S-2-Hydroxyacylglutathione Hydrolase (Glyoxalase II): Active-Site Mapping of a Nonserine Thiolesterase†

James C. Ball and David L. Vander Jagt*

ABSTRACT: *S*-2-Hydroxyacylglutathione hydrolase (glyoxalase II) from rat erythrocytes is a specific thiolesterase. Chemical modification studies with phenylmethanesulfonic acid, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoate) suggest that glyoxalase II does not have a serine or a cysteine residue at the active site. The effect of pH on the rate of hydrolysis of *S*-lactoylglutathione indicates the existence of an active-site residue, pK = 8.87, essential for binding of the substrate. Inactivation studies with trinitrobenzenesulfonic acid suggest that this binding residue is an amine. Inactivation studies with phenylglyoxal implicate the existence of an active-site arginine residue that also is essential for binding of the substrate. The effects of pD on the rate of hydrolysis of *S*-lactoylglutathione in D_2O give no evidence for general acid–general base catalysis. ^1H NMR studies of the glyoxalase II catalyzed hydrolysis of *S*-mandeloylglutathione show no evidence for a carbanion (E1cB) mechanism. The catalytic role of glyoxalase II appears to involve direct nucleophilic attack of the thiol ester by an active-site histidine residue, based upon inactivation experiments using diethyl pyrocarbonate and photoinactivation with methylene blue.

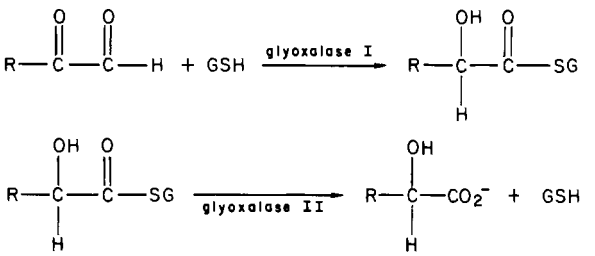
The susceptibility of thiol esters to attack by nitrogen nucleophiles has been recognized for many years. Of the nitrogen nucleophiles, the imidazoyl group is especially important as a potential active-site residue of protein-bound histidine. The high nucleophilicity of the imidazoyl group toward thiol esters, both in intermolecular and in intramolecular reactions, is well-known (Bender & Turnquest, 1957; Bruice, 1959).

Numerous proteases exist that efficiently catalyze the hydrolysis of peptides and amides. Many of these are serine proteases, characterized by the participation of active-site serine and histidine residues (Kraut, 1977). The general mode of action of the serine proteases appears to involve an active-site imidazoyl group as a general acid–general base rather than as a nucleophile (Bender & Kezdy, 1965). These serine proteases also can catalyze the hydrolysis of certain oxygen esters and thiol esters, presumably by mechanisms similar to those that are involved in the protease activities.

Numerous thiolesterases are known that are specific for thiol esters. Some of these contain active-site serine residues and, presumably, fall into the same general category of mechanism as the serine proteases. However, there are some specific thiolesterases that appear not to have active-site serine residues. One of these is *S*-2-hydroxyacylglutathione hydrolase (glyoxalase II) which catalyzes the hydrolysis of a number of thiol esters of glutathione, such as *S*-lactoylglutathione (Scheme I). Glyoxalase II from human liver is not inactivated by diisopropyl phosphorofluoridate, nor does it appear to require an active-site metal ion for activity (Uotila, 1973).

We wished to examine a nonserine thiolesterase in detail in order to test for the possible participation of a nitrogen base in the active site acting as a nucleophile rather than as a general acid–general base in the catalytic reaction. We report here results of a study of glyoxalase II isolated from rat erythrocytes (Ball & Vander Jagt, 1979).

Scheme I



Experimental Procedures

Materials

Phenylmethanesulfonyl fluoride, tetranitromethane, trinitrobenzenesulfonic acid, D_2O, 2-hydroxy-5-nitrobenzyl bromide, diethyl pyrocarbonate (Sigma Chemical Co.), *N*-ethylmaleimide, *p*-chlorophenacyl bromide, *p*-bromobenzyl bromide (Aldrich), and methylene blue (Nutritional Biochem) were used directly. Glyoxalase II from rat erythrocytes was purified essentially to homogeneity as described earlier (Ball & Vander Jagt, 1979). The enzyme used in the present study showed a specific activity of 780 μmol min^{-1} mg^{-1} and represented a 9000-fold purification of the erythrocyte enzyme.

Methods

Syntheses. The synthesis of α-deuteriophenylglyoxal was described previously (Vander Jagt & Han, 1973). The synthesis of *S*-methylglutathione, *S*-(*p*-chlorophenacyl)glutathione, and *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione was carried out as described by Vince et al., (1971). α-Deuteriomandelic acid was synthesized by the base-catalyzed disproportionation of α-deuteriophenylglyoxal in D_2O. The synthesis of *S*-lactoylglutathione has been described (Ball & Vander Jagt, 1979). *S*-α-Deuteriomandeloylglutathione was synthesized as follows: α-deuteriophenylglyoxal, 0.10 g (0.67 mmol), glutathione, 0.22 g (0.72 mmol), and K_2HPO_4, 0.3 g (1.7 mmol) were dissolved in 160 mL of D_2O. Glyoxalase I (20 units) isolated from rat erythrocytes (Han et al., 1976) was desalted into D_2O on a Sephadex PD-10 column and then was added to the reaction mixture. When the reaction was essentially complete, as determined by sulfhydryl titration, the solution was lyophilized, and the powder was used directly

† From the Departments of Biochemistry and Chemistry, University of New Mexico, Albuquerque, New Mexico 87131. *Received August 1, 1980.* This work was supported by U.S. Public Health Service Grant GM 25295. This work was taken from the Ph.D. thesis of J. C. Ball, submitted to the Graduate School of the University of New Mexico, 1980.

