Fluorescence and Nuclear Relaxation Enhancement Studies of the Binding of Glutathione Derivatives to Manganese-Reconstituted Glyoxalase I from Human Erythrocytes. A Model for the Catalytic Mechanism of the Enzyme Involving a Hydrated Metal Ion†

Siv Sellin, L. E. Göran Eriksson, and Bengt Mannervik*

ABSTRACT: The apoenzyme of glyoxalase I (EC 4.4.1.5) from human erythrocytes was prepared by removal of Zn^{2+} with ethylenediaminetetraacetic acid (EDTA). Methanol was used as a stabilizing agent. Extended dialysis was required to remove EDTA from the resulting solution of apoenzyme. Reconstitution with Mn^{2+} was followed by measuring enzyme activity, electron paramagnetic resonance of free Mn^{2+} ions, and nuclear magnetic resonance of water protons. The holoenzyme contained two Mn^{2+} per protein dimer and had approximately 50% of the catalytic activity of the native enzyme. The binding of the cosubstrate glutathione (γ-L-glutamyl-L-cysteinylglycine), the product S-D-lactoylglutathione, and the competitive inhibitor S-(p-bromobenzyl)glutathione was monitored by the quenching of the intrinsic tryptophan fluorescence and by the proton relaxation enhancement of water bound to Mn^{2+} in the active site of the enzyme. The dissociation constants were 1.1 mM, 0.42 mM, and 0.54 μM for glutathione, S-D-lactoylglutathione, and S-(p-bromobenzyl)glutathione, respectively. The temperature and frequency dependences of the longitudinal and transverse paramagnetic relaxation rates, $1/T_{1p}$ and $1/T_{2p}$, were studied for water. The results were analyzed in terms of correlation and exchange times. In addition proton and deuteron relaxation rates were measured in parallel at two different magnetic fields. Good agreement between the two approaches of analysis was noticed. The data show that two water molecules are bound in the first coordination sphere of Mn^{2+} in the active site of glyoxalase I. When S-(p-bromobenzyl)glutathione or S-D-lactoylglutathione is bound to the enzyme, only one exchangeable water molecule could be detected, indicating occlusion of the second water molecule. An enediol mechanism involving the metal-bound water is proposed for the catalysis effected by glyoxalase I.

Glyoxalase I (EC 4.4.1.5) catalyzes the formation of S-D-lactoylglutathione from methylglyoxal and glutathione (Ekwall & Mannervik, 1973). The enzyme was found to be inhibited by metal ion chelators and reactivated by bivalent metal ions (Davis & Williams, 1966), and it was concluded that the inactivation should be ascribed to removal of metal ions and not to direct inhibition by binding of the chelating agent to the enzyme (Mannervik et al., 1972). In view of the effectiveness of magnesium ions to restore catalytic activity to inactivated enzyme, it was originally believed that Mg was the metal cofactor of the native enzyme. Later studies, in which the metal content was determined by atomic absorption and mass spectrometry, showed the presence of stoichiometric amounts of Zn in glyoxalase I from several sources (Aronsson et al., 1978). The identification of Zn as the metal essential for activity in native glyoxalase I was further corroborated by studies of the effects of various metal substitutions on the binding properties and the kinetics of the enzyme (Aronsson et al., 1981). Even if the presence of an essential Zn per subunit appears well established, the location of the metal in the protein and its role in catalysis remain to be clarified. For example, no direct proof for the presence of the metal in the active site has been obtained, even if this localization is commonly assumed. Clarification of spatial relationship between the essential metal and the substrate bound to the active site of the enzyme obviously would help decide whether the role of Zn in catalysis is direct or only indirect (e.g., structural). In the present study, Mn-substituted glyoxalase I was used to monitor the interaction between enzyme and some glutathione analogues and to demonstrate that the essential metal is bound to the active site. The ligands used in the study were glutathione, which can be regarded as a cosubstrate, S-D-lactoylglutathione, the product of the reaction, and S-(p-bromobenzyl)glutathione, a strong reversible competitive inhibitor of glyoxalase I (Aronsson et al., 1981). Their structures are shown in Figure 1. A preliminary report of this study has been presented (Sellin et al., 1981).

†From the Department of Biochemistry (S.S. and B.M.) and the Department of Biophysics (L.E.G.E.), Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden. *Received March 1, 1982.* This work was supported by the Swedish Natural Science Research Council.

Experimental Procedures

Enzyme Preparation and Assay. Glyoxalase I from human erythrocytes was purified as described earlier (Aronsson et al., 1979). The preparation consists of a mixture of three isoenzymes, which was used in all studies reported here. No difference in the enzymatic and binding properties of the isoenzymes has been noted (Aronsson et al., 1981). Furthermore, preliminary experiments with isolated isoenzymes gave the same results as those obtained with the mixture. The spectrophotometric method of Racker (1951) was used for activity measurements under conditions detailed by Marmstål et al. (1979). Protein concentration was measured by a microbiuret method (Goa, 1953).

The apoenzyme was prepared by treatment of the enzyme with 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM imidazole, and 10% methanol (v/v) at pH 6.8 and with a subsequent dialysis against 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer containing 10% methanol (v/v) at pH 7.8 for 9 h (Uotila & Koivusalo, 1975; Sellin et al., 1980). An extended dialysis (50 h) was used in some of the experiments. The procedure gave an apoenzyme with only about 1% residual activity. It was possible to restore the enzymatic activity completely by adding

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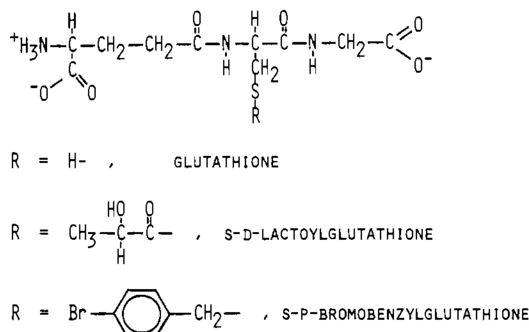

FIGURE 1: Structures of glutathione derivatives binding to the active site of glyoxalase I.

Zn^{2+} or Mg^{2+}, whereas Mn^{2+} gave approximately 50% of the activity of native enzyme. Excess metal ions were removed by dialysis. Electron paramagnetic resonance (EPR) measurement showed that solutions of glyoxalase I reconstituted with Mn^{2+} did not contain any detectable free Mn^{2+} ions. All buffers used were treated with Chelex-100 (Willards et al., 1969). Glassware and plastic vessels were rinsed as described earlier (Aronsson & Mannervik, 1977). The concentration of the enzyme used for the NMR experiments was 20–80 μM, for EPR 40–70 μM, and for the fluorometric studies approximately 0.5 μM. Most solutions also contained approximately 10% (v/v) methanol, which has been found to stabilize the enzyme (Sellin et al., 1980).

