Involvement of Ionizable Groups in Catalysis of Human Liver Glycolate Oxidase†
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Running head: pH Effects on Human Liver Glycolate Oxidase
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Glycolate oxidase is a flavin-dependent, peroxisomal enzyme that oxidizes \( \alpha \)-hydroxy acids to the corresponding \( \alpha \)-keto acids, with reduction of oxygen to \( \text{H}_2\text{O}_2 \). In plants, the enzyme participates in photorespiration. In humans, it is a potential drug target for treatment of primary hyperoxaluria, a genetic disorder where overproduction of oxalate results in the formation of kidney stones. In this study, steady-state and pre steady-state kinetic approaches have been used to determine how pH affects the kinetic steps of the catalytic mechanism of human glycolate oxidase. The enzyme showed a Ping-Pong Bi-Bi kinetic mechanism between pH 6.0 and 10.0. Both the overall turnover of the enzyme (\( k_{\text{cat}} \)) and the rate constant for anaerobic substrate reduction of the flavin were pH-independent at pH values above 7.0 and decreased slightly at lower pH, suggesting the involvement of an unprotonated group acting as a base in the chemical step of glycolate oxidation. The second-order rate constant for capture of glycolate (\( k_{\text{cat}}/K_{\text{glycolate}} \)) and the \( \text{app}K_d \) for the formation of the enzyme-substrate complex suggested the presence of a protonated group with apparent \( pK_a \) of 8.5 participating in substrate binding. The \( k_{\text{cat}}/K_{\text{oxygen}} \) values were an order of magnitude faster when a group with \( pK_a \) of 6.8 was unprotonated. These results are discussed in the context of the available three dimensional structure of GOX.

Glycolate oxidase (EC 1.1.3.15; glycolate:oxygen oxidoreductase; GOX\(^1\)) catalyzes the FMN-mediated oxidation of glycolate to glyoxylate with reduction of oxygen to hydrogen peroxide (Scheme 1) (1). The enzyme has been identified in plants and mammals and contains tightly, but not covalently, linked FMN. GOX has been grouped in the superfamily of the \( \alpha \)-hydroxy acid oxidases, which includes among others long-chain hydroxy acid oxidase, lactate oxidase, mandelate dehydrogenase, and the flavin-binding domain of yeast flavocytochrome \( b_2 \) (2-6). In plants, GOX is localized in the glyoxysome of photosynthetic tissues where it participates in photorespiration (7,8). In humans and other vertebrates such as pig, the enzyme is found in the peroxisomes of liver and kidney and is involved in the production of oxalate (9-11). The latter finding recently prompted considerable interest in the study of the mechanistic and structural properties of human GOX for the potential development of therapeutic agents targeting the treatment of primary hyperoxaluria (12), a genetic disorder in which overproduction of oxalate results in large deposits of calcium oxalate.

To date the GOX that is best characterized in its structural, kinetic, and biochemical properties is the one from spinach leaves (13-19). Recently, the structure of the enzyme from human liver has been solved in complex with a number of ligands to resolutions \( \leq 1.95 \text{ Å} \) (20). In the active site of the human enzyme (Figure 1), and based on mutational studies on
the spinach enzyme, mandelate dehydrogenase, or flavocytochrome $b_2$, Tyr26, Arg167 and Arg263 have been proposed to participate in the binding of the carboxylate group of glyoxylate (19-22). Based on its spatial location in the active site, His260 has been proposed to initiate catalysis by acting as the proton acceptor for the deprotonation of the hydroxyl group or the $\alpha$-carbon of the substrate (19,20). However, mechanistic studies on human GOX have yet to address such proposals. Apparent steady-state kinetic parameters with glycolate as substrate for human liver GOX have been reported by other research groups for pH 7.0 and 7.5 at atmospheric concentration of oxygen (1,23). The enzyme has been reported to be inhibited by chloride ions, oxalate and mandelate, with IC$_{50}$ values of 280 mM, 1.8 mM and 3.7 mM, respectively, at pH 7.0 and 25 °C, and to form a reversible covalent FMN-adduct with sulfite, with a $K_d$ value of ~60 μM at pH 7.0 and 25 °C (23).

