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Diffusion-Dependent Rates for the Hydrolysis Reaction Catalyzed by Glyoxalase II from Rat Erythrocytes†

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ABSTRACT: Glyoxalase II from rat erythrocytes is a near optimal catalyst for the hydrolysis of *S*-D-lactoylglutathione in the sense that the magnitude of k_{cat}/K_m is limited, in large part, by the rate constant for diffusion-controlled encounter between substrate and active site. The experimental basis for this conclusion is derived from the dependencies of the kinetic properties of the enzyme on solution viscosity (pH 7, I_c = 0.1 M, 25 °C). When sucrose is used as a viscogenic agent, k_{cat}/K_m for *S*-D-lactoylglutathione (8.8×10^5 M^{-1} s^{-1}) decreases markedly with increasing solution viscosity. This effect appears not to be due to a sucrose-induced change in the intrinsic kinetic properties of the enzyme, since k_{cat}/K_m for the slow substrate *S*-acetylglutathione (3.7×10^4 M^{-1} s^{-1}) is nearly independent of solution viscosity. Quantitative treatment of the data using Stoke's law indicates that the rate of hydrolysis of *S*-D-lactoylglutathione will be ~50% diffusion limited when [substrate] $\ll K_m$; the encounter complex between enzyme and substrate partitions nearly equally between product formation and dissociation to form free enzyme and substrate. The same conclusion is reached when glycerol is used as a viscogenic agent, once the apparent activation effect of glycerol on the intrinsic activity of the enzyme is taken into account. Finally, the rate of formation of the encounter complex between substrate and active site may be governed to a significant extent by charge–charge interactions. This conclusion is based upon a Debye–Hückel treatment of the ionic strength dependency of k_{cat}/K_m for *S*-D-lactoylglutathione in which $k_{cat}/K_m \simeq 4 \times 10^7$ M^{-1} s^{-1} at zero ionic strength.

The widely distributed glyoxalase enzyme system composed of glyoxalases I (Glx I,[1] EC 4.4.1.5) and II (Glx II, EC 3.1.2.6) catalyzes the net glutathione (GSH) dependent conversion of methylglyoxal to D-lactate (eq 1). A proposed physiological function of the pathway is to rapidly remove from cells cytotoxic methylglyoxal that arises either as a normal or as an

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[1] Abbreviations: Glx I, glyoxalase I; Glx II, glyoxalase II; GSH, glutathione; H_R and H_S, thiohemiacetals formed from GSH and phenylglyoxal in which the chirality at the thiohemiacetal carbon is *R* and *S*, respectively; I_c, ionic strength.

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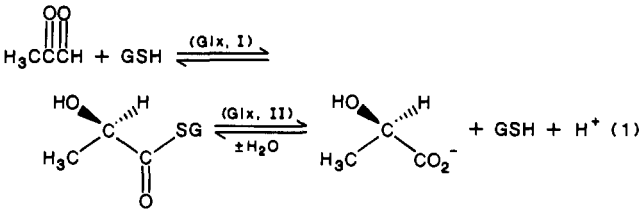

aberrant product of metabolism (Creighton & Pourmotabbed, 1988). In support of a detoxification role for the glyoxalase pathway, a mutant strain of the yeast *Saccharomyces cerevisiae*, defective in glyoxalase I, is eventually killed by exposure to glycerol and excretes methylglyoxal into the growth medium (Penninckx et al., 1983). In addition, *Escherichia coli* cells amplified with the glyoxalase I gene exhibit enhanced resistance to the growth-inhibitory effect of methylglyoxal in the growth medium (Rhee et al., 1987). In mammalian systems, the glyoxalase pathway appears to be an important, if not the exclusive, means of chemically removing methylglyoxal from cells (Pourmotabbed & Creighton, 1986).