$MnSO_4$ of analytical grade was used to prepare reconstituted glyoxalase I. Synthesis of the inhibitor *S*-(*p*-bromobenzyl)-glutathione was carried out by method A of Vince et al. (1971). *S*-D-Lactoylglutathione was prepared by an enzymatic method (Ball & Vander Jagt, 1979).

Fluorescence. Measurements were made on a Jasco Fp-4 spectrofluorometer at 22 °C. The quenching of the intrinsic fluorescence of glyoxalase I was studied as described elsewhere (Aronsson et al., 1981). When the effect of *S*-D-lactoylglutathione on the fluorescence of the enzyme was observed, an excitation wavelength of 300 nm was used in order to minimize the inner-filter effect, and the emission was measured at 347 nm.

Magnetic Resonance Methods. Electron paramagnetic resonance (EPR) spectra were recorded at 20 °C with a Varian Model E-9 X-band spectrometer operating at 100-mW power. A 100-kHz modulation with 10-G amplitude was used. The concentration of free Mn^{2+} ions in the 1-mm i.d. capillary was estimated by comparison with the amplitudes of the outermost low-field hyperfine component using an aqueous Mn^{2+} standard. Ions bound to the protein were not detectable by EPR at room temperature.

Measurements of proton relaxation times were carried out at 100.0 MHz with a Varian Model XL-100A NMR spectrometer with a deuterium internal lock. Measurements at 50.0 and 14.0 MHz were carried out with a Bruker CXP-90 spectrometer, operating at reduced magnetic field. A single transient allowed longitudinal relaxation time (T_1) measurements on water by the 180°–τ–90° sequence. No less than ten different delay times (τ) were used. At 100 MHz a waiting time of $>5T_1$ was applied, and T_1 was calculated by a linear least-squares fit. The 14-MHz data were treated by a nonlinear regression program. Transverse relaxation times (T_2) were estimated at 100 MHz from the minimal line width at half-height of the continuous wave, absorption signal, recorded well below radio frequency power saturation. At 14 MHz T_2 was measured by a Carr–Purcell–Meiboom–Gill pulse sequence.

For application of the proton–deuteron relaxation method (Burton et al., 1979), observations of deuteron relaxation rates were carried out. At 15.4 MHz (100-MHz proton frequency) the XL-100A spectrometer was operating in the Gyrocode mode and with an external proton lock. At 7.7 MHz (50-MHz proton frequency) the Bruker CXP spectrometer was employed. The samples contained 12 or 50% D_2O.

A Varian variable temperature regulator was employed in conjunction with the XL-100A spectrometer. The temperature on the probe was kept at ±0.5 °C and intermittently checked by a Pt resistance, before and after each relaxation measurement. With the Bruker spectrometer room temperature (22 °C) was allowed.

The samples (0.4 mL) were contained in standard 5-mm o.d. NMR tubes with air present. In the case of internal deuterium lock about 12% D_2O was added.

The paramagnetic relaxation rates ($1/T_{ip}$; $i = 1, 2$) were obtained from the measured values ($1/T_{i,\text{obsd}}$) by correcting for the diamagnetic contributions ($1/T_{i,\text{dia}}$). As diamagnetic (blank) samples, either native glyoxalase I, the apoenzyme, or the buffer was used. The proton relaxation enhancement (PRE) is defined as

$$\epsilon_i = \frac{1/T_{ip}^*}{1/T_{ip}} = \frac{1/T_{i,\text{obsd}}^* - 1/T_{i,\text{dia}}^*}{1/T_{i,\text{obsd}} - 1/T_{i,\text{dia}}} \qquad i = 1, 2 \quad (1)$$

The asterisk indicates that the rates are measured in the presence of protein.

The experimentally determined paramagnetic relaxation rates, $1/T_{ip}$, are related to the contribution from the protons within the first hydration sphere of Mn^{2+}, $1/T_{iM}$, by the Swift–Connick equation (Dwek, 1973).

$$1/T_{ip} = \frac{qp_M}{T_{iM} + \tau_M} \qquad i = 1, 2 \quad (2)$$

Here $p_M = [Mn^{2+}]/[H_2O]$, where the water concentration is 55.6 M in the absence of methanol. No corrections for any effects of heavy water on this concentration were applied. An ideal mixture was assumed with a small volume of methanol present. No isotopic effects of deuterons were assumed. q is the number of exchangeable water molecules, on the NMR time scale, within the Mn^{2+} hydration sphere. τ_M is the residence lifetime of protons of the ligands. Any contribution to the relaxation rates from the outer sphere protons is not included. When $T_{iM} > \tau_M$, fast exchange prevails and eq 2 is simplified to $1/T_{ip} = p_M q/T_{iM}$; if $T_{iM} \sim \tau_M$, the exchange conditions are intermediates between fast and slow. The effective correlation time τ_c characterizing the dipolar interaction is given by

$$1/\tau_c = 1/\tau_r + 1/\tau_s + 1/\tau_M \quad (3)$$

where τ_r is the rotational correlation time of the Mn^{2+}–proton vector and τ_s is the longitudinal relaxation time of the Mn^{2+}–electron spin. The fastest rate process has the dominating contribution to τ_c. As seen, τ_M is involved in eq 3, as well as in eq 2. When the temperature and frequency dependence of T_{1p} and T_{2p} are studied, it may be possible to elucidate the actual rate conditions. If the relaxation rates within the hydration sphere, $1/T_{iM}$, are expressed by the simplified Solomon–Bloembergen equations, the following expression is obtained (James, 1975):

$$T_{1p}/T_{2p} = (7 + 4\omega_I^2\tau_c^2)/6 \quad (4)$$

where ω_I is the proton Larmor angular frequency. In this equation it is assumed that the outer sphere and scalar contributions to the relaxation rates are negligible. Other con-

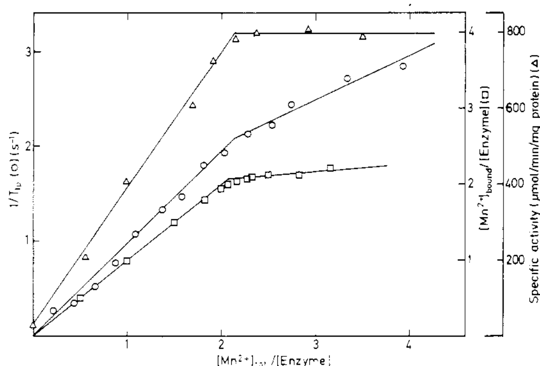

FIGURE 2: Titration of apoglyoxalase I with Mn^{2+} ions in 10 mM Tris-HCl (pH 7.8) containing 10% (v/v) methanol: (Δ) specific enzyme activity (30 °C); (O) longitudinal relaxation rates of water protons (43 μM protein); (□) concentration of bound Mn^{2+} per protein molecule (60 μM) determined by EPR. Temperature 20 °C.

ditions that should be fulfilled are fast exchange and $\omega_s^2\tau_c^2 > 1$, where ω_s is the corresponding angular frequency for the electron. When these prerequisites are met, a value for τ_c may be calculated from eq 4 at a given Larmor frequency.