In this study, we have determined the steady-state and pre-steady state kinetic parameters with glycolate as substrate for human liver GOX in the pH range from 5.0 to 10.0. The enzyme showed a Ping-Pong Bi-Bi steady-state kinetic mechanism throughout the pH range considered, with the requirement of a protonated group for binding of the substrate in the active site. The kinetic step in which substrate oxidation results in the reduction of the flavin was independent of pH above pH 7.0, with a slight decrease at lower pH values, consistent with the presence of an unprotonated group participating in the chemical step of glycolate oxidation. The $k_{cat}/K_{oxygen}$ values increased between two limiting values with increasing pH, identifying an unprotonated group with $pK_a$ of 6.8 that is important for flavin oxidation. Finally, the overall turnover of the enzyme is limited by the chemical step of flavin reduction at high pH, and by another kinetic step at low pH.

**Experimental Procedures**

**Materials.** The plasmid pTrcHisB-HAOXI harboring the gene encoding for human liver GOX was a gift from Prof. Peracchi, University of Parma, Italy. Escherichia coli strain Rosetta(DE3)pLysS was from Novagen (Madison, WI). DNase was from Roche. Bovine serum albumin, chloramphenicol, DMSO, isopropyl $\beta$-d-thiogalactopyranoside (IPTG), phenylmethanesulfonyl fluoride (PMSF), lysozyme, glycolate, glucose, glucose oxidase from Aspergillum niger, Luria–Bertani agar and broth were from Sigma (St. Louis, MO). EDTA was from Fisher. Ampicillin was from ICN Pharmaceutical Inc. Nickel Sepharose High Performance and PD-10 columns were from GE Healthcare. All other reagents were of the highest purity commercially available.

**Expression of Human GOX in E. coli.** GOX was expressed following a modification of the procedure previously published (23,24). *E. coli* Rosetta(DE3)pLysS cells were transformed with pTrcHisB-HAOXI and permanent stocks of these transformants were stored at -80 °C. Colonies of transformants, which were selected on Luria-Bertani plates supplemented with 50 µg/mL ampicillin and 34 µg/mL chloramphenicol were transferred in 50 mL of Luria-Bertani broth medium containing the same antibiotics. The culture was grown at 37 °C under orbital shaking (220 rpm). After 3 h, 10 mL of the starter culture were used to inoculate 5 x 1 L of the same liquid culture medium at 37 °C, 220 rpm. When the culture reached an optical density at 600 nm of 0.6, typically after 5 h, IPTG was added to a final concentration of 0.5 mM. Expression of GOX was induced at 37 °C for 16 h. Cells were harvested by centrifugation at 5,000 × g for 20 min at 4 °C and stored at −20 °C until used for enzyme purification.

**Purification of Recombinant Human GOX.** All the purification steps were carried out at 4 °C. The cell paste, typically 20 g, was suspended in 4 volumes of 50 mM sodium
phosphate, pH 7.4, containing 500 mM NaCl, 20 mM imidazole, 10% glycerol, 0.2 mg/mL lysozyme and 1 mM PMSF. After sonication for a total of 15 min in a Fisher Scientific Sonifier, the cell-free extract was obtained by centrifugation for 30 min at 20,000 × g. The soluble fraction was loaded onto a Nickel Sepharose High Performance (GE Healthcare) column (25 mL) connected to an Äkta Prime Amersham-Pharmacia Biotech system equilibrated in 50 mM sodium phosphate, pH 7.4, containing 500 mM NaCl, 20 mM imidazole and 10% glycerol. Unbound proteins were removed by washing the column with 100 mL of equilibrating buffer, followed by a linear gradient from 20 to 500 mM imidazole developed over 10 column volumes. The fractions with the highest purity as judged by UV-visible absorbance spectroscopy were pooled together and were concentrated with the addition of 70% ammonium sulfate saturation followed by centrifugation. The pellet was solubilized and dialyzed against 50 mM sodium phosphate, pH 7.0, containing 100 mM NaCl2 and 10% glycerol. Aliquots of the solution containing the enzyme, after removal of the precipitated protein by centrifugation, were stored at −20 °C.