* Correspondence should be addressed to this author at the Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, NM 87131.

0006-2960/81/0420-0899$01.00/0

for studies using glyoxalase II.

Kinetics. Glyoxalase II activity generally was measured by following the initial rate of hydrolysis of *S*-lactoylglutathione at 240 nm, $\Delta\epsilon = 3100\ M^{-1}\ cm^{-1}$, in 0.05 M Tris[1] buffer, pH 7.4, 25 °C (Uotila, 1973). Initial rates also were measured at 412 nm by titrating the liberated glutathione with 0.15 mM 5,5′-dithiobis(2-nitrobenzoic acid), $\Delta\epsilon = 13\,600\ M^{-1}\ cm^{-1}$ (Ellman, 1959).

The pH-rate profiles were determined in 5 mM Tris–citrate buffers containing 0.25 M KCl, except above pH 9 where 5 mM ethanolamine–citrate was used. At each pH, values for V_{max} and K_m were determined from Eadie–Hofstee plots of the initial rate data. For kinetic studies in D_2O, the exchangeable protons on *S*-lactoylglutathione and on the buffer salts were replaced with deuterium by repeated lyophilization in D_2O. pD was adjusted with 40% KOD or 37% DCl.

Chemical Modification Studies. The amount of glyoxalase II used in chemical modification experiments ranged from 2 to 5 units, depending upon the experiment. Glyoxalase II was desalted on a Sephadex PD-10 column to remove mercaptoethanol, salts, and glycerol present in the storage buffer (Ball & Vander Jagt, 1979). Chemical modification studies generally were carried out at room temperature.

Chemical modification of glyoxalase II with trinitrobenzenesulfonic acid, 1 mM, was carried out at several pH values with 5 mM sodium phosphate–borate buffer containing 0.25 M KCl, except at pH 10.5 where 5 mM sodium carbonate buffer was used. Samples were assayed periodically and were compared to a control without trinitrobenzenesulfonic acid. Protection of the enzyme from inactivation was evaluated at pH 8.41 in the presence of the competitive inhibitor *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione (K_i = 2 mM), 50 mM, and 0.1 M KCl.

Chemical modification of glyoxalase II with *N*-ethylmaleimide, 10 mM, was carried out in the presence of or absence of *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione, 20 mM, in 0.01 M sodium phosphate buffer, pH 7.0. The competitive inhibitor interferes with the normal enzyme assay at 240 nm, and *N*-ethylmaleimide interferes with the assay at 412 nm. Therefore, after glyoxalase II, inhibitor, and *N*-ethylmaleimide were incubated for 1 h, the reaction was quenched by adding mercaptoethanol to react with *N*-ethylmaleimide. The reaction mixture then was desalted on a Sephadex PD-10 column after which the normal assay for activity could be carried out. A control was worked up similarly.

Chemical modification of glyoxalase II with phenylglyoxal, 20 mM, was carried out in 0.2 M *N*-ethylmorpholine acetate, pH 8, with and without the competitive inhibitor *S*-(*p*-chlorophenacyl)glutathione (K_i = 0.14 mM), 50 mM. Takahashi has reported that amino groups on short peptides can be deaminated by phenylglyoxal (Takahashi, 1968). Therefore, the reaction between phenylglyoxal and *S*-(*p*-chlorophenacyl)glutathione was evaluated by titrating the free amino group with trinitrobenzenesulfonic acid (Fields, 1972). There was no significant loss of the amino group of *S*-(*p*-chlorophenacyl)glutathione during the time required for the chemical modification of glyoxalase II.

Chemical modification of glyoxalase II with diethyl pyrocarbonate was carried out in 0.01 M sodium phosphate buffer, pH 6.0, with and without *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione, 50 mM, as will be discussed under Results.

Photoinactivation of glyoxalase II by singlet oxygen, generated with methylene blue, was carried out at 20 °C in a temperature-controlled vessel. A solution of 0.01% methylene blue in sodium phosphate buffer, 0.05 M, pH 7.0, containing KCl, 0.05 M, and glyoxalase II was stirred rapidly enough to ensure adequate oxygen exchange while being irradiated with a 150-W spot light. A control was stirred similarly, but in the dark. Protection of the enzyme from photoinactivation was evaluated by using *S*-(*p*-chlorophenacyl)glutathione, 50 mM.

Chemical modification of glyoxalase II with tetranitromethane (Riordan & Vallee, 1972) was carried out in 0.1 M Tris buffer, pH 8.0, with a 5.5-fold molar excess of reagent. Modification with phenylmethanesulfonyl fluoride, 2 mM, was carried out in 0.05 M sodium phosphate buffer, pH 7.4. Modification of glyoxalase II with 2-hydroxy-5-nitrobenzyl bromide, 5 mM, was carried out in 0.1 M sodium phosphate buffer, pH 6.5 (Horton & Koshland, 1967).

^1H NMR Studies. *S*-α-Deuteriomandeloylglutathione prepared as above was dissolved in 170 mL of H_2O and was hydrolyzed with 10 units of glyoxalase II. When hydrolysis was complete, the pH was lowered to 2, and the solvent was removed by rotary evaporation. The mandelic acid product was crystallized from benzene. ^1H NMR spectra were obtained in D_2O with DSS as an internal standard by using a Varian FT-80 spectrometer. The spectrum of the product was compared with the spectra of mandelic acid and α-deuteriomandelic acid.