The substrate specificities and kinetic properties of the glyoxalase enzymes are consistent with their proposed detoxification role in the cell. Glyoxalase I from both yeast and porcine erythrocytes has the unusual ability of being able to use directly as substrates both diastereotopic thiohemiacetals (H_R and H_S), formed by preequilibrium addition of glutathione to the substrate analogue phenylglyoxal (Griffis et al., 1983). This may be an adaptation toward more efficient metabolic usage in that catalysis is not limited by the nonenzymic rate of interconversion of H_R and H_S. The enzyme from yeast is a highly active catalyst for which $k_{cat}/K_m \simeq 4 \times 10^6$ M^{-1} s^{-1} with phenylglyoxal–thiohemiacetals (Vander Jagt & Han, 1973); for the enzyme from human erythrocytes, $k_{cat}/K_m = 1 \times 10^7$ M^{-1} s^{-1} per subunit with methylglyoxal–thiohemiacetals (Sellin & Mannervik, 1983). Glyoxalase II from human liver has a broad substrate specificity for both aliphatic and aromatic thioesters of glutathione (Uotila, 1973). Consistent with its proposed role in the glyoxalase pathway, the enzyme is most active with α-hydroxythioesters of glutathione. The homogeneous enzyme from rat erythrocytes is highly active with *S*-D-lactoylglutathione, $k_{cat}/K_m = 1.6 \times 10^6$ M^{-1} s^{-1} (Tris buffer, 50 mM, pH 7.4, 25 °C) (Ball & Vander Jagt, 1981).

As part of a general effort to understand precisely how well the properties of the glyoxalase enzymes are adapted to the removal of methylglyoxal from cells, we report here on the viscosity dependencies of the kinetic properties of glyoxalase II from rat erythrocytes. This study was undertaken in order to evaluate the extent to which the magnitude of k_{cat}/K_m is limited by the bimolecular rate constant for diffusion-controlled encounter between substrate and active site, one criterion for an optimally efficient catalyst (Albery & Knowles, 1976).

Experimental Procedures

Materials. A homogeneous preparation of glyoxalase II from rat erythrocytes was stored at −10 °C in buffered solutions (pH 7) containing 50% glycerol (v/v) (Ball & Vander Jagt, 1979). *S*-D-Lactoylglutathione (Sigma) was separated from trace amounts of contaminating glutathione by isocratic elution of the commercial material from a Waters μBondapak C_{18} column (7.8-mm i.d. × 30 cm, 10 μm) using a running solvent composed of 0.25% acetic acid in water. The effluent from the column was monitored at 206 nm: glutathione (elution volume ≃ 19 mL); *S*-D-lactoylglutathione (elution volume ≃ 44 mL). *S*-Acetylglutathione was synthesized by a published method and twice recrystallized from hot water (Kielley & Bradley, 1954). The product migrated as a single peak (elution volume ≃ 24 mL) in the reverse-phase column chromatography system described above. Both *S*-acetyl- and *S*-D-lactoylglutathione were stored as lyophilized powders at −10 °C. Sucrose (Aldrich, 99+%, gold label) was used without further purification. Glycerol (Aldrich, 99.5+%, spectrophotometric grade) was purified by vacuum distillation before use. Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] was an Aldrich product. All other materials were of the highest purity commercially available.

Viscogenic Buffers and Reagent Solutions. Viscogenic buffers were prepared by dissolution of the required amounts of either sucrose or glycerol in phosphate buffer for which the ionic strength was adjusted by use of either NaCl or KCl. The buffers were then filtered through sintered glass to remove any suspended particles. The viscosities of these solutions were measured with an Ostwald viscometer (25 °C). Relative viscosity (η_{rel}) was calculated by dividing the viscosity of the viscogenic buffer (η) by that for the corresponding buffer without viscogenic agent (η^0).

All reagent solutions were prepared immediately before use in the kinetic experiments. Dilute enzyme solutions were prepared by dilution into ice-cold phosphate buffer (100 mM, pH 7) containing 50% glycerol (v/v) and 2% bovine serum albumin. Under these conditions, the enzyme retains constant activity over 24 h. Stock solutions of *S*-D-lactoyl- and *S*-acetylglutathione were prepared in distilled water. The analytical concentration of *S*-D-lactoylglutathione was determined on the basis of ΔOD_{230} in the presence of glyoxalase II by using an extinction coefficient of 3100 M^{-1} cm^{-1} (Ball & Vander Jagt, 1981); that for *S*-acetylglutathione was determined on the basis of the absolute OD_{230} by using an extinction coefficient of 4500 M^{-1} cm^{-1} (Uotila, 1973).