Site Directed-mutagenesis and Purification of Human Liver His260Ala and His260Gln Enzymes. The enzyme variants where His260 is replaced with glutamine or alanine were prepared using the QuikChange site-directed mutagenesis kit (Stratagene), following the manufacturer’s instructions. After confirming the mutations by DNA sequencing at the DNA core Facility at Georgia State University, the mutated enzymes were expressed (at 37 ºC or 20 ºC) and purified as described above for the wild-type enzyme.

Enzyme Assays. GOX activity was measured with the method of the initial rates (25) using a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 30 ºC. Steady-state kinetic parameters for GOX were determined at varying concentration of glycolate between 0.05 and 20 mM and oxygen between 0.1 and 1 mM. The pH dependences of the steady-state kinetic parameters of GOX were determined by measuring enzymatic activity at varying concentration of both glycolate and oxygen. Sodium phosphate (100 mM final) was used in the pH range from 6.0 to 8.0, and sodium pyrophosphate (100 mM final) was used in the pH range from 8.5 to 10.0. When both glycolate and oxygen were varied, the assay reaction mixture was equilibrated at the desired concentration of oxygen by bubbling the appropriate O2/N2 gas mixture for 10 min before the reaction was started by the addition of the enzyme.

The effects of solvent viscosity on the $k_{cat}/K_{glycolate}$ values were measured in air-saturated 100 mM sodium phosphate, pH 7.0, at 30 ºC, using glycerol as viscosigen. The values for the relative viscosities at 30 ºC were calculated according to the values at 25 ºC from Lide (26).

Stopped-flow studies of the reductive half reaction catalyzed by GOX were performed at 30 ºC using a Hi-Tech Scientific SF-61 DX2 stopped-flow spectrophotometer under anaerobic condition. GOX aliquots were gel filtered through a PD-10 column (GE Healthcare) against 100 mM piperazine at pH 5.0, 100 mM sodium phosphate in the pH range from 6.0 to 8.0, and 100 mM sodium pyrophosphate in the pH range from 9.0 to 10.0. The solution of enzyme was made anaerobic in a tonometer by repeated cycles of evacuation and flushing with oxygen-free argon (pretreated with an oxygen scrubbing cartridge, Agilent, Palo Alto, CA). Next, the tonometer containing the anaerobic enzyme was mounted onto the stopped-flow instrument, which had been subjected to an overnight incubation with an oxygen scrubbing system containing glucose (5 mM) and glucose oxidase (3600 units). Glycolate solution were prepared in the same buffer systems used for GOX at concentrations.
ranging from 0.125 to 40 mM, and were made anaerobic by flushing with oxygen-free argon for at least 15 minutes before mounting the syringe with the substrate onto the stopped-flow instrument. To scavenge possible trace amounts of oxygen in the enzyme and substrate, glucose (2 mM) and glucose oxidase (0.5 µM) were present. Equal volumes of GOX and substrate were mixed anaerobically in the stopped-flow apparatus, yielding a final enzyme concentration of ~ 20 µM. Triplicate measurements differed typically by less than 2%.

Data Analysis. Kinetic data were fit with KaleidaGraph software (Synergy Software, Reading, PA) and GraFit 5 (Erythacus Software Ltd, Staines, UK). The steady-state kinetic parameters were determined by fitting the initial rates of reactions to eq. 1, which describes a Ping-Pong Bi-Bi kinetic mechanism. $K_a$ and $K_b$ are the Michaelis constants for glycolate (A) and oxygen (B), respectively, and $k_{cat}$ is the turnover number of the enzyme ($e$) when saturated with both substrates.

\[
\frac{v}{e} = \frac{k_{cat} A B}{K_a B + K_b A + A B} \quad (1)
\]