Results

Glyoxalase II from human liver is not sensitive to diisopropyl phosphorofluoridate, suggesting that this enzyme is not a serine-dependent thiolesterase (Uotila, 1973). To test this further, using the erythrocyte enzyme, we treated glyoxalase II from rat erythrocytes with phenylmethanesulfonyl fluoride, 2 mM, which also is a serine reagent. The enzyme retained 95 ± 4% (n = 4) of its activity over a period of 1 h, leading to the conclusion that glyoxalase II is not a serine thiolesterase.

Glyoxalase II from human liver was not inhibited by EGTA or by 8-hydroxyquinoline. EDTA did not immediately affect the enzyme, although it labilized the enzyme on prolonged storage. For rat erythrocyte glyoxalase II, dialysis against EDTA, 1,10-phenanthroline, or 8-hydroxyquinoline-5-sulfonic acid inactivated the enzyme. However, in no instance was any reactivation observed with a variety of metal ions. In addition, glyoxalase II in the absence of chelators is not very stable to dialysis, even in the presence of 2 mM mercaptoethanol which stabilizes the enzyme during storage at −20 °C. Consequently, it is not clear whether glyoxalase II has an essential metal ion.

Inactivation of Glyoxalase II with Sulfhydryl Reagents. Glyoxalase II retained 100% of its activity over a 1-h period upon treatment with 5,5′-dithiobis(2-nitrobenzoic acid), 10 mM. This suggests that a sulfhydryl group on the enzyme is not important for catalysis. However, since this reagent is charged and bulky, it may not have access to the active site or to a sterically hindered thiol group. Therefore, a smaller sulfhydryl reagent was used. The inactivation of glyoxalase II was carried out using *N*-ethylmaleimide, 10 mM. A plot of the rate of inactivation gave a pseudo-first-order rate constant $k = 2.3 \times 10^{-4}\ s^{-1}$. A similar curve was not generated with competitive inhibitor present because neither the assay at 240 nm nor the assay at 412 nm could be used directly. Instead, glyoxalase II was treated with *N*-ethylmaleimide in the presence of the competitive inhibitor *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione, 20 mM, for 1 h, after which the enzyme was isolated by gel filtration. The activity of glyoxalase II was 54% of the control activity compared to 44% of the activity measured for glyoxalase II treated similarly but

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

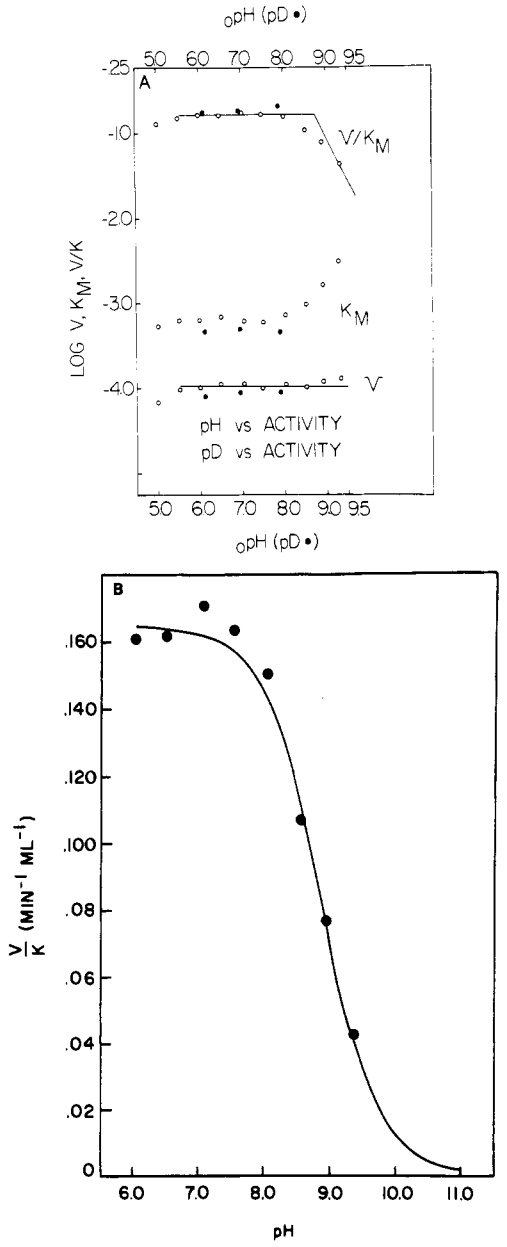


FIGURE 1: (A) Effects of pH on kinetic parameters of glyoxalase II using *S*-lactoylglutathione as substrate. All parameters were measured at 25 °C. The solid circles represent the parameters measured in D_2O. V is the maximum velocity; K_m is the Michaelis constant. (B) Plot of the experimental V/K_m values and a theoretical titration curve for a dissociating group with $pK_a = 8.87$.

without inhibitor. Thus, the presence of near saturating levels of competitive inhibitor did not afford any significant protection against the slow inactivation by *N*-ethylmaleimide. It appears that *N*-ethylmaleimide reacts slowly with a sulfhydryl group that is not in the active site but which is important for the conformational integrity of the enzyme. The observation that a thiol, such as mercaptoethanol, is necessary to stabilize the enzyme during purification is consistent with this idea (Ball & Vander Jagt, 1979).

Therefore, we conclude from these preliminary chemical modification studies that glyoxalase II is a thiolesterase whose hydrolase activity does not mimic that of serine proteases, cysteine proteases, or metalloproteases. Glyoxalase II should be a good candidate for an enzyme whose mode of catalysis may utilize the special chemical properties of the thiol ester substrate.

Active-Site Residues Involved in Substrate Binding

pH–Rate Profiles in H_2O and D_2O. The effect of pH on the kinetic parameters V, K_m, and V/K_m is shown in Figure 1A. The maximum velocity does not vary from pH 6 to pH 9.3. Below pH 6, the activity begins to fall off. However, the enzyme stability also falls off below pH 6. Consequently, it is not possible to analyze the kinetics of glyoxalase II below pH 6 with any accuracy. The loss of activity, however, was slow enough that it could not account for all of the decrease in the activity observed for glyoxalase II in the pH studies between pH 5 and pH 6. Thus, there undoubtedly is a critical ionization at low pH.