According to the simplified Solomon–Bloembergen equation for fast exchange conditions

$$T_{1p} = \frac{r^6}{Cp_Mq\tau_c}(1 + \omega_I^2\tau_c^2) \quad (5)$$

where $C = {}^6/_{15}\,S(S + 1)\gamma_I^2g^2\beta^2$. For Mn^{2+} it is assumed that $S = {}^5/_2$ and $g = 2$. Measurements of T_{1p} at two frequencies may also provide a value of τ_c:

$$T_{1p}(1)/T_{1p}(2) = \frac{1 + \tau_c^2\omega_I(1)^2}{1 + \tau_c^2\omega_I(2)^2} \quad (6)$$

τ_c should be independent of frequency. In the present study measurements at frequencies 14, 50, and 100 MHz were carried out for protons.

In an application of the "proton/deuteron relaxation rate method" of Burton et al. (1977), T_{1p} was measured for identical samples at two different magnetic fields (B_1 and B_2) with both proton NMR and deuteron NMR. For both nuclei it follows from eq 2 and 5 that

$$p_MT_{1p} = \frac{Dr^6}{q}\left(\frac{1 + \omega_I^2\tau_c^2}{\omega_I^2\tau_c}\right)B^2 + \frac{\tau_M}{q} \quad (7)$$

where $D = {}^{15}/_6[1/[g^2\beta^2S(S + 1)]]$. This gives four equations with the unknowns $\tau_c(B_1)$, $\tau_c(B_2)$, τ_M, and q. The system of equations was solved by an iterative computer program.

Results

Titration of Apoglyoxalase I with Mn^{2+}. The binding of Mn^{2+} to the apoenzyme of glyoxalase I from human erythrocytes was studied with EPR by observing the free $Mn(H_2O)_6^{2+}$. Upon binding to the protein, Mn^{2+} was no longer detectable at room temperature owing to the zero-field splitting in an anisotropic environment. At liquid nitrogen temperature, the protein-bound Mn^{2+} gave an irregular spectrum. Figure 2 shows that the metal-binding sites of the protein were saturated after addition of 2 equiv of Mn^{2+} per enzyme molecule. Since the enzyme is a dimeric protein (Aronsson et al., 1979), this stoichiometry corresponds to one Mn atom per subunit. The concomitant restoration of enzymatic activity indicates that Mn^{2+} is bound to the active site of glyoxalase I (Figure 2). The titration was also followed by measuring the NMR relaxation rate $1/T_{1p}$ of water protons. At a stoichiometry of 2 equiv of Mn^{2+} per enzyme molecule, a break point occurs

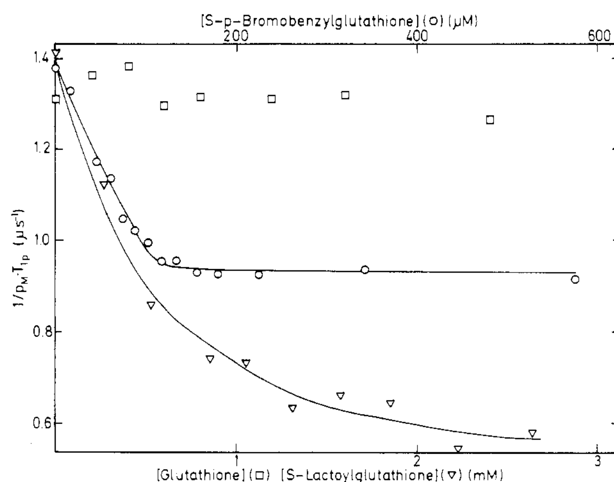

FIGURE 3: Titration of Mn–glyoxalase I with some glutathione derivatives monitored by the paramagnetic proton relaxation rate of water [$1/(p_MT_{1p})$] at 100 MHz. 60 mM Tris-HCl (pH 7.8) at 20 °C was used. The symbols show the experimental points, and the graphs are obtained by nonlinear regression. (O) *S*-(*p*-Bromobenzyl)glutathione. The enzyme concentration was 56 μM. (▽) *S*-Lactoylglutathione. Enzyme concentration was 38 μM. (□) Glutathione. Enzyme concentration was 63 μM.

above which the contribution of $Mn(H_2O)_6^{2+}$ sets in (Figure 2). The affinity of the apoenzyme for Mn^{2+} is high, but a direct determination of the dissociation constant could not be made owing to the lack of sufficient sensitivity of the EPR and NMR methods. The tight binding of Mn^{2+} to the protein in the binary Mn–enzyme complex allowed the calculation of an enhancement factor of $\epsilon = 3.4$ for the Mn–holoenzyme.

In titration experiments with some of the first preparations of apoenzyme, it was found that considerably more than 2 (up to 20) equiv of Mn^{2+} had to be added to achieve saturation. The EPR measurements gave the same curve shape as in Figure 2, but the NMR and activity measurements yielded sigmoid titration curves. Evidently, Mn^{2+} was bound to something distinct from the active site of the enzyme. After it was established that no unspecific ion interaction with the protein existed, the effect was traced to chelation of Mn^{2+} by EDTA, which gives a complex lacking a detectable EPR signal at room temperature. The EDTA remained after preparation of the apoenzyme, and it was found that dialysis for 50 h was required to completely remove the interfering EDTA. A similarly slow rate of diffusion was found (with several dialysis membranes) for EDTA in the absence of any protein, suggesting that the long escape time was not caused by binding of EDTA to the apoenzyme. Any significant amount of EDTA remaining in the apoenzyme preparation causes an initial "lag phase" in the proton relaxation titration curve, because the enhancement factor for water protons in the EDTA–Mn^{2+} complex is only 0.5 (King & Davidson, 1958) as compared to $\epsilon = 3.4$ for Mn–glyoxalase I.

