic Mn^{2+} as an indication that the metal ion is located in the vicinity of the active site.

Figure 2 shows plots of the kinetics of inactivation of glyoxalase I from rat erythrocytes using varying concentrations of dansyl chloride. This inactivation can be prevented by *N*-acetyl-(*S*-*p*-chlorobenzyl)glutathione (Figure 2, plot E). Most *S*-alkylglutathione derivatives are competitive inhibitors of glyoxalase I (Vince et al., 1971). Acetylation of the free amino group on the inhibitor was necessary in order to prevent it from reacting with dansyl chloride. This modification weakens the inhibitory properties of these compounds. Consequently, rather high concentrations of the acetylated inhibitors are required to protect the enzyme from inactivation by dansyl chloride. If the slopes of the plots in Figure 2 are plotted against slope/[inactivator] in a secondary plot one observes a linear relationship with supports the idea that an inactivator–enzyme complex forms prior to the inactivation. The dissociation constant for the dansyl chloride–glyoxalase I complex determined from this secondary plot is about 2 mM. Consequently, it is reasonable to conclude that dansyl chloride forms a complex by binding to the active site of glyoxalase I and subsequently covalently modifies and inactivates glyoxalase I.

Apoglyoxalase I was treated with dansyl chloride until the amount of reactivation possible with Mg^{2+} was less than 1% of the starting value. Rates of inactivation of apoglyoxalase I are similar to those for inactivation of Mg^{2+}-glyoxalase I. The dansylated apoenzyme, after purification (see Methods), was examined by fluorescence emission spectroscopy for the effects of Mg^{2+} and Mn^{2+} on the emission spectrum. Addition of 2 mM Mg^{2+} causes an initial small shift in the spectrum which then over a period of 2–3 h changed slowly until the spectrum was almost identical with that of dansylated apoenzyme. Figure 3 shows the effects of 2 mM Mn^{2+} on the emission spectrum of dansylated apoglyoxalase I. Again there

is an initial small shift in the spectrum, but this is followed over a 2–3-h period by extensive quenching of the spectrum, along with the appearance and partial loss of a new peak at longer wavelength. The time required for these changes is similar to the time needed for activation of apoglyoxalase-I by Mg^{2+} or Mn^{2+} at the concentrations used in this study. To test further that the effects of Mn^{2+} are the result of its binding to a specific metal ion binding site, Mn^{2+} was added to the sample of dansylated apoglyoxalase I which had been treated with Mg^{2+}. Over a period of several hours, only a slight change is observed in the emission spectrum. This slow change also reflects the appearance of the high wavelength fluorescence band observed in Figure 3. Consequently, the slow change in the spectrum probably represents the replacement of Mg^{2+} by Mn^{2+}. To measure the stoichiometry of dansylation, a sample of the dansylated enzyme was treated with HCl to pH 1 to denature the protein. After adjustment to pH 7, the fluorescence spectrum was compared with a standard curve prepared using dansylated glycine. The fluorescence intensity suggested the presence of about 0.8 dansyl group per protein molecule (mol wt 50 000). Thus it appears that the dansylation reaction is quite selective under the experimental conditions used. It appears reasonable to conclude that the metal ion in glyoxalase I is located near the substrate binding site and is in a position to participate in the catalytic reaction.

Effects of Pressure on V_{max} for the M^{2+}-Glyoxalase I. In order to obtain some information regarding any changes in mechanism which might take place as the metal ion in glyoxalase I is changed, the effects of pressure on the V_{max} of the M^{2+}-glyoxalase I reactions were determined in the range 1–700 atm, and the activation volumes, $\Delta V^{\ddagger}$, were calculated. For all of the M^{2+}-glyoxalase I, increased pressure has a similar effect such that both the sign and the magnitude of $\Delta V^{\ddagger}$ are comparable (14 ± 2 cm^3/mol) for all four M^{2+}-glyoxalase I. This suggests that exchange of the metal ion does not result in any fundamental change in mechanism.