Table I: Solvent Deuterium Isotope Effects for Glyoxalase II Catalyzed Hydrolysis of *S*-Lactoylglutathione

parameter	av ± SD
$V(H_2O)/V(D_2O)$	1.24 ± 0.04
$K_m(H_2O)/K_m(D_2O)$	1.42 ± 0.14
$(V/K)(H_2O)/(V/K)(D_2O)$	0.88 ± 0.02

A break is observed in the V/K_m profile as a result of changes in K_m with pH. This break in the V/K_m plot represents a group on the enzyme or free substrate that has a pK_a of 8.87, determined by least-squares analysis of the data using a linear form of eq 1. This equation describes the pH dependence of V/K_m in response to the ionization of a single group; $(V/K_m)_{lim}$ is the limiting pH-independent value (Figure 1A) at lower pH. The pK_a of 8.87 was analyzed further by calculating a theoretical titration curve using this value. Figure 1B shows a plot of the experimental data and the theoretical curve, demonstrating an adequate fit of the data.

$$\frac{(V/K_m)_{lim}}{1 + K_a/[H^+]} = V/K_m \qquad (1)$$

The pK_a of the amino group of glutathione has been determined to be 9.46 (Martin & Edsall, 1958) and 9.42 (Vander Jagt et al., 1972) by using two different methods. The possibility exists that the pK_a of 8.87 determined in the pH studies represents the amino group of *S*-lactoylglutathione. Since this substrate hydrolyzes at high pH, a direct titration to determine the pK_a of this amino group was not possible. The pK_a of the amino group of *S*-methylglutathione was measured by titrating this model compound, 0.12 g in 25 mL of 0.25 M KCl, with NaOH in 0.25 M KCl. The ionic strength of this solution of *S*-methylglutathione is close to the average ionic strength of the buffer system used in the pH studies. Least-squares analysis of the titration data gave a pK_a of 9.34 ± 0.01 for *S*-methylglutathione. We conclude that the pK_a value of 8.87 should be assigned to glyoxalase II.

The kinetic properties of glyoxalase II were investigated by using D_2O as the solvent with Tris–citrate, 5 mM, and KCl, 0.25 M, at 3 pD values. The results (Figure 1A) show that the solvent has very little effect on the kinetic parameters (Table I). The replacement of H_2O by D_2O is known to shift pK values. Since the kinetic parameters at the three pD values did not vary, it was concluded that the experiments were conducted at pD values significantly removed from any shifted pK_a. The isotope effects, Table I, are consistent with those that would be expected from changing the solvent. The values do not suggest that general acid–general base catalysis is important in the rate-determining step of the glyoxalase II catalyzed reaction.

Inactivation of Glyoxalase II with Trinitrobenzenesulfonic Acid. *S*-Lactoylglutathione has two carboxylate groups as well as an amino group. The possibility exists that the pK_a value of 8.87 for glyoxalase II, determined from the pH studies, represents the pK_a of an amino group required for binding one of the carboxylate groups of the substrate. This was tested by measuring the rate of inactivation of glyoxalase II with

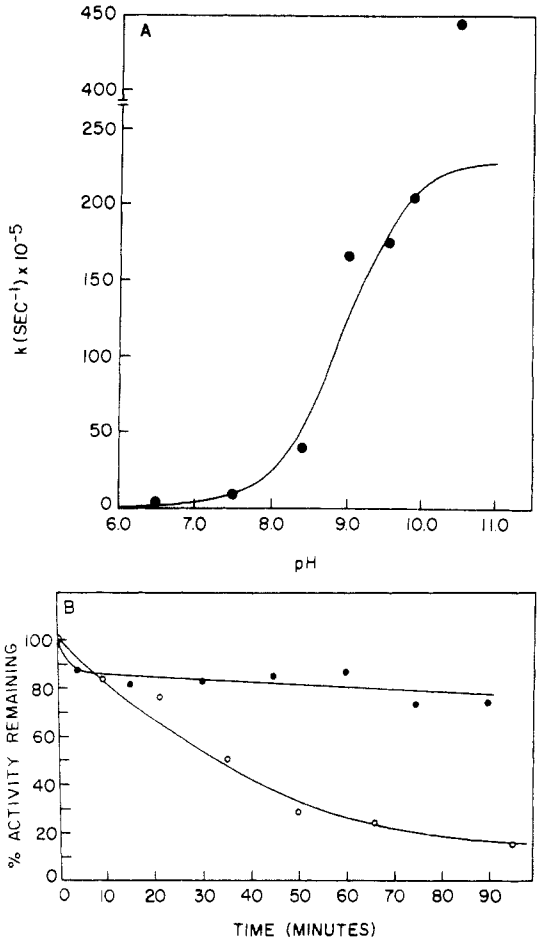


FIGURE 2: (A) Effects of pH on pseudo-first-order rate constant for inactivation of glyoxalase II with 1 mM trinitrobenzenesulfonic acid. (B) Inactivation of glyoxalase II with 1 mM trinitrobenzenesulfonic acid in the absence (O) and presence (●) of the competitive inhibitor *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione, 50 mM, pH 8.41.

trinitrobenzenesulfonic acid, 1 mM, as a function of pH. Figure 2A is a plot of the pseudo-first-order rate constants for inactivation of glyoxalase II along with a theoretical titration curve for a group with $pK_a = 8.95$. The rate constant at high pH did not fit the titration curve, presumably as a result of more than one enzyme group reacting with trinitrobenzenesulfonic acid at high pH.