Product inhibition studies were performed by varying the concentrations of both glycolate (from 0.02 mM to 1.0 mM) and glyoxylate (0, 0.5, 1.0 and 2.5 mM) at a fixed atmospheric concentration of oxygen. The data were fit to eq. 2, which describes an uncompetitive inhibition pattern of the product and the organic substrate. $A$ is the concentration of glycolate, $P$ is the concentration of glyoxylate, and $K_{ii}$ is the inhibition constant for binding of glyoxylate to GOX.

\[
\frac{v}{e} = \frac{k_{cat} A}{K_a + A \left[ 1 + \left( \frac{P}{K_{ii}} \right) \right]} \quad (2)
\]

The pH profile of the $k_{cat}/K_{glycolate}$ values was determined by fitting the data to eq. 3, which describes a curve with a slope of -1 and a plateau region at low pH. $C$ is the pH-independent value of the kinetic parameter of interest. The pH profile of the $k_{cat}/K_{oxygen}$ value was determined by fitting the data to eq. 4, where $Y_L$ and $Y_H$ are the limiting values at low and high pH, respectively, and $K_a$ is the dissociation constant for the ionization of groups which are relevant to catalysis.

\[
\log Y = \log \left( \frac{C}{1 + 10^{pH - pK_a}} \right) \quad (3)
\]

\[
\log Y = \log \left( \frac{Y_L + Y_H 10^{-pK_a}}{1 + 10^{-pK_a}} \right) \quad (4)
\]

Stopped-flow traces at individual wavelengths were fit to eq. 5, which describes a double-exponential process, using KinetAsyst 3 software (Hi-Tech Scientific). $A$ is the value of absorbance at the specific wavelength of interest, $k_{obs1}$ and $k_{obs2}$ are the observed first-order rates for the phases of the reaction, $B_1$ and $B_2$ are the corresponding amplitudes for the change in absorbance associated with the phase of interest, and $C$ is an offset value to account for a non-zero final absorbance value. Pre-steady state kinetic parameters were determined by using eq. 6 and eq. 7, where $k_{obs}$ is the observed first-order rate for the reduction of the enzyme-bound flavin at any given concentration of substrate, $k_3$ is the limiting first-order rate constant for flavin reduction at saturating substrate concentration, $k_{rev}$ is the rate constant for the reverse step in catalysis and $K_{d}^{app}$ is the apparent dissociation constant for the enzyme-substrate complex, which is equal
to \((k_2+k_3)/k_1\) (see Results). The pH profile of the \(K_d\) values for glycolate was determined by fitting the individual \(K_d\) values to eq. 8, which describes a curve with a slope of +1 and a plateau region at low pH. \(C\) is the pH independent value of \(K_d\).

\[
A = B_1 \exp(-k_{obs1} t) + B_2 \exp(-k_{obs2} t) + C \quad (5)
\]

\[
k_{obs} = \frac{k_3 \text{[Glycolate]}}{K_d + \text{[Glycolate]}} + k_{rev} \quad (6)
\]

\[
k_{obs} = \frac{k_3 \text{[Glycolate]}}{K_d + \text{[Glycolate]}} \quad (7)
\]

\[
\log Y = \log \left[ C \left( 1 + 10^{-pK_a + pH} \right) \right] \quad (8)
\]

The viscosity effects data on the \(k_{cat}/K_{glycolate}\) values were fit to eq. 9, where \((k_{cat}/K_{glycolate})_o\) and \((k_{cat}/K_{glycolate})_\eta\) are the values in the absence and presence of viscosigen, \(S\) is the degree of viscosity dependence and \(\eta_{rel}\) is the relative viscosity.

\[
\frac{(k_{cat}/K_{glycolate})_o}{(k_{cat}/K_{glycolate})_\eta} = S(\eta_{rel} - 1) + 1 \quad (9)
\]