Titration of Mn–Glyoxalase I with Glutathione and Glutathione Derivatives As Followed by Proton Relaxation and Fluorescence. Kinetic studies have demonstrated that *S*-(*p*-bromobenzyl)glutathione is a strong competitive inhibitor of glyoxalase I from human erythrocytes (Aronsson et al., 1981). Several lines of evidence indicate that this inhibitor binds to the active site of the enzyme (Aronsson et al., 1981). The titration of Mn–glyoxalase I with this glutathione derivative was monitored by the proton relaxation rate (Figure 3) and by the quenching of the intrinsic fluorescence of the protein (Figure 4). Similar titrations had been carried out in some preliminary experiments (Sellin et al., 1981), but since these earlier experiments had been carried out in 10% (v/v) meth-

Table I: Binding Parameters of Glutathione Derivative Ligands of Mn–Glyoxalase I

ligand	methanol (%)	K_d (μM) fluorometry	K_d (μM) proton relaxation	no. of binding sites[a]	ϵ_t/ϵ_b [b]
S-(p-bromobenzyl)glutathione	8	1.6 ± 0.3[c]	ND[d]	1.7 ± 0.3[c]	0.59
S-(p-bromobenzyl)glutathione	0	0.54 ± 0.09[e]	ND	1.9 ± 0.1	0.64
glutathione	0	1100 ± 100	no effect	ND	
S-D-lactoylglutathione	0	no effect	420 ± 80	ND	0.29

[a] From proton relaxation data. [b] Ratio of the enhancement of the ternary ligand–Mn–glyoxalase I complex and the enhancement of the binary Mn–glyoxalase I complex. [c] Sellin et al. (1981). [d] ND, not determined; the data are consistent with the K_d values determined by fluorometry, but owing to the high protein concentrations used for the proton relaxation measurement, independent values cannot be estimated accurately. [e] Aronsson et al. (1981).

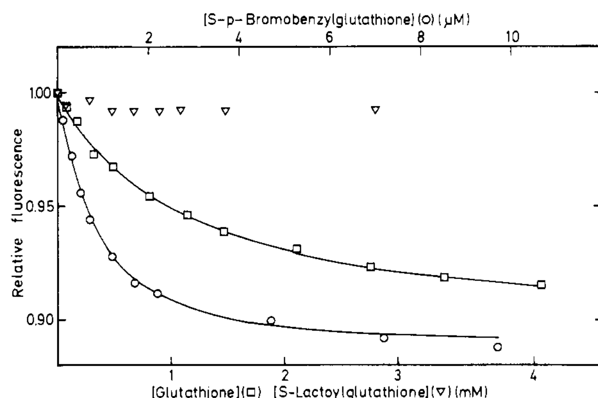

FIGURE 4: Titration of the intrinsic tryptophan fluorescence of Mn–glyoxalase I with glutathione derivatives at 22 °C. (O) 0.27 μM S-(p-bromobenzyl)glutathione and 10 mM Tris-HCl (pH 7.8). λ_{ex} = 280 nm and λ_{em} = 344 nm. (□) 0.89 μM glutathione and 50 mM Aces buffer (pH 6.3). λ_{ex} = 280 nm and λ_{em} = 344 nm. (▽) 0.46 μM S-lactoylglutathione and 50 mM Aces buffer (pH 6.9). λ_{ex} = 300 nm and λ_{em} = 346 nm. The curves are fitted by nonlinear regression (Aronsson et al., 1981). The binding parameters obtained are K_d = 0.54 ± 0.09 μM [S-(p-bromobenzyl)glutathione] and K_d = 1.1 ± 0.1 mM (glutathione).

anol, it was desirable to repeat the experiments. (Meanwhile it had been found that glyoxalase I preparations were stable enough for these titrations in the absence of methanol.) The fluorescence titration curve was fitted by use of nonlinear regression as previously described (Aronsson et al., 1981). A dissociation constant (K_d) of 0.54 ± 0.09 μM was obtained for a binding stoichiometry (n) of 2 mol of ligand per mol of enzyme. Table I shows that the affinity for S-(p-bromobenzyl)glutathione is 3-fold lower in the presence of 10% methanol. In the case of the proton relaxation titration, the protein concentration was 2 orders of magnitude higher, and the data were not suitable for an accurate estimate of the K_d value, but quite adequate for an estimate of n. Therefore, the nonlinear regression was restricted by setting K_d equal to the value (0.54 μM) determined previously by the fluorescence titration. Thus, a value for the binding stoichiometry of n = 1.9 ± 0.1 mol/mol was obtained from the proton relaxation experiments in agreement with the titration curves of Figure 3. The enhancement factor for the ternary inhibitor–Mn–protein complex (ϵ_t) was about 40% lower than that for the binary Mn–protein complex (ϵ_b).

Equilibrium binding studies were also performed with free glutathione and the product of the enzymatic reaction S-D-lactoylglutathione. Glutathione had no significant effect on the proton relaxation of water molecules bound to Mn^{2+} in the enzyme (Figure 3), whereas excess of S-D-lactoylglutathione decreased the proton relaxation enhancement by approximately 70% (Figure 3). The dissociation constant for the binding of S-D-lactoylglutathione was estimated as K_d = 0.42 ± 0.08 mM from these experiments.

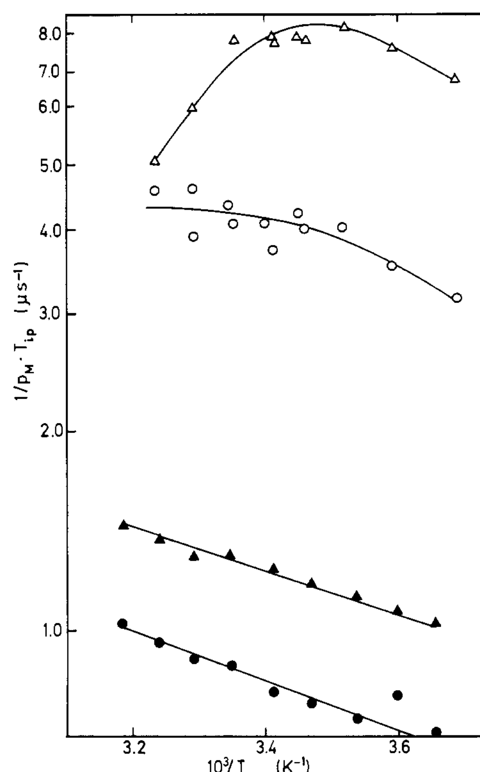

FIGURE 5: Temperature dependence of the normalized water proton relaxation rates at 100 MHz with Mn–glyoxalase [65 μM; (△, ▲)] and with the complex by S-(p-bromobenzyl)glutathione [76 μM; (O, ●)]. The values of $1/(p_M T_{1p})$ are denoted by filled symbols and those of $1/(p_M T_{2p})$ by open symbols. The solutions contained 50 mM Tris-HCl (pH 7.8), 8% methanol, and 12% D_2O.