Effects of Metal Ion Exchange on the Stereochemistry of the Glyoxalase I Reaction. To test further that metal ion exchange does not alter the basic mechanism of the glyoxalase I reaction, the stereochemistry of the reaction for the various M^{2+}-glyoxalase I was determined. If metal ion exchange does not alter the stereochemistry, the data would support the conclusion that metal ion exchange does not alter the mechanism of the reaction. Within experimental error, there is no alteration of the stereospecificity, regardless of which metal ion is activating the enzyme. The lactic acid product formed after hydrolysis of the thiol ester is about $95 \pm 2\%$ D-lactate. The same results are obtained if glyoxalase II is used instead of hydroxide to hydrolyze the thiol ester. Consequently, the 95% D-lactate presumably reflects lack of complete stereospecificity in the glyoxalase I reaction. Both the stereochemistry studies and the pressure studies suggest that the mechanism of the reaction is unchanged when the metal ion is exchanged.

Discussion

Two general theories concerning the role of metal ions in metalloenzymes have been suggested. One proposal regards the efficient catalysis associated with metalloenzymes as a property of the strain resulting from unusual metal ion coordination in the enzyme. This proposal is the result of studies of spectral data of some metalloproteins compared with simple complexes of the same metal ions (Vallee and Williams, 1968). The other proposal views metalloenzymes as superacid catalysts which utilize the Lewis acid properties of the metal ions (Westheimer, 1955). The observation of little or no sensitivity of specific activity to the particular metal ion present in a metalloenzyme is most easily explained by the superacid theory (Dixon et al., 1976). However, for those metalloenzymes which appear to utilize the Lewis acid properties of the metal ion in order to polarize bonds and thereby to provide an efficient pathway for a particular reaction, there is the additional question of whether the metal ion participates directly such that ligand-exchange reactions between the substrate and the metal ion occur, or whether the metal ion interacts through one or more intervening solvent molecules in polarizing a given bond of the substrate. A number of metalloenzymes have been suggested to function by way of this second sphere coordination (Hsu et al., 1976).

The results from the present study on the role of the divalent metal ion in glyoxalase I from rat erythrocytes suggest that the metal ion participates as a superacid in the intramolecular disproportionation of the hemimercaptal of glutathione and an α-ketoaldehyde by polarizing the α-ketone group. This conclusion is based mainly on the observation that the series of metal ions used to activate glyoxalase I, namely, Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}, gave rather similar V_{max} values using methylglyoxal as substrate. Lack of sensitivity to the particular metal ion present in the enzyme is most easily explained by the superacid theory. However, the question of direct coordination of the substrate by the metal ion cannot be answered from the results of this study. Purified Mg^{2+}-glyoxalase I from rat erythrocytes has a turnover number (k_3, Scheme I) of about 1.2×10^5 min^{-1} using methylglyoxal as substrate (Han et al., 1976). The isotope efects on V_{max} show that the rate-determining step, k_3, involves the breaking of the carbon–hydrogen bond of the hemimercaptal. Consequently, any ligand-exchange reactions between metal ion and substrate would have to be considerably greater than k_3 in order not to be rate determining. For the four metal ions used to activate glyoxalase I, Ni^{2+} has the slowest water exchange rate (ca. 6×10^5 min^{-1}) which is still greater than k_3 (Hughes, 1972). In principle, if we can find a substrate which is considerably better than methylglyoxal, then the use of isotope effects may allow us to determine whether direct coordination occurs. This would provide a method which does not require the use of relaxation techniques to "measure" distances.