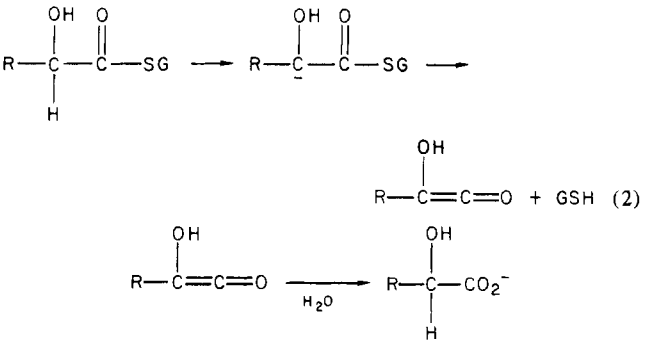
Figure 2B shows a plot of the inactivation of glyoxalase II by trinitrobenzenesulfonic acid at pH 8.41 compared with the same reaction in the presence of the competitive inhibitor *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione, 50 mM. Clearly glyoxalase II is protected by the competitive inhibitor, suggesting that the critical amino group being modified by trinitrobenzenesulfonic acid resides at the active site. The pK_a value for this amine ($pK_a = 8.95$) agrees well with the pK_a value determined in Figure 1 ($pK_a = 8.87$). This also suggests that the pK_a determined in the pH studies (Figure 1) is a true pK_a. One assumption that is generally made in pH studies is that the dissociating protons can rapidly equilibrate. This assumption is not always true, nor is it easy to prove (Knowles, 1976).

Inactivation of Glyoxalase II with Phenylglyoxal. *S*-Lactoylglutathione has two carboxylate groups. If one of these interacts electrostatically with the active-site amino group of glyoxalase II observed in the pH–rate profile and in the inactivation studies with trinitrobenzenesulfonic acid, the other carboxylate may also interact with a cationic group of glyoxalase II. Since the pH studies and the inactivation studies with trinitrobenzenesulfonic acid implicated a single amino group, the most likely candidate for a second binding cation would be an arginine residue. Glyoxalase II was treated with phenylglyoxal, 20 mM, pH 8. The enzyme is inactivated fairly rapidly. Significant protection was afforded by the competitive inhibitor *S*-(*p*-chlorophenacyl)glutathione, 50 mM. The results suggest that an active-site arginine is present, presumably involved in binding although a catalytic role cannot be ruled out.

Table II: Substrate Specificity of Glyoxalase II

substrate	molecular act. (MA) (min^{-1})	K_m (μM)	MA/K_m
S-lactoylglutathione	17 000	180	94
S-mandeloylglutathione	340	14	24

^1H *NMR Studies of Glyoxalase II.* If glyoxalase II utilizes a mode of catalysis that is fundamentally different than those utilized by serine proteases, cysteine proteases, and metalloproteases, then three possible pathways seem most probable. (1) One is general acid–general base catalysis, where active-site residues aid in the nucleophilic attack of water on the thiol ester. The pH studies in D_2O do not support this pathway. (2) A second is a carbanion (E1cB) mechanism whereby an active-site residue removes the α hydrogen of the thiol ester to generate a carbanion. This may then eliminate glutathione to generate a reactive ketene intermediate (eq 2).



(3) A third is a mechanism involving direct nucleophilic attack of an active-site nucleophile on the thiol ester to generate an acyl enzyme intermediate. To test for the involvement of an E1cB type of mechanism, we studied the glyoxalase II reaction by using *S*-α-deuteriomandeloylglutathione as the substrate. This substrate is not as good as *S*-lactoylglutatione based upon maximum velocity, but this is offset by a smaller Michaelis constant (Table II). The hydrolysis of *S*-α-deuteriomandeloylglutathione in H_2O should produce mandelic acid if the α hydrogen is abstracted from the substrate. On the other hand, α-deuteriomandelic acid should form if there is no removal of the α hydrogen. Figure 3A shows the ^1H NMR spectra of α-deuteriomandelic acid and mandelic acid. Figure 3B shows the ^1H NMR spectrum of the product of the glyoxalase II reaction using α-deuteriomandeloylglutathione. There is no detectable isotope exchange. Thus, there is no evidence to support the E1cB mechanism.

Active-Site Residues Involved in Catalysis

Inactivation of Glyoxalase II with Diethyl Pyrocarbonate. Diethyl pyrocarbonate is reasonably specific for histidine residues at low pH. Therefore, inactivation experiments were carried out at pH 6, the lowest pH where glyoxalase II is stable. Figure 4 shows the results from inactivation of glyoxalase II with diethyl pyrocarbonate, 5.6 mM, compared with inactivation of glyoxalase II in the presence of two different