When the binding was followed by fluorescence quenching, the reverse results were obtained: S-D-lactoylglutathione did not cause any significant effect, but glutathione gave quenching of similar amplitude as that induced by S-(p-bromobenzyl)-glutathione (Figure 4). The dissociation constant for glutathione was estimated as K_d = 1.1 ± 0.1 mM. Even if S-D-lactoylglutathione did not quench the protein fluorescence, it was nevertheless found to compete with S-(p-bromobenzyl)-glutathione bound to the enzyme and abolish the quenching caused by the latter ligand. Thus, both glutathione and S-D-lactoylglutathione bind to the enzyme with dissociation constants in the millimolar range, but the inhibitor S-(p-bromobenzyl)glutathione has an affinity for the enzyme that is about 3 orders of magnitude larger. The binding parameters are collected in Table I.

Temperature and Frequency Dependence of $1/T_{1p}$ and $1/T_{2p}$. The dependence of the proton relaxation rate on temperature and frequency was studied with Mn–glyoxalase I and its complex with S-(p-bromobenzyl)glutathione in order to elucidate the physical mechanisms behind the effects on the relaxation. A positive temperature dependence in the interval

Table II: Relaxation Rates, Correlation Time (τ_c), and Rapidly Exchangeable Water Molecules (q) in Mn–Glyoxalase I and an Inhibitor Complex[a]

sample	$1/(p_M T_{1p}) \times 10^{-6}$ (s^{-1})			$1/(p_M T_{2p}) \times 10^{-6}$ (s^{-1})		τ_c (ns)		
	100 MHz	50 MHz	14 MHz	100 MHz	14 MHz	1[b]	2[c]	q[d]
Mn–glyoxalase I	1.54	4.09	6.51	9.2	7.0	3.6	4.4	1.8 ± 0.4
Mn–glyoxalase I + S-(p-bromobenzyl)glutathione[e]	0.829	2.09	2.91	4.2	3.3	3.3	3.9	0.9 ± 0.2

[a] The measurements of relaxation rates were carried out at 20 °C in 50 mM Tris-HCl (pH 7.8) containing 12% (v/v) D_2O and 8% (v/v) methanol. The concentration of bound Mn in the two samples was 130 and 152 μM, respectively. [b] Calculated from frequency dependence, eq 4. [c] Calculated from T_{1p}/T_{2p}, eq 6. [d] Calculated from averages of the corresponding τ_c values of sets 1 and 2 and assuming $r = 2.87 \pm 0.05$ Å. Errors of q were calculated from the estimated errors of T_{ip} (1–6%) and τ_c (5–6%) and that of r. [e] The concentration of S-(p-bromobenzyl)glutathione was 0.8 mM.

0–40 °C was obtained for $1/T_{1p}$ at 100 MHz for both Mn–glyoxalase I and its inhibitor complex (Figure 5). The activation energies for these species were calculated as 5.8 and 7.1 kJ/mol, respectively. The samples contained 10% (v/v) methanol as a stabilizing agent, but control experiments carried out in the absence of methanol showed that the activation energies were essentially the same. Experiments with S-D-lactoylglutathione gave a positive temperature dependence for $1/T_{1p}$ with an activation energy of 9.9 kJ/mol. The fact that $d(1/T_{1p})/dT > 0$ would appear to indicate a condition of slow exchange (James, 1975), but additional findings indicate that on the contrary, a condition of fast exchange prevails at 20 °C (cf. James, 1975): (1) The values of the activation energies are considerably lower than the value of 20 kJ/mol expected for a slow-exchange condition. (2) The ratios of T_{1p}/T_{2p} are 6.2 for Mn–glyoxalase I and 5.1 for its inhibitor complex, respectively; consequently $\omega_I\tau_c > 1$. (3) $1/T_{1p}$ increased when the frequency was changed from 100 to 14 MHz (Table II); no frequency dependence is expected for a pure slow exchange.

The value of $1/T_{2p}$ for Mn–glyoxalase I decreased with temperature above about 15 °C (Figure 4), which may indicate fast exchange in this temperature region. A similar temperature dependence of $1/T_{2p}$ was obtained for the complex between S-D-lactoylglutathione and Mn–glyoxalase I. In the case of the S-(p-bromobenzyl)glutathione–Mn–glyoxalase I complex, $1/T_{2p}$ increases slowly with temperature. A pure slow-exchange condition can be excluded since the activation energy is significantly lower than expected for this case (James, 1975). However, some slow-exchange contribution leading to a state of an intermediate exchange cannot be ruled out for $1/T_{2p}$ of the ternary complex.

Determination of Correlation Time (τ_c) and Number of Exchangeable Water Molecules (q) in Mn–Glyoxalase I from Proton Relaxation Rates. Values of τ_c were calculated from the ratio of T_{1p}/T_{2p} according to eq 4 (Table II). Fast-exchange conditions were assumed for both relaxation rates even though the temperature dependence (Figure 5) indicated some slow-exchange contribution to $1/T_{2p}$. It has been pointed out that if $1/T_{2p} \gg 1/T_{1p}$, the presence of some slow-exchange contribution to $1/T_{2p}$ will not significantly affect the value calculated for q (Burton et al., 1979). Values of τ_c were also calculated according to eq 5. Only the T_{1p} values of the two highest frequencies were used, because a plot of T_{1p} vs. ω_I^2 appeared to deviate slightly from linearity at low frequencies, indicating that τ_c is frequency dependent and dominated by τ_s in the domain of low frequencies (Dwek, 1973). The τ_c values obtained by the two methods agree resonably well (Table II) and are in the range expected for macromolecular Mn complexes (Burton et al., 1979).