Results

Expression and Purification of Recombinant Human Liver GOX. Heterologous expression of GOX was performed using Rosetta(DE3)pLysS as the host strain, by inducing the cultures at 37 °C overnight with 0.5 mM IPTG. GOX was purified on nickel affinity chromatography after optimizing the conditions in order to maximize the recovery of the enzyme in holoprotein form. Indeed, initial attempts yielded significant amounts of free FMN originating from the enzyme during the washing of the column. Glycerol, salts and neutral pH turned out to substantially stabilize the native conformation of GOX without loss of bound FMN. Consequently, 10% glycerol was included in all the solutions used throughout the purification procedure, while the enzyme storage buffer was supplemented with 100 mM NaCl². Following this procedure, ~8 mg of GOX were routinely purified to high levels (Figure 2) out of 1 g of wet cell paste, with a specific activity of ~4.0 U/mg using 10 mM glycolate and atmospheric oxygen at pH 7.0 and 30 °C.

Kinetic Mechanism and Parameters at pH 7.0. The steady-state kinetic mechanism and the associated kinetic parameters of the enzyme were determined using the method of the initial rates by measuring the rates of oxygen consumption at varying concentrations of both glycolate and oxygen in 100 mM sodium phosphate, pH 7.0, at 30 °C. Initial velocity data yielded parallel lines in double reciprocal plots (Figure 3), suggesting a Ping-Pong Bi-Bi kinetic mechanism in which the enzyme-bound FMN is alternately reduced by glycolate and reoxidized by oxygen. Fitting of the kinetic data to eq. 1 yielded an overall turnover number \((k_{cat})\) of 15.7 ± 0.4 s⁻¹, a second-order rate constant for the capture of glycolate \((k_{cat}/K_{glycolate})\) of 68300 ± 3500 M⁻¹s⁻¹ and a second-order rate constant for reaction of the reduced flavin with oxygen \((k_{cat}/K_{oxygen})\) of 24500 ± 1300 M⁻¹s⁻¹. In contrast to a recent report, we did not observe enzyme inhibition at concentrations of glycolate as high as 20 mM (23).

As a complementary approach to establish the order of binding of substrates and products to the enzyme, the inhibition pattern of the product of the reaction, glyoxylate, versus glycolate was determined in 100 mM sodium phosphate, pH 7.0, using the steady-state kinetics approach at atmospheric oxygen concentration. The inhibitor concentrations were varied between 0.5 and 2.5 mM, which is below the \(K_m\) value for glyoxylate of 3.4 mM reported for the same enzyme at pH 7.0 (23). As shown in Figure 4, a double reciprocal plot of the initial rates of reaction as a function of the substrate concentration at fixed concentrations of product yielded parallel lines. The observed kinetic pattern
establishes that glycolate and glyoxylate bind to two forms of enzymes that are interconnected by the presence of at least one irreversible step (27). This immediately rules out product release occurring after reaction of the reduced enzyme with oxygen, since if this were the case glyoxylate would compete with glycolate for binding to the same oxidized enzyme species. As illustrated in the minimal kinetic mechanism of Scheme 2, the inhibition data are consistent with release of glyoxylate from the reduced enzyme occurring prior to the oxidation of the reduced enzyme by molecular oxygen and with the chemical step of glycolate oxidation being irreversible (27).

The relevant kinetic parameters determined were: \( k_{cat}^{app} = 3.32 \pm 0.03 \) s\(^{-1}\), \( K_{glycolate}^{app} = 0.07 \pm 0.01 \) mM and \( K_{ii}^{app} = 1.05 \pm 0.02 \) mM (\( R^2 = 0.998 \)).