Kinetic Measurements. Initial rate kinetics were obtained by using a Gilford response spectrophotometer equipped with a thermostatically controlled cuvette carriage. Kinetic runs were initiated by introducing 10–20 μL of ice-cold, dilute enzyme solution into 1 mL of buffer solution containing substrate and 0.15 mM Ellman's reagent. Initial rates were obtained by computer fit of the time-dependent increase in OD_{412} (ϵ = 13 600 M^{-1} cm^{-1}, pH 7), due to the appearance of glutathione, over the first 3–5% of reaction. This indirect method of assay has the advantage of being applicable to high substrate concentrations, where direct monitoring of the loss of substrate (OD_{230}) is inaccurate because of the high absorbancy of the thioester function (Ball & Vander Jagt, 1979). Both methods give comparable rates when equivalent concentrations of substrate are used. The magnitudes of k_{cat}, K_m, and k_{cat}/K_m, as well as their standard errors, were obtained from a hyperbolic fit of the initial rate data to the Michaelis–Menten equation using the HYPER computer program described by Cleland (1967). Initial rates were obtained for five to seven different substrate concentrations spanning the following ranges: *S*-D-lactoylglutathione, ~5 K_m to ~0.3 K_m; *S*-acetylglutathione, ~2 K_m to ~0.3 K_m.

Results and Discussion

The Viscosity Variation Method. The variation of the kinetic parameters of an enzyme with solution viscosity can be used to evaluate the extent to which the magnitude of k_{cat}/K_m is determined by the rate constant for diffusion-controlled encounter between substrate and active site. Several enzymes have proved to be amenable to this experimental approach, including the hydrolases chymotrypsin (Brouwer & Kirsch, 1982), β-lactamase I (Hardy & Kirsch, 1984), and acetylcholinesterase (Bazelyansky et al., 1986). This approach

Table I: Viscosity Dependencies of the Kinetic Parameters of Glyoxalase II with *S*-D-Lactoyl- and with *S*-Acetylglutathione[a]

viscogen (% w/v)	η_{rel}	*S*-D-lactoylglutathione $10^{-2}k_{cat}$ (s^{-1})	10^4K_m (M)	$10^{-5}(k_{cat}/K_m)$ (M^{-1} s^{-1})	*S*-acetylglutathione $10^{-2}k_{cat}$ (s^{-1})	10^4K_m (M)	$10^{-5}(k_{cat}/K_m)$ (M^{-1} s^{-1})
				Sucrose			
0	1	2.83 ± 0.05	3.23 ± 0.16	8.76 ± 0.14	0.649 ± 0.008	17.5 ± 0.51	0.371 ± 0.007
16	1.60	3.06 ± 0.09	4.83 ± 0.36	6.34 ± 0.31	0.677 ± 0.011	19.0 ± 0.71	0.357 ± 0.008
24	2.08	3.04 ± 0.10	5.52 ± 0.43	5.51 ± 0.27	0.649 ± 0.013	17.9 ± 0.85	0.363 ± 0.010
32	2.87	3.25 ± 0.14	7.15 ± 0.67	4.55 ± 0.25	0.672 ± 0.009	19.0 ± 0.57	0.354 ± 0.006
				Glycerol			
16	1.49	3.02 ± 0.08	3.81 ± 0.28	7.93 ± 0.40	0.677 ± 0.016	15.9 ± 0.94	0.426 ± 0.006
24	1.85	3.06 ± 0.05	4.38 ± 0.20	6.99 ± 0.21	0.697 ± 0.009	15.1 ± 0.49	0.462 ± 0.010
32	2.35	2.87 ± 0.08	4.93 ± 0.35	5.82 ± 0.27	0.711 ± 0.005	15.3 ± 0.27	0.465 ± 0.005

[a] In sodium phosphate (35 mM), NaCl (18.4 mM), pH 7, 25 °C; ionic strength = 0.1 M.

has now been applied to rat erythrocyte glyoxalase II by using sucrose and glycerol as viscogenic agents.

The theoretical basis for this method and its potential limitations have been discussed elsewhere (Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984; Blacklow et al., 1988). For an enzyme-catalyzed reaction obeying Michaelis–Menten kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} E + P \qquad (2)$$

k_{cat}/K_m is defined by eq 3. In the simple case where k_1 and

$$\frac{k_{cat}}{K_m} = \frac{k_1k_2}{k_{-1} + k_2} \qquad (3)$$

k_{-1} reflect diffusional processes and k_2 does not, Stokes' law requires that $k_1^0/k_1 = \eta/\eta^0$ and that $k_{-1}^0/k_{-1} = \eta/\eta^0$, where k_1^0 and k_{-1}^0 are rate constants obtained in water solvent with a viscosity of η^0 and k_1 and k_{-1} are rate constants obtained in buffers containing the viscogenic agents (η). Combining these relationships with eq 3 gives