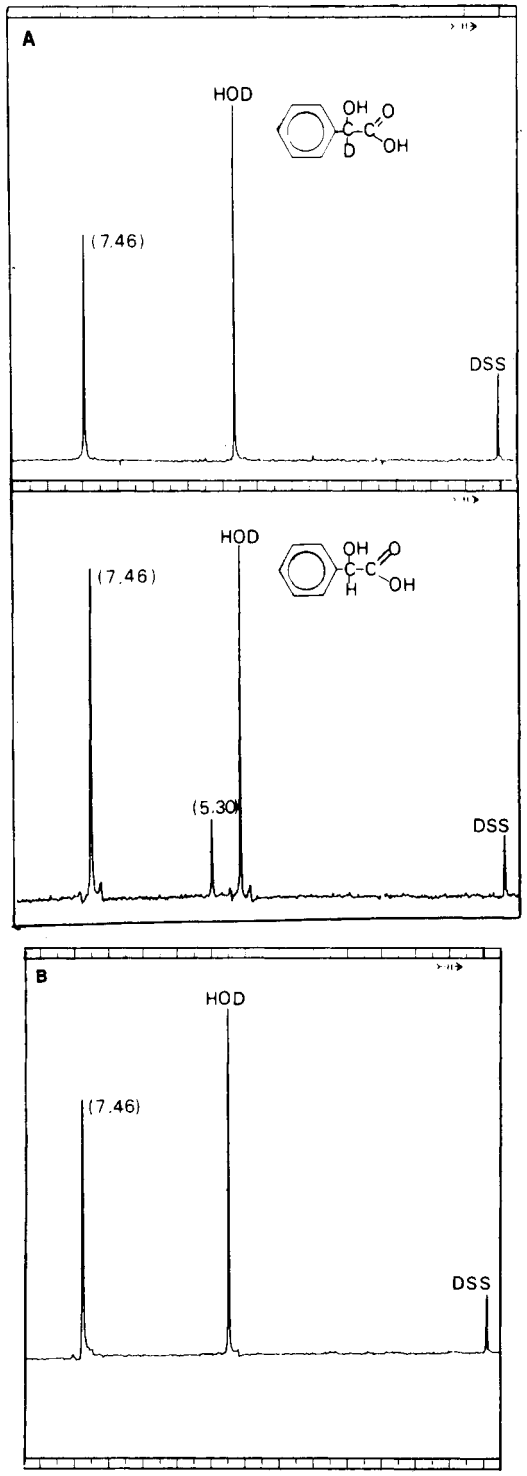
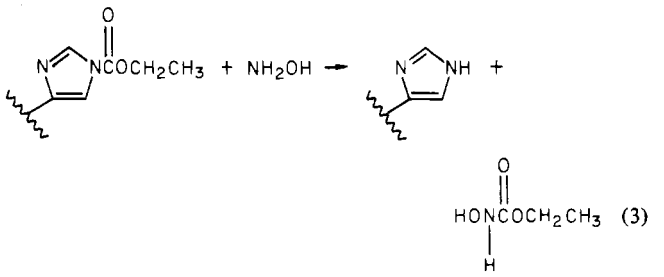


FIGURE 3: (A) ^1H NMR spectra of mandelic acid and α-deuteriomandelic acid in D_2O with DSS as internal standard. (B) ^1H NMR spectrum of the product isolated from the glyoxalase II catalyzed hydrolysis of α-deuteriomandeloylglutathione.

concentrations of *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione. The protection afforded by the competitive inhibitor is similar for both concentrations, suggesting that the active site is saturated. However, there is still a significant rate of inactivation indicating that residues away from the active site are also being modified. Nevertheless, since some protection is obtained in the presence of the inhibitor, it appears that diethyl pyrocarbonate does react with a critical residue at the active site.

If histidine is modified by diethyl pyrocarbonate, it is sometimes possible to reactivate a modified enzyme by treating it with hydroxylamine (eq 3). Glyoxalase II, inactivated to



(3)

2% of the control activity with 21 mM diethyl pyrocarbonate, was treated with hydroxylamine, 0.2 M. The enzyme was separated from the smaller molecular weight compounds by gel filtration. No reactivation was observed. The process was repeated with 5 μM diethyl pyrocarbonate, which required a longer incubation time to inactivate glyoxalase II to an appreciable extent. Again, no reactivation was observed with hydroxylamine. Since protection of the active site of glyoxalase II (Figure 4) merely retards but does not prevent inactivation of the enzyme with diethyl pyrocarbonate, it is probable that the modification of some nonactive-site residues leads to irreversible damage such that reactivation with hydroxylamine is not possible. However, the conclusion that an active-site histidine was modified by diethyl pyrocarbonate (Figure 4) is tenuous in the absence of any reactivation.

Photoinactivation of Glyoxalase II with Methylene Blue. The photoinactivation of glyoxalase II with singlet oxygen, generated with methylene blue as the sensitizer, shows a half-life of ~9 min under the conditions used (Figure 5). Protection of glyoxalase II with *S*-(*p*-chlorophenacyl)glutathione, 50 mM, is complicated, as shown in Figure 5. Initially, the enzyme appears to be activated if the competitive inhibitor is present. The activity then falls off somewhat leading to a stable state where the enzyme retains its activity very well. Thus, good protection is afforded by the competitive inhibitor. This protection does not result from reactions involving the competitive inhibitor directly. If *S*-(*p*-chlorophenacyl)glutathione, 50 mM, was exposed to the photoinactivation conditions for 1 h and then was compared to the inhibitor that had been kept in the dark, both showed identical properties as inhibitors of glyoxalase II, suggesting that no photochemical modification of the inhibitor had occurred. In addition, *S*-(*p*-chlorophenacyl)glutathione did not alter the rate of photoinactivation of lactate dehydrogenase under the same conditions used for the photoinactivation of glyoxalase II, suggesting that the inhibitor does not protect glyoxalase II by consuming the singlet oxygen. Thus, the protection of glyoxalase II by the competitive inhibitor appears to involve protection of an active-site residue, presumably histidine, from singlet oxygen.

The cause of the initial activation observed in Figure 5 in the presence of inhibitor is not clear. Conceivably, initial photoreactions of non-active-site residues results in the formation of a modified glyoxalase II with slightly better catalytic properties than the native enzyme.