Upon mixing anaerobically GOX with glycolate in a stopped-flow spectrophotometer at pH 7.0 and 30 °C, reduction of the enzyme-bound flavin to the anionic hydroquinone state was observed as bleaching of the absorbance signal at 450 nm in a biphasic pattern (Figure 5). Accordingly, the rapid kinetic data were fit best with a double exponential process (eq. 5). The observed rates for the first observable kinetic phase (\( k_{obs1} \)), which accounted for ~90% of the total absorbance change, were hyperbolically dependent on glycolate concentration. Fitting of the kinetic data to eq. 6, which describes a mechanism with a reversible chemical step of flavin reduction, yielded a limiting rate constant for flavin reduction (\( k_3 \)) of 54 \pm 1 \) s\(^{-1}\), an \( K_d^{app} \) value of 0.29 \pm 0.02 mM, and a limiting rate constant for the reverse of the chemical step of flavin reduction (\( k_{rev} \)) that was not significantly different from zero (i.e., \( k_3 \) is comparable to \( k_{rev} \)). These data also indicate that \( K_d^{app} \) measured in the reductive half reaction is equal \( (k_2 + k_3)/k_1 \).

\[ pH \text{ Dependences of Kinetic Parameters.} \]

The steady-state and pre steady-state kinetic parameters were determined in the pH range from pH 5.0 to 10.0, in order to gain insights on whether ionizable groups participate in the kinetic steps of the mechanism of GOX. As for the case of pH 7.0, at any given pH value the steady-state kinetic mechanism was Ping-Pong Bi-Bi (data not shown) and the stopped-flow traces describing the anaerobic substrate reduction of the flavin were biphasic, with the first phase accounting for more than 85% of
the total change in absorbance (see SI). The kinetic parameters determined at different pH values using the steady-state kinetic approach are summarized in Table 1. Those for the anaerobic reductive half-reactions are listed in Table 2. Below pH 7.0 the enzyme was not saturated with oxygen at concentrations as high as 1 mM, thereby preventing the determination of the $k_{\text{cat}}$ and $K_{\text{oxygen}}$ values. Both the $k_{\text{cat}}$ and $k_3$ values were independent of pH above pH 7.0, but showed slight decreases at pH 6.0 ($k_{\text{cat}}$) and pH 5.0 ($k_3$) (Figure 7), consistent with the requirement of an unprotonated group acting as a base in the chemical step of glycolate oxidation.

As shown in Figure 7, the $k_{\text{cat}}/K_{\text{glycolate}}$ was maximal at low pH, with a limiting value of 74000 ± 8500 M$^{-1}$s$^{-1}$, and decreased with increasing pH values defining an apparent $pK_a$ value of 8.5 ± 0.1 for a group that needs to be protonated. In contrast, the $pK_d$ value for binding of glycolate to the enzyme was maximal at low pH, with a limiting value of 0.35 ± 0.04 mM, and increased with increasing pH values defining an apparent $pK_a$ value of 8.5 ± 0.1 for a group that needs to be protonated for substrate binding (Figure 7). Finally, the pH profile for the $k_{\text{cat}}/K_{\text{oxygen}}$ value increased from a poorly defined limiting value of 6700 ± 2000 M$^{-1}$s$^{-1}$ at low pH to a well defined limiting value of 57400 ± 4700 M$^{-1}$s$^{-1}$ at high pH, identifying a $pK_a$ value of 6.8 ± 0.2 for a group that needs to be unprotonated for efficient oxidation of the reduced flavin by oxygen (Figure 7). Interestingly, at any given pH comprised between 5.0 and 10.0 the UV-visible absorbance spectrum of the enzyme seen at the end of the substrate reduction process showed a well-defined peak at ~340 nm (Figure 6 and S1 in SI), consistent with the enzyme-bound reduced flavin being present in the anionic form throughout the pH range considered.

**Attempt at Investigating the Mechanistic Role of His260.** The His260Gln and His260Ala enzymes were expressed and purified to high levels following the protocol employed for the wild-type enzyme. Although ~2 mg of purified enzymes were obtained out of 1 g of wet cell paste, both enzymes variants did not show any absorbance in the 350-500 nm region, suggesting lack of enzyme-bound flavin. In agreement with this observation the apoenzymes did not show any oxygen consumption when assayed at final concentrations as high as 20 µM with 10 mM glycolate at pH 7.0 and 30 °C, irrespective of whether exogenous FMN as high as 400 µM was present in the enzyme reaction mixture. Independent attempts to measure oxygen consumption during catalytic turnover of the variant enzymes with glycolate as substrate upon pre-incubating the His260Gln or His260Ala apoenzymes with 1 mM FMN were also unsuccessful. Thus, the mechanistic investigation of the role of His260 in the reaction catalyzed by human liver GOX could not be carried out.