$$\frac{k_{cat}}{K_m} = \frac{k_1^0}{\eta_{rel} + k_{-1}^0/k_2} \qquad (4)$$

where $\eta_{rel} = \eta/\eta^0$. This simple function will apply as long as the viscogenic agent does not inhibit the activity of the enzyme or otherwise alter its intrinsic kinetic properties. The presence of such aberrant effects on the enzyme can be tested for by determining the viscosity dependencies of the kinetic parameters for a slow substrate that should not be diffusion limited, on the basis of the small magnitude of k_{cat}/K_m. This strategy was first employed by Kirsch and co-workers [e.g., Brouwer and Kirsch (1982)]. In the present study, *S*-acetylglutathione was selected for this purpose, since it was reported to be a poor substrate for glyoxalase II from human liver (Uotila, 1973).

Using Sucrose as a Viscogenic Agent. That eq 4 adequately describes the viscosity dependence, due to sucrose, of k_{cat}/K_m for the hydrolysis of *S*-D-lactoylglutathione is supported by the following observations (Table I): First, the magnitude of K_m increases markedly with increasing viscosity, while k_{cat} ($=k_2$) remains essentially invariant within experimental error. Second, for the hydrolysis of *S*-acetylglutathione, wherein k_{cat}/K_m is ~24-fold smaller than that for *S*-D-lactoylglutathione, the magnitudes of *both* K_m and k_{cat} are insensitive to solution viscosity. This observation argues against both competitive inhibition of the enzyme by the viscogenic agent and/or alterations in the intrinsic activity of the enzyme that could arise from a viscosity-dependent conformational change in the enzyme protein. Third, the ratios of the kinetic parameters for *S*-D-lactoylglutathione in the presence and in the absence of sucrose are invariant with pH (Table II). This observation, together with those on *S*-acetylglutathione, indicates that the effect of the viscogen is not a result of altering a kinetically important pK_a due either to the substrate or to the enzyme. Regression analysis of the data of Table I for *S*-D-lactoylglutathione according to eq 4 gives $k_1^0 = (1.64 \pm 0.19) \times 10^6$ M^{-1} s^{-1} and a partition ratio $k_{-1}^0/k_2 = 0.90 \pm 0.26$.

Table II: pH Dependencies of the Kinetic Parameters of Glyoxalase II with *S*-D-Lactoylglutathione in the Presence and Absence of Sucrose[a]

pH	sucrose[b] (% w/v)	$10^{-2}k_{cat}$ (s^{-1})	10^4K_m (M)	$10^{-5}(k_{cat}/K_m)$ (M^{-1} s^{-1})
6.5	0	2.97 ± 0.04	7.25 ± 0.21	4.10 ± 0.07
6.5	32	2.91 ± 0.05	12.84 ± 0.41	2.27 ± 0.04
7.0	0	3.16 ± 0.05	6.82 ± 0.25	4.63 ± 0.11
7.0	32	3.33 ± 0.06	11.97 ± 0.42	2.78 ± 0.05
7.5	0	3.62 ± 0.13	8.37 ± 0.65	4.33 ± 0.02
7.5	32	3.75 ± 0.06	12.42 ± 0.37	3.02 ± 0.05

[a] Phosphate buffer (21 mM); NaCl (250 mM); 25 °C. [b] At 32% (w/v) sucrose, $\eta_{rel} = 2.87$.

A useful way of comparing the data for *S*-D-lactoylglutathione with that for *S*-acetylglutathione is by use of the normalized plot shown in Figure 1 (Brouwer & Kirsch, 1982). The plot shows the relative effect of viscosity on k_{cat}/K_m according to eq 5, derived from eq 4, where $(k_{cat}/K_m)^0$ applies

$$\frac{(k_{cat}/K_m)^0}{(k_{cat}/K_m)} = \frac{k_{-1}^0/k_2}{1 + k_{-1}^0/k_2} + \frac{1}{1 + k_{-1}^0/k_2}\eta_{rel} \qquad (5)$$

at $\eta_{rel} = 1$. Thus, the slopes of the lines in the plot can be directly compared with a slope of unity that obtains at the diffusion limit when $k_2 \gg k_{-1}^0$. On this basis, k_{cat}/K_m for *S*-D-lactoylglutathione is ~50% diffusion controlled; i.e., $k_2 \simeq k_{-1}^0$. For *S*-acetylglutathione, k_{cat}/K_m contains only a small diffusion-dependent component (<3%), in accordance with the smaller comparative magnitude of k_{cat}/K_m for this substrate versus that for *S*-D-lactoylglutathione.