Other Chemical Modifications of Glyoxalase II. Although the photochemical inactivation of proteins with singlet oxygen, generated by sensitizers such as methylene blue, is suggestive of the presence of critical histidine residues, other amino acid residues may also react with singlet oxygen; these include tryptophan and tyrosine. The inactivation of glyoxalase II with a 5.5-fold molar excess of tetranitromethane showed an initial loss of ~20% activity, after which the modified glyoxalase II remained stable for at least 90 min with no further change. Tetranitromethane is quite specific for tyrosine if a large excess of the reagent is not used (Riordan & Vallee, 1972). Thus, there is no indication of a tyrosine residue that is essential for

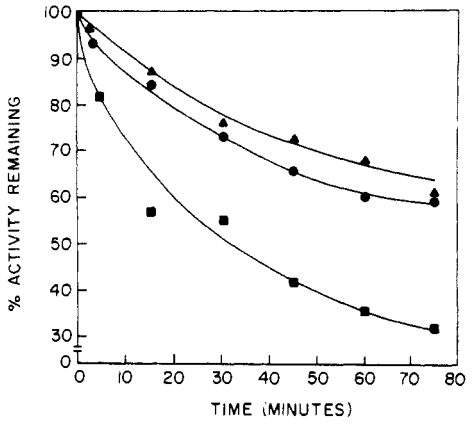


FIGURE 4: Inactivation of glyoxalase II with 5.6 mM diethyl pyrocarbonate, pH 6: (■) no inhibitor present; (●) 20 and (▲) 55 mM competitive inhibitor *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione present.

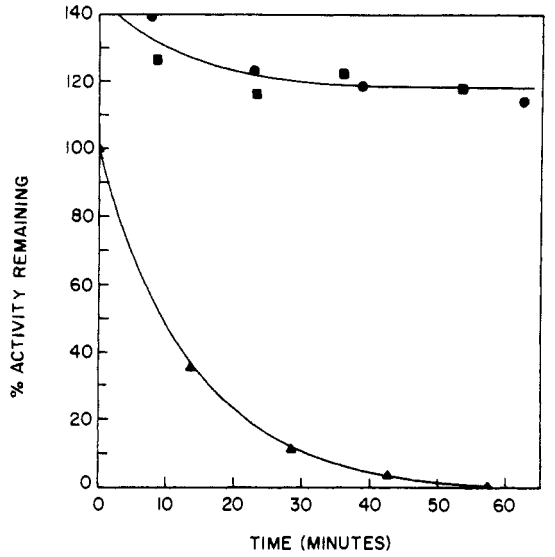


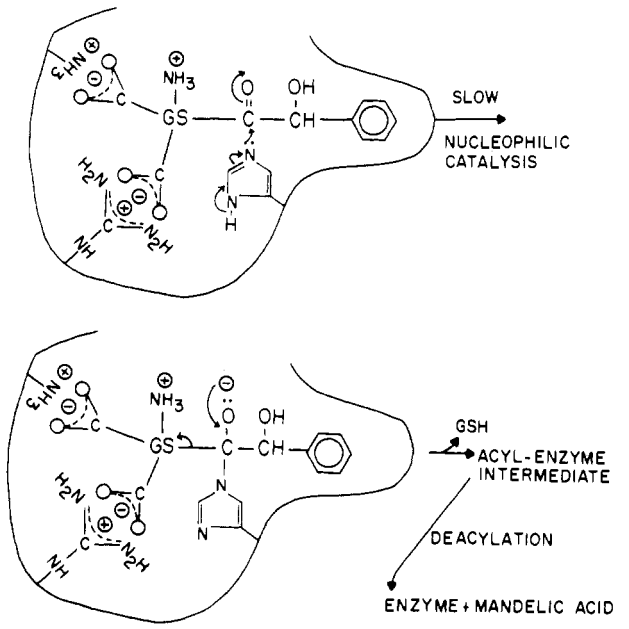
FIGURE 5: Photinactivation of glyoxalase II, pH 7, 20 °C, in the presence of 0.01% methylene blue. The inactivation in the absence of competitive inhibitor (▲) showed a half-life of 9 min. Two separate experiments (● and ■) were carried out in the presence of 50 mM *S*-(*p*-chlorophenacyl)glutathione.

activity. Glyoxalase II, treated with the tryptophan reagent 2-hydroxy-5-nitrobenzyl bromide, 5 mM, showed no loss of activity suggesting that there is no critical tryptophan residue. Thus, these chemical modification studies support the conclusion that the photoinactivation experiments indicate the existence of a critical active-site histidine. This is also consistent with the inactivation studies using diethyl pyrocarbonate.

Discussion

On the basis of a number of experiments that either rule out alternative mechanisms or give support to a given mechanism, the mechanism shown in Scheme II is proposed as one that is consistent with all of the following: (1) An active-site amine, pK_a 8.87–8.95, is essential for the binding of the substrate, presumably by electrostatic interaction with one of the carboxylate groups of the glutathione moiety. The assignment of this pK_a is based upon the results of the pH–rate profile (Figure 1) and the inactivation of glyoxalase II with trinitrobenzenesulfonic acid (Figure 2). The formation of electrostatic bonds between carboxylate groups of glutathione and positively charged residues on enzymes that utilize glutathione may be a general phenomenon. A group with pK_a