The number of rapidly exchangeable water molecules (q) bound to Mn in the enzyme was calculated as $q = 1.8 \pm 0.4$

Table III: Relaxation Parameters of Mn–Glyoxalase I Obtained by the Proton/Deuteron Relaxation Rate Method[a]

B (kG)	ν (MHz) proton	ν (MHz) deuteron	T_{1p} (s) proton	T_{1p} (s) deuteron	τ_c (ns)	τ_M (ns)	q
23.5	100	15.4	0.135	1.142	3.14	129	1.9
11.8	50	7.7	0.0636	1.079	3.13		

[a] The sample contained 0.124 mM Mn–glyoxalase I, 50 mM Tris-HCl, pH 7.8 (uncorrected), 50% D_2O, and 5% methanol. Temperature 22 °C.

according to eq 5 by adopting a value of $r = 2.87 \pm 0.05$ Å (James, 1975) for the distance between Mn and a water proton in its first hydration sphere. The choice of the distance was based on the assumption of an octahedral coordination of ligands around the metal (Sellin et al., 1982a). The values of τ_c used to estimate q were averages of the values given in Table II. In one experiment in which no methanol was added, the q value was only marginally affected ($q = 2.0 \pm 0.4$), indicating that the presence of methanol (8% v/v) should have no influence on the qualitative interpretation of the data. Thus, an integral value of 2 appears most plausible for the number of exchangeable water molecules in the first coordination sphere of Mn–glyoxalase I. The value of q for the inhibitor–Mn–glyoxalase I complex appears to be one unit lower ($q = 1$). Addition of S-D-lactoylglutathione to Mn–glyoxalase I decreased the value of q from 1.8 to 0.5 (or to 0.7, if intermediate exchange conditions are assumed).

Determination of τ_c, τ_M, and q by the Proton/Deuteron Relaxation Rate Method. The proton/deuteron relaxation rate method, earlier used for Gd^{3+} (Burton et al., 1977), was applied to the study of Mn–glyoxalase I. With this method no assumption about the time scale for the exchange is required. For the same sample, containing 50% D_2O, $1/T_{1p}$ was measured at a field of 23.5 and 11.8 kG for both the aqueous protons and deuterons bound to Mn^{2+} in glyoxalase I. The choice of the deuterium concentration was not crucial, since almost identical relaxation rates were observed with 12% D_2O. Owing to the comparatively low sensitivity of $1/T_{1p}$ in the deuteron relaxation measurements, the enzyme concentration was kept high (124 μM).

As seen from Table III the deuteron relaxation rates are roughly an order of magnitude less than the corresponding proton rates. This is to be expected because of the lower value of the resonance frequency for deuterons as compared with that for protons. The T_{1p} increases with increasing frequency as can be expected for τ_c values of the estimated order and under fast-exchange conditions. Analysis of the data, assuming $r = 2.87$ Å, gave τ_c values which were almost identical (3.1 ns) at the two magnetic fields. The values are in fair agreement with those obtained with the conventional proton re-

laxation rate method (Table II). The value of $\tau_M = 129$ ns implies that τ_M has a negligible contribution to τ_c at 22 °C; neither does it have any major contribution to T_{1p} in eq 2. Hence, the assumption of fast exchange at 100 MHz for $1/T_{1p}$ with protons is validated. The value of $q = 1.9$ is in good agreement with the values obtained previously (Table II). The estimated experimental error in T_{1p} of 5 and 10% for the protons and deuterons, respectively, would correspond to a maximal error in q of about 0.2 unit.

Effects of Mn–Glyoxalase I on the Proton NMR Spectra of Glutathione and S-D-Lactoylglutathione. Some preliminary studies of the effect on the 100-MHz ^{1}H NMR spectra of glutathione and *S*-D-lactoylglutathione from binding of these ligands to glyoxalase I were carried out. By comparison of the spectra obtained in the presence of native or Mn–glyoxalase I, differential paramagnetic broadening of the individual line width was detected. The proton resonances of the glutamyl and cysteinyl residues of glutathione were broadened more markedly than the peak of the methylene protons of glycine, indicating that the glycine residue is more remote from Mn than are the other residues of the tripeptide. These studies have been extended in collaboration with Drs. A. S. Mildvan and P. R. Rosevear (Sellin et al., 1982b).

Discussion

The present study focuses on glyoxalase I in which the essential Zn of the native protein (Aronsson et al., 1978) has been replaced by Mn. This substitution has previously been found to yield Mn–glyoxalase I with approximately 50% of the catalytic activity of the native enzyme [see Sellin et al. (1980) and references cited in this paper]. Mn^{2+} furnishes the metal-binding site with a paramagnetic probe which can be used to explore the vicinity of this site. In particular, Mn^{2+} is suitable as a relaxation probe since it may cause pronounced NMR effects. The various assumptions underlying the use of the Solomon–Bloembergen equation (cf. Burton et al., 1979) are more easily fulfilled for Mn^{2+} than for alternative paramagnetic probes.

Substitution of Co for Zn in glyoxalase I also gives a catalytically active protein with a paramagnetic center in the metal-binding site. Co(II)–glyoxalase I has been prepared, but, as expected, the observed paramagnetic effect on the NMR relaxation was small in comparison with that of Mn–glyoxalase I. EPR spectrometry showed that the electron g tensor was anisotropic. The results of some spectroscopic studies of Co–glyoxalase I will be reported elsewhere (S. Sellin, L. E. G. Eriksson, and B. Mannervik, unpublished data).

Metal analysis by atomic absorption spectrometry has previously shown that native glyoxalase I from human erythrocytes contains two Zn per enzyme molecule (i.e., one Zn per subunit) (Aronsson et al., 1978). A content of two metal ions per enzyme molecule was also demonstrated when the apoenzyme was reactivated with Mg^{2+}, Mn^{2+}, or Co^{2+} (Sellin et al., 1980). This stoichiometry was confirmed in the present investigation (Figure 2) by the results of titrations of apoglyoxalase I with Mn^{2+}, as monitored by the catalytic activity, concentration of free Mn^{2+} (measured by EPR), and increase in the longitudinal relaxation rate of water protons, $1/T_{1p}$ (measured by NMR). The titrations also indicate a tight binding of the Mn^{2+} ions ($K_d < 10^{-7}$ M) typical of metalloenzymes.