Using Glycerol as a Viscogenic Agent. The variation of the kinetic parameters for *S*-D-lactoylglutathione when glycerol is used as a viscogenic agent cannot be simply explained by eq 4 (Table I). In apparent contradiction to the above observations, K_m increases by ~40% at $\eta_{rel} = 2$ (interpolated), while sucrose produces an increase of ~70% at the same interpolated relative viscosity. Moreover, k_{cat}/K_m is concluded to be ~30% diffusion controlled on the basis of the increase in this parameter with viscosity according to eq 4 (Figure 1). On the other hand, the intrinsic kinetic properties of glyoxalase II are not independent of glycerol concentration, as evidenced by the 25% increase in k_{cat}/K_m for *S*-acetylglutathione at $\eta_{rel} = 2.35$.

A qualitatively similar phenomenon has been reported for the effect of sucrose on the magnitudes of k_{cat}/K_m for acetylcholinesterase with the slow substrates *p*-nitrophenyl acetate and propionyl-β-methylthiocholine (Bazelyansky et al., 1986).

Table III: Buffer, Salt, and Ionic Strength (I_c) Dependencies of the Kinetic Parameters of Glyoxalase II with *S*-D-Lactoylglutathione (pH 7, 25 °C)

ionic strength contribution due to							
sodium phosphate (M)	NaCl (M)	KCl (M)	I_c (total) (M)	$10^{-2}k_{cat}$ (s^{-1})	10^4K_m (M)	$10^{-5}k_{cat}/K_m$ (M^{-1} s^{-1})	symbol[a]
0.0408	0.0092		0.05	3.00 ± 0.03	1.91 ± 0.09	15.71 ± 0.56	△
0.0816	0.0184		0.10	2.83 ± 0.05	3.23 ± 0.16	8.76 ± 0.30	△
0.0816	0.1184		0.20	2.85 ± 0.10	5.49 ± 0.49	5.19 ± 0.30	△
0.0816	0.3184		0.40	2.82 ± 0.03	9.88 ± 0.23	2.85 ± 0.04	△
0.0816	0.5184		0.60	2.62 ± 0.05	11.56 ± 0.46	2.27 ± 0.05	△
0.0816		0.3184	0.40	2.88 ± 0.09	10.53 ± 0.64	2.73 ± 0.08	□
0.200			0.20	3.00 ± 0.06	6.14 ± 0.31	4.89 ± 0.16	○
0.400			0.40	3.09 ± 0.04	11.37 ± 0.29	2.72 ● 0.04	○

[a] The data to the left of each symbol were used to calculate the data points represented by the same symbol in Figure 2.

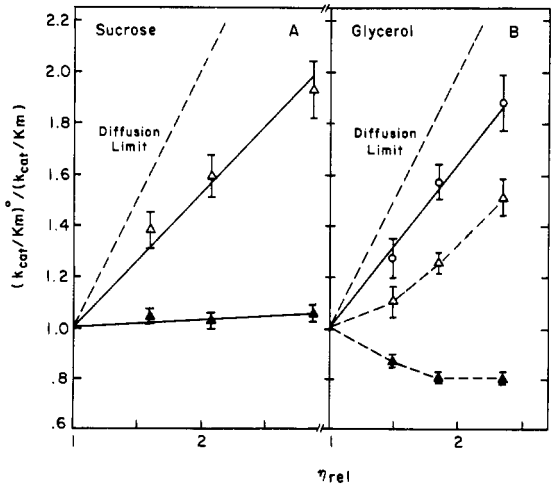

FIGURE 1: (A) Viscosity dependencies of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ for *S*-D-lactoylglutathione (open triangles) and *S*-acetylglutathione (filled triangles) when sucrose was used as a viscogenic agent. The solid lines through the data were calculated by using the best-fit values of k_1^0 and k_{-1}^0/k_2 (see text) in eq 5. (B) Corresponding viscosity dependencies of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ when glycerol was used as a viscogenic agent (dashed lines). Also shown is the viscosity dependency of $(k_{cat}/K_m)^0/[(k_{cat}/K_m)/C]$ (open circles) where $C = (k_{cat}/K_m)/(k_{cat}/K_m)^0$ for *S*-acetylglutathione. The solid line through the data was calculated by using the best-fit values of k_1^0 and k_{-1}^0/k_2 (see text), obtained from regression analysis of $(k_{cat}/K_m)/C = k_1^0/(\eta_{rel} + k_{-1}^0/k_2)$ in eq 5.