**Discussion**

The renewed interest in human GOX arises from the potential for using the enzyme as drug target for the treatment of primary hyperoxaluria, a hereditary disorder in which large deposits of calcium oxalate produced enzymatically by GOX result in kidney stones (12). Despite a number of biochemical and structural studies on GOX from human and plants (9,13-19,23,30-32), investigations aimed at the understanding of the mechanistic properties of the human enzyme have not been reported to date. Here, we have determined the effects of pH on the steady-state and pre-steady state kinetic parameters of human liver GOX. The information that has emerged from these studies allowed us to establish that the enzyme shows a Ping-Pong Bi-Bi catalytic mechanism throughout the pH range from 6.0 to 10.0. The requirements for ionizable groups or lack of thereof in all of the kinetic steps that define the minimal steady-state kinetic
mechanism of the enzyme in turnover with glycolate have also been established.

The oxidation of glycolate to glyoxylate catalyzed by human liver GOX involves the flavin-mediated transfer of a hydride equivalent from the α-hydroxy acid substrate to the final electron acceptor oxygen. A Ping-Pong Bi-Bi mechanism is consistent with the kinetic data observed with glycolate as substrate for the enzyme, as illustrated in the minimal steady-state kinetic mechanism of Scheme 2. After the reversible formation of a Michaelis complex involving the oxidized enzyme and the substrate (E-FMN_{ox}\cdot S), the enzyme reacts with glycolate irreversibly to generate glyoxylate with concomitant reduction of the enzyme-bound flavin (E-FMN_{red}\cdot P). The catalytic cycle is then completed with the release of the glyoxylate product from the active site of the enzyme \((k_5\) step in Scheme 2), followed by the second-order reaction of the reduced enzyme-bound FMN with molecular oxygen to generate hydrogen peroxide and oxidized enzyme \((k_7\) step). Evidence supporting the mechanism of Scheme 2 comes from the patterns with parallel lines that were observed in double reciprocal plots of the initial rates of reaction and the concentrations of either glycolate or oxygen. The Ping-Pong Bi-Bi kinetic mechanism was further confirmed with inhibition studies with glyoxylate. Indeed, the uncompetitive inhibition pattern observed is consistent with glycolate and glyoxylate binding to two forms of GOX, the oxidized and reduced enzymes, respectively, interconnected by an irreversible kinetic step of catalysis, i.e., the oxidation step where glycolate is converted to glyoxylate \((k_3\) step). Further evidence for the chemical step of glycolate oxidation \((k_3\) being irreversible comes from the rapid kinetics data showing a negligible \(y\)-intersect of the saturation curve that fits the reductive half reaction data in a plot of \(k_{obs}\) versus [glycolate]. While a Ping-Pong Bi-Bi steady-state kinetic mechanism was recently reported for human liver GOX at pH 7.0 and 7.5 (11,23), and for the spinach enzyme at pH 8.3 (17) the investigation reported here shows that the relative order of the kinetic steps in the kinetic mechanism of the enzyme remains the same throughout the pH range from 6.0 to 10.0. In this respect, GOX has a behavior that is different from that previously reported for pyranose 2-oxidase, another flavin-dependent enzyme that oxidizes alcohols. In that case, the enzyme switches from a Ping-Pong Bi-Bi mechanism where flavin oxidation occurs after the release of the product at pH <7.0 to a Ternary complex mechanism where oxidation of the flavin occurs while the product of the reaction is still bound at the active site at pH >7.0 (33).

A protonated group with an apparent \(pK_a\) value of 8.5 is required for the formation of the enzyme-substrate complex. Evidence supporting this conclusion comes from the pH profiles determined with glycolate as substrate for the enzyme using the steady-state and rapid kinetic approaches. Indeed, the