Scheme II



of 8.4–8.9, required for binding, has also been shown for glyoxalase I (Vander Jagt & Han, 1973). (2) An active-site arginine residue also is required for binding of the substrate. The presence of this amino acid was shown with the use of phenylglyoxal as a group-specific reagent. (3) The much lower Michaelis constant for *S*-mandeloylglutathione compared with *S*-lactoylglutathione (Table II) suggests that a hydrophobic pocket is present to accept the acyl moiety of the substrate. This same property was reported for glyoxalase II isolated from human liver (Uotila, 1973). However, since both k_{cat} and K_m are lower for *S*-mandeloylglutathione than for *S*-lactoylglutathione, it may well be that nonproductive modes of binding occur for *S*-mandeloylglutathione. This would result in lower k_{cat} and K_m values for this substrate. (4) The argument that glyoxalase II does not utilize the mechanisms of catalysis utilized by serine proteases or cysteine proteases is based upon the insensitivity of glyoxalase II to phenylmethanesulfonic acid and the lack of protection afforded by a competitive inhibitor to inactivation by *N*-ethylmaleimide. In addition, the studies of glyoxalase II isolated from human liver support this argument (Uotila, 1973). Thus, Scheme II does not indicate any role for serine or cysteine. (5) The absence of general acid–general base catalysis in the rate-determining step of the glyoxalase II catalyzed hydrolysis of *S*-lactoylglutathione is indicated by the lack of a significant solvent isotope effect in the pH studies (Figure 1, Table I). (6) ^{1}H NMR studies appear to rule out any contribution of an E1cB type of mechanism whereby a protein base aids in the removal of the α hydrogen of the thiol ester (Figure 3). However, it is possible that a highly shielded general base is involved in abstracting the α hydrogen, in which case the absence of solvent exchange of hydrogen would not prove that the α hydrogen had not been abstracted. (7) The use of histidine-specific reagents suggests the presence of a histidine at the active site. These studies utilized diethyl pyrocarbonate at low pH and photoinactivation with singlet oxygen, generated by using methylene blue as a sensitizer (Figures 4 and 5). Additional modification studies ruled out the importance of tyrosine or tryptophan at the active site. Consequently, the best interpretation of available data would appear to be that nucleophilic catalysis by the imidazoyl group of histidine is

the essential feature of the mechanism of glyoxalase II.

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Physical Map of the Ribosomal Ribonucleic Acid Gene from *Tetrahymena pyriformis*†

Edward G. Niles* and Rakesh K. Jain

ABSTRACT: A physical map of the rRNA gene from the ciliated protozoan *Tetrahymena pyriformis* has been determined. The isolated rDNA palindrome was labeled by nick translation and digested with the sequence-specific endonucleases *Kpn*I, *Bcl*I, *Pvu*II, *Pst*I, *Sst*I, *Ava*I, *Ava*II, *Hpa*I, *Bam*HI, *Bgl*II, or *Hin*dIII, and the molecular weights of the products were determined by gel electrophoresis. The DNA fragments generated by digestion with each endonuclease were ordered into a unique sequence either by analyzing the products produced by concomitant cleavage with one or more additional restriction enzymes or by hybridizing to 17S and 25S rRNA. Hybridization of the 35S rRNA primary transcription product to *Hin*dIII and *Bam*HI restriction fragments localizes the promoter to the left of 16.9% and the terminator to the region between 71.6% and 78% on the physical map.

Tetrahymena is a genus of ciliated protozoa which possesses multiple nuclei: a diploid micronucleus found only in conjugating strains and a polyploid macronucleus present in all strains, which is the site of gene expression during vegetative growth. The rRNA gene is isolated from the macronuclei as an extrachromosomal element of unique size (Gall, 1974; Engberg et al., 1974). The gene is a perfect palindrome which possesses two transcription units for the 35S rRNA precursor (Karrer & Gall, 1976; Engberg et al., 1976) and represents 1%–2% of the total DNA (Yao et al., 1974).

In the micronucleus, the rRNA gene is present in an integrated, nonrepeated linear form (Yao & Gall, 1977). The palindromic structure must be a product of the amplification process which occurs after conjugation, during the production of the new macronucleus. In the nucleoli, the rRNA gene possesses a nucleosomal structure (Mathis & Gorovsky, 1976; Piper et al., 1976). Recent evidence indicates that the nucleosomes in the transcribed region of the rDNA from *T. thermophilia* exist in an altered conformation (Cech & Karrer, 1980). rDNA replication initiates at the center of the palindrome and proceeds bidirectionally to the termini (Truett & Gall, 1977). The termini of the palindrome are heterogeneous in size due to the presence of a variable repeat of the hexanucleotide C_4A_2. The ends of the molecule are unavailable for either 3′ or 5′ enzymatic labeling, suggesting either a hairpin loop or a chemical block (Blackburn & Gall, 1978).

Transcription is initiated near the center of the palindrome and proceeds outward (Gall et al., 1977; Niles, 1978; Engberg & Klenow, 1978), generating a 35S rRNA precursor which is processed to the mature 17S rRNA, 5.8S rRNA, and 25S rRNA (Kumar, 1970; Prescott et al., 1971; Pousada et al., 1975; Eckert et al., 1978; Niles, 1978). The 35S rRNA is a primary transcription product, at least at the 5′ end since high yields of pppAp can be isolated (Niles, 1978).

In *T. pigmentosa* (Wild & Gall, 1979; Din & Engberg, 1979) and *T. thermophila* (Cech & Rio, 1979; Din et al., 1979; Din & Engberg, 1979), an intron is found in the 25S rRNA region. The intron is transcribed, and its removal may be the first step in the processing of the 35S rRNA (Cech & Rio, 1979; Din & Engberg, 1979). Processing of the 35S rRNA proceeds by a cleavage into a pre-17S rRNA and pre-25S rRNA followed by subsequent nucleolytic steps to generate the mature forms. Within the ribosome, the 25S rRNA is clipped, forming two stable species of about 16 S in size (Bostock et al., 1971; Eckert et al., 1978).

The positions of the 5′ and 3′ termini of the 35S rRNA from *T. thermophilia* have been mapped at about 17% and 79%, respectively, by Cech & Rio (1979) and at 30% and 75%, respectively, by Din et al. (1979) from the center of the

† From the Biochemistry Department, State University of New York, Buffalo, New York 14214. *Received May 14, 1980.* This work was supported by National Institute of Medical Sciences Grant GM 23259. Reprints of this report will not be available.

0006-2960/81/0420-0905$01.00/0