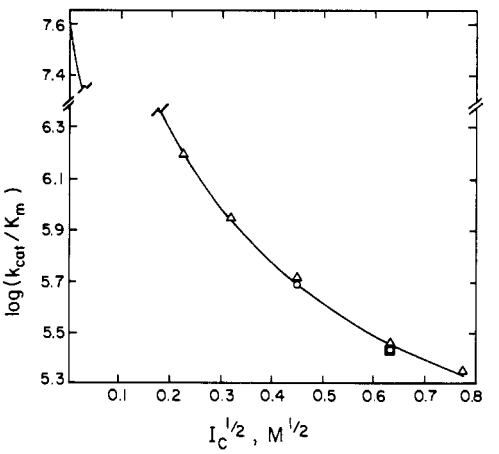

FIGURE 2: Dependence of log (k_{cat}/K_m) for *S*-D-lactoylglutathione on (ionic strength)$^{1/2}$, pH 7, 25 °C. The numerical values of the plotted parameters were calculated from the data to the left of the corresponding symbols shown in Table III. The solid line through the data was calculated from eq 7, where log $[(k_{cat}/K_m)'] = 7.59 \pm 0.13$, $Z = 11.3 \pm 0.1$, and $N = 1.2$ ● 1.0 nm.

These workers were able to rationalize this effect in terms of a "competitive activator" model in which sucrose favorably perturbs the equilibrium distribution of putative enzyme forms containing blocked and unblocked active sites, thus increasing the apparent magnitude of k_1. In this case, k_{cat}/K_m for fast substrates is related to viscosity by eq 6, where C equals

$$k_{cat}/K_m = Ck_1^0/(\eta_{rel} + k_{-1}^0/k_2) \quad (6)$$

$(k_{cat}/K_m)/(k_{cat}/K_m)^0$ for the slow substrate at different known viscosities. Applying this relationship to the hydrolysis of *S*-D-lactoylglutathione by regression analysis of the calculated values of $(k_{cat}/K_m)/C$ (Table I) with the rearranged form of eq 6 gave best-fit values of $k_1^0 = (1.37 \pm 0.12) \times 10^6$ M^{-1} s^{-1} and a partition ratio $k_{-1}^0/k_2 = 0.55 \pm 0.16$ (Figure 1). These values are in approximate agreement with those obtained by using sucrose as a viscogenic agent.[2]

Ionic Strength Dependence. During the course of these studies, the kinetic properties of glyoxalase II with *S*-D-lactoylglutathione were found to be exceedingly sensitive to the ionic strength of the buffers employed in the kinetic measurements. A systematic investigation of this phenomenon revealed that K_m increases markedly with ionic strength while k_{cat} is invariant with ionic strength (Table III). Evidence could not be found for special ion effects on these parameters either by replacing NaCl with KCl or by varying the relative ionic strength contributions due to phosphate ion versus NaCl. Figure 2 shows a log plot of k_{cat}/K_m versus the square root of ionic strength (I_c) according to eq 7, a relationship previously

$$\log (k_{cat}/K_m) = \log [(k_{cat}/K_m)'] + \frac{2AZ_EZ_LI_c^{1/2}}{1 + BNI_c^{1/2}} \quad (7)$$

used to analyze the ionic strength dependency of k_{cat}/K_m for acetylcholinesterase (Nolte et al., 1980), where $(k_{cat}/K_m)'$ applies at zero ionic strength; A and B are constants that apply at 25 °C in aqueous solution having values of 0.509 $M^{-1/2}$ and 3.29 $M^{-1/2}$, respectively; Z_E and Z_L are the apparent charges on the enzyme and substrate, respectively; and N is the mean distance of closest approach between substrate and enzyme. Regression analysis of the data, using the net charge on *S*-D-lactoylglutathione at pH 7 ($Z_L = -1$) gave best-fit values of $(k_{cat}/K_m)' = (3.9 \pm 1.4) \times 10^7$ M^{-1} s^{-1}, $Z_E = 11.3 \pm 0.1$, and $N = 1.2 \pm 1.0$ nm. The values of Z and N are best viewed as empirical constants having uncertain physical significance, as discussed by Smissaert (1981). The physical meaning of these constants is ambiguous because eq 7 is derived from

[2] An alternative model was considered by us, as well as by Bazelyansky et al. (1986), in which the viscogenic agent decreases the partition ratio k_{-1}^0/k_2. In this case, $k_{cat}/K_m = k_1^0/[\eta_{rel} + C^{-1}(k_{-1}^0/k_2)]$. However, there was little difference in the quality of the fit of the data to this relationship versus to that of eq 6. Therefore, both models represent essentially equivalent explanations for the glycerol dependence of k_{cat}/K_m.