In these studies, we have assumed that the disproportionation of the hemimercaptal involves the intramolecular migration of a hydride to the α-ketone. Recently, it was reported that the glyoxalase reaction, when run in D_2O, results in the formation of lactic acid which has some deuterium incorporated onto the α carbon (Hall et al., 1976). This would support a reaction scheme involving removal of the hemimercaptal hydrogen as a proton, formation of an enediol intermediate, and then protonation at the α carbon. These data conflict with an earlier study (Rose, 1957) which reported little or no solvent hydrogen incorporation when the glyoxalase I reaction was carried out in tritiated water. Thus the question of an enediol intermediate vs. a hydride migration in the glyoxalase I reaction remains open. The results from the present study showing rate-determining C–H bond cleavage do not necessarily favor one or the other of these alternative mechanisms. However, the observation that the V_{max} values for substituted phenylglyoxals (Table II) show a consistent trend for all four M^{2+}-glyoxalase I such that electron-withdrawing groups accelerate and electron-donating groups slow down the reaction appears to favor a hydride transfer mechanism whereby the polarity of the α-ketone group to which the hydride is migrating is influenced both by the metal ion of the enzyme and by the electronic properties of the substrate.

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Design of Potent Competitive Inhibitors of Angiotensin-Converting Enzyme. Carboxyalkanoyl and Mercaptoalkanoyl Amino Acids†

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ABSTRACT: A hypothetical model of the active site of angiotensin-converting enzyme has been utilized to guide the design and synthesis of specific inhibitors. By analogy to bovine carboxypeptidase A, the active site of angiotensin-converting enzyme was proposed to contain three important groups that participate in binding of peptide substrates: a carboxyl-binding group, a group with affinity for the C-terminal peptide bond, and a tightly bound zinc ion that could coordinate with the carbonyl of the penultimate (scissile) peptide bond. According to the model, a succinyl amino acid could interact with each of these binding groups via its amino acid carboxyl, amide bond, and succinyl carboxyl, respectively, and thus act as a specific competitive inhibitor of the enzyme. Succinyl-L-proline was found to be such an inhibitor (I_{50} = 330 μM), and attempts to optimize its interaction with the active site of the enzyme as proposed in the model led to the synthesis of D-2-methylsuccinyl-L-proline (*R,S*) (K_i = 2.5 μM), and D-2-methylglutaryl-L-proline (*R,S*) (K_i = 0.8 μM). Replacement of the succinyl carboxyl group of these compounds by a sulfhydryl group led to a series of extremely potent competitive inhibitors of angiotensin-converting enzyme, including 3-mercaptopropanoyl-L-proline (SQ 13 863, K_i = 0.012 μM) and D-3-mercapto-2-methylpropanoyl-L-proline (*S,S*) (SQ 14 225, K_i = 0.0017 μM). These compounds are also potent orally active inhibitors of angiotensin-converting enzyme and have great potential as antihypertensive agents.

Angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) is an exopeptidase that cleaves dipeptides from the carboxyl-terminal end of various peptide substrates (Piquilloud et al., 1970; Cushman and Cheung, 1971; Massay and Fessler, 1976; Angus et al., 1972; Yang et al., 1971; Elisseeva et al., 1971); like the similar carboxypeptidases, it is a zinc containing enzyme (Das and Soffer, 1975). Two reactions catalyzed by angiotensin-converting enzyme may play a role in blood pressure regulation: "conversion" of the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II (Skeggs et al., 1956) and inactivation of the vasodepressor nonapeptide bradykinin (Yang et al., 1971; Elisseeva et al., 1971; Dorer et al., 1974). The importance of one or both of these reactions in hypertensive disease has been greatly clarified in recent years by the development of a potent and specific inhibitor of angiotensin-converting enzyme, the nonapeptide SQ 20 881 (Ondetti et al., 1971, 1972c; Cushman and Cheung, 1972; Cheung and Cushman, 1973; Engel et al., 1972; Keim et al., 1972; Bianchi et al., 1973). SQ 20 881 lowers blood pressure in animal models of renovascular hypertension (Engel et al., 1973; Muirhead et al., 1974), in most patients with renovascular or malignant hypertension, and in many patients with essential hypertension (Gavras et al., 1974, 1975; Johnson et al., 1975; Case et al., 1976). In spite of these outstanding actions, however, the therapeutic utility of SQ 20 881 is limited by its lack of oral activity. Our understanding of the binding of peptide inhibitors to angiotensin-converting enzyme, based on extensive studies with SQ 20 881 and its analogues (Ondetti et al., 1972a,b; Pluščec et al., 1973; Cushman et al.,

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