A practical consequence of the high affinity of apoglyoxalase I for bivalent metal ions is that the apoprotein will readily bind trace amounts of such ions in reaction vessels and the air. In the presence of EDTA the catalytic activity of the apoenzyme preparation can be kept ≤1% of the specific activity of the native enzyme, but when EDTA is removed by extended dialysis (>50 h), the activity rises to 5–10% in spite of rigorous attempts to maintain metal-free conditions. Therefore, Mn–glyoxalase I was always prepared by addition of Mn^{2+} to an apoenzyme preparation which contained a small amount of EDTA (that remaining after 9-h dialysis; see Results) in order to avoid the binding of extraneous metal ions occurring in the absence of EDTA. The Mn–glyoxalase I obtained by this procedure was found to contain ≥1.9 g-atoms of Mn per mol of enzyme and ≤0.05 g-atom of Zn per mol of enzyme as determined by atomic absorption spectrometry.

A significant result of the present investigation is the definitive demonstration that the essential metal of glyoxalase I is part of the active site of the enzyme. Since the discovery of the metal dependence of glyoxalase I (Davis & Williams, 1966), it has been explicitly or tacitly assumed that the essential metal was located in the active center, but the evidence has so far only been indirect. An example, in the case of the human enzyme, is the finding that the steady-state kinetic parameters and, even more markedly, the affinity of the competitive inhibitor *S*-(*p*-bromobenzyl)glutathione were clearly dependent on the nature of the bivalent metal ion used to reactivate the apoenzyme (Aronsson et al., 1981). The perturbation of the fluorescence spectrum of a tryptophan residue, whose position was ascribed to the active center of the enzyme, is another indication for the presence of the metal in the active site (Aronsson et al., 1981). Although these, and other, findings give support for this location of the essential metal, they do not rigorously exclude the possibility that the metal has a purely structural role, in which it serves only to stabilize a catalytically active conformation of the enzyme. The effects of glutathione derivatives on the proton relaxation rates of water molecules bound to Mn in Mn–glyoxalase I, which are reported here, add to the body of such indirect, but not fully conclusive, evidence for the location of the metal in the active site. However, the paramagnetic effects of Mn^{2+} on the protons of glutathione and *S*-D-lactoylglutathione prove that these ligands do bind in the vicinity of the metal. Since glutathione is a part of the hemimercaptal substrate and *S*-D-lactoylglutathione, the product of the enzymatic reaction, it appears evident that they bind to the active site. The fact that only one molecule of glutathione and one Mn are bound per subunit (two per enzyme molecule) rules out the possibility of interaction at a secondary binding site. Thus, the above findings seem to provide definitive evidence for a role of the essential metal in the active site of glyoxalase I.

The results of the studies of the binding of glutathione, *S*-D-lactoylglutathione, and *S*-(*p*-bromobenzyl)glutathione give some insight into the interaction of these ligands and the substrate to the active site of glyoxalase I. Human glyoxalase I, like the enzyme from other mammalian sources, is composed of two subunits (Marmstål et al., 1979). Since no cooperativity in binding has been detected and only one molecule of glutathione derivative is bound per subunit (Aronsson et al., 1981), it is, in the present context, sufficient to discuss events at a single binding site in one of the subunits. Table I shows that, although the three ligands all bind to Mn–glyoxalase I, only the strong competitive inhibitor *S*-(*p*-bromobenzyl)glutathione causes both quenching of the intrinsic tryptophan fluorescence of the protein and a decrease of the proton relaxation enhancement. The product of the enzymatic reaction, *S*-D-lactoylglutathione, gives a clear effect only on the proton relaxation enhancement, whereas unsubstituted glutathione affects only the tryptophan fluorescence. On the basis of kinetic and binding studies, it has been concluded that *S*-(*p*-

bromobenzyl)glutathione binds to the active site of the enzyme (Aronsson et al., 1981). S-D-Lactoylglutathione and glutathione, being product and part of the substrate of the enzymatic reaction, respectively, also must be capable of binding to the active site. In view of the structural similarities of the three ligands (Figure 1), we therefore assume that they have basically the same binding site and that their different spectroscopic effects are caused by different modes of interaction with the protein.

The quenching of the intrinsic protein fluorescence by glutathione and S-(p-bromobenzyl)glutathione is interpreted as being caused by a conformational change of the protein. This interpretation has support from earlier studies with S-(p-bromobenzyl)glutathione (Aronsson et al., 1981), as well as from the finding that also glutathione itself gives a similar (approximately 10%) effect on the fluorescence (Figure 4). The S substituent is evidently not required for quenching even if it confers a higher affinity of binding to the glutathione moiety. The explanation for the lack of quenching by S-D-lactoylglutathione would consequently be that this ligand binds to another conformational state of the protein, similar to or identical with that of the free (unliganded) protein. The binding modes of the three ligands cannot be completely different, since both S-(p-bromobenzyl)glutathione and S-D-lactoylglutathione affect the proton relaxation enhancement (Figure 3). Evidently, for interaction with the inner sphere ligands of the essential metal, an S substituent on glutathione is necessary. The binding parameters of S-(p-bromobenzyl)glutathione obtained by the fluorescence quenching and the proton relaxation measurements are fully consistent, indicating that both methods monitor the same binding process. The ratio of the proton relaxation enhancements ϵ_t/ϵ_b was 0.59–0.64 for S-(p-bromobenzyl)glutathione and 0.29 for S-D-lactoylglutathione (Table I). The results of the fluorescence studies in combination with the paramagnetic effects on the protons of the glutathione ligands indicate strongly that the effects of the S substituents on the relaxation rates of water protons are caused by positioning of the S substituents in the vicinity of the essential metal and not by conformational change of the protein. It is noteworthy that the lactoyl group has a larger effect than the p-bromobenzyl substituent in spite of the fact that the latter confers a very significantly higher affinity to the glutathione moiety (Table I).

Several alternative explanations for the effects of the glutathione derivatives on the relaxation rates of water protons exist: (1) displacement of water molecules from the coordination sphere of the paramagnetic metal ion by direct or indirect substitution and (2) immobilization of water molecules or change of the correlation time for the metal–H_2O interaction.

In the absence of ligands the number (q) of rapidly exchangeable water molecules bound to Mn was calculated to be close to two (Tables II and III). The calculation is critically dependent on the assumed value (2.87 Å) for the distance between the metal and a water proton in its hydration sphere, but a distance short enough to give $q = 1$ would appear too small, judging from the range of distances reported. Furthermore, the decrease in q upon addition of S-(p-bromobenzyl)glutathione (Table II) or S-D-lactoylglutathione suggests that the smallest values of q in the presence and absence of ligand are 1 and 2, respectively, assuming that integral numbers apply. The possibility of half-site reactivity of the dimeric protein, leading to lower q values, is ruled out by the binding stoichiometry and lack of negative cooperativity in the binding isotherm.

The metal ion in Co(II)–glyoxalase I appears to have an octahedral coordination (Sellin et al., 1982a), and it appears likely that the coordination scheme of Mn–glyoxalase I would be similar. Thus, a value of $q = 2$ would imply that the protein provides four ligands in addition to the two water molecules. Pyruvate kinase, which has been found to have an octahedral coordination (Mildvan & Cohn, 1965), is an example of a protein giving a q value of 2 (Navon, 1970). Carboxypeptidase, which has a tetrahedral metal coordination with one water ligand and three amino acid side chains (Lipscomb et al., 1969), was found to have $q = 1$ as determined by proton relaxation rates (Navon, 1970).

The determination of the number of exchangeable water molecules in Mn–glyoxalase I as being $q = 2$ has strong support by the proton/deuteron relaxation method (Table III). The simplicity of this method makes it an attractive alternative to the somewhat ambiguous interpretation of temperature and frequency studies used in analysis of conventional proton relaxation measurements (Burton et al., 1977, 1979). Burton et al. (1977, 1979) have earlier applied the method to Gd^{3+} bound to immunoglobulins. The present study demonstrates that it is also suitable for studies of Mn^{2+} bound to proteins. However, it should be noted that deuteron NMR measurements require higher enzyme concentrations than do proton NMR measurements, which may limit the use of the method.

The q value of Mn–glyoxalase I calculated from measurements in the presence of S-(p-bromobenzyl)glutathione was 0.9, assuming fast-exchange conditions. Since the dependence of $1/T_{2p}$ on temperature (Figure 5) indicated the possibility of some slow-exchange contribution, an estimation of q in the case of intermediate-exchange conditions ($T_{2M} = \tau_M$) was undertaken. For this case, a value of $q = 1.2$ was calculated as compared to 0.9 in Table II, demonstrating that the lower q value obtained in the presence of S-(p-bromobenzyl)glutathione cannot originate merely from a change in the correlation time (τ_M). Thus, the ligand appears to reduce the number of exchangeable water molecules from two to one.

The analysis of the proton relaxation data cannot discriminate between the occlusion or the expulsion of one water ligand on Mn in the active site of Mn–glyoxalase I. However, for steric reasons it appears more likely that one water molecule is immobilized rather than being displaced by the bulky p-bromobenzyl group, if an octahedral coordination to the metal is to be retained. Furthermore, the tight binding of S-(p-bromobenzyl)glutathione probably arises from hydrophobic interactions rather than from coordination of the p-bromobenzyl group to the metal. In the case of S-D-lactoylglutathione a direct coordination of the lactoyl group to the metal would, a priori, seem more probable. However, since the enhancement of the proton relaxation rate is far from eliminated ($\epsilon_t/\epsilon_b = 0.29$, Table I), both water molecules bound to Mn cannot be displaced, even if the value of $q = 0.5$ is somewhat smaller than that obtained with S-(p-bromobenzyl)glutathione.

Thus, also in the case of S-D-lactoylglutathione it is for steric reasons less likely that the S substituent is directly coordinated to Mn in a position made vacant by displacement of a water molecule. Displacement of any of the protein ligands would probably cause conformational changes, but no indications of such changes were obtained by the fluorescence studies with S-D-lactoylglutathione. We therefore propose that both glutathione derivatives cause their effect on the proton relaxation rate by immobilizing a water molecule.

It is possible to propose a mechanism for the catalytic events in the active site of glyoxalase I involving the water molecules

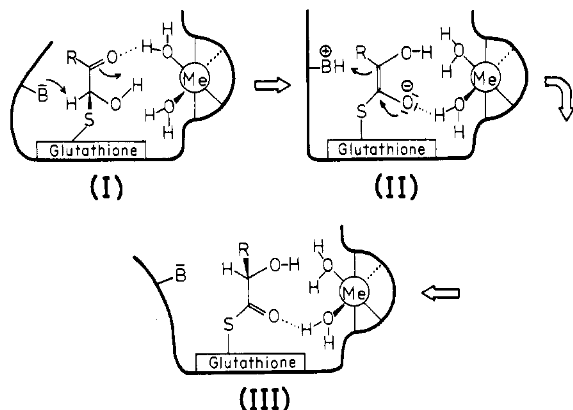

FIGURE 6: Proposed catalytic mechanism of glyoxalase I involving water bound to the active-site metal.

bound to the essential metal (Figure 6). Recent investigations indicate strongly that the mechanism involves a shielded proton transfer from C1 to C2 in the 2-oxoaldehyde bound as a hemimercaptal adduct (I) to the thiol group of glutathione (Hall et al., 1976; Chari & Kozarich, 1981). An enediol (II) is a proposed intermediate in the catalytic pathway leading to the *S*-(2-hydroxyacyl)glutathione (III). A base (B) is required for abstraction of the Cl proton, but its chemical nature remains unidentified. Figure 6 shows three steps in the proposed mechanism. In the first step the hemimercaptal (I) is bound in the active site, forming a hydrogen bond between the keto group and one of the water molecules. The hydrogen bond facilitates the protonation of the keto group and the concomitant abstraction of the proton from Cl. The resulting enediol (II) has a negative charge on the oxygen on Cl which may in part be neutralized by hydrogen bonding to the second water molecule. The next step involves formation of a C–H bond at C2, by recovery of the proton from the base, and concomitant formation of the carbonyl group of the thiol ester (II) that is the product of the overall reaction. It is possible that a conformational change of the protein, triggered by the formation of the two charges, provides the driving force for this step and that the product is released more readily from the new conformation. The fluorescence studies suggest that the hemimercaptal substrate, like glutathione and *S*-(*p*-bromobenzyl)glutathione (but unlike the product *S*-D-lactoylglutathione), might induce a conformational change upon binding to the enzyme. The conformational change proposed to be associated with the product formation would convert the enzyme into its original state. The function of the base in the transfer of the proton from C1 to C2 seems to require two discrete locations of the base in relation to the two carbon atoms. Free enzyme and the enzyme product complex would have the base in the position corresponding to C2, whereas the substrate, glutathione, and *S*-(*p*-bromobenzyl)-glutathione, which induce a conformational change upon binding, would have the base in the position corresponding to C1.

The mechanism proposed accounts for the new findings of water ligands bound to the essential metal and the conformational changes indicated by the fluorescence studies. The octahedral coordination scheme involving two water molecules provides an explanation for the unusual finding that Mg^{2+} gives the same catalytic activity as Zn^{2+} in the active site of the enzyme [see Sellin et al. (1980) and references cited in this paper].

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