Debye–Hückel theory using the problematic assumption that the dielectric constant of the active site is equivalent to that of bulk solvent. To the extent that the dielectric constant of the active site may be overestimated, Z_E will be correspondingly overestimated. In spite of these theoretical limitations, this analysis suggests that k_{cat}/K_m is significantly larger at low ionic strength and that charge–charge interactions are likely to be important in the formation of the initial encounter complex between enzyme and substrate. The association rate constant (k_1^0) is calculated to be $\sim 8 \times 10^7$ M^{-1} s^{-1} at zero ionic strength on the basis of $(k_{cat}/K_m)' \simeq 3.9 \times 10^7$ M^{-1} s^{-1} and $k_{-1}/k_2 = 0.9$.[3]

Comparisons with Other Hydrolases. For glyoxalase II, the magnitudes of the partition ratio (k_{-1}^0/k_2) and the association rate constant (k_1^0) at zero ionic strength are comparable to those for two serine hydrolases with their most active substrates, obtained by the use of the viscosity variation method. For the acylation of chymotrypsin by *N*-(methoxycarbonyl)-L-tryptophan *p*-nitrophenyl ester (pH 8, 25 °C), $k_{-1}^0/k_2 = 2.5$ and $k_1^0 = 9 \times 10^7$ M^{-1} s^{-1} (Brouwer & Kirsch, 1982); for the hydrolysis of benzylpenicillin by β-lactamase (pH 7, 25 °C, $I_c = 0.63$ M), $k_{-1}^0/k_2 = 2$ and $k_1^0 = 7.6 \times 10^7$ M^{-1} s^{-1} (Hardy & Kirsch, 1984). There is, as yet, no evidence for the involvement of seryl or cysteinyl residues in the catalytic reaction mechanism of glyoxalase II, since phenylmethanesulfonic acid, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoate) ion fail to chemically modify the enzyme (Ball & Vander Jagt, 1981). This raises the interesting possibility that glyoxalase II represents a highly efficient solution to the problem of substrate hydrolysis by a reaction mechanism fundamentally different from that used by the serine- and sulfhydryl-dependent hydrolases, known to involve the formation of acyl-enzyme intermediates.

Conclusion. Glyoxalase II is in one sense a near optimal catalyst for the hydrolysis of *S*-D-lactoylglutathione in that the overall free energy of the transition states leading from bound substrate to free enzyme and product are similar in magnitude to that for diffusion-controlled encounter between substrate and active site, as reflected by the near unity value of k_{-1}^0/k_2. Under conditions where [substrate] $\ll K_m$ the rate of hydrolysis will be determined to a significant extent (~50%) by the encounter frequency of enzyme and substrate. The concept of optimality can be applied here in the sense that further improvements in the efficiency of the chemical transformation step(s) (k_2), via evolutionary selection for a better enzyme, would lead to only small increases (no more than 2-fold) in the rate of hydrolysis of substrate when [substrate] $\ll K_m$ (Albery & Knowles, 1976). Thus, glyoxalase II seems to be well adapted to its proposed role in the glyoxalase pathway at low steady-state concentrations of *S*-D-lactoylglutathione.

Registry No. Glx, 9025-90-5; *S*-D-lactoylglutathione, 41656-56-8.

[3] Given the large apparent charge on the enzyme, the decrease in the dielectric constant of water ($\epsilon = 78.5$) due to the presence of sucrose ($\epsilon = 70.9$ for 30% sucrose/water; Åkerlöf, 1932) could potentially increase the rate constant for association between enzyme and substrate according to the Debye–Hückel theory. However, the calculated increase is only minor (~10%), using $Z_E = 11.3$, $Z_L = -1$, and $N = 1.2$ nm with the mathematical functions given in Nolte et al. (1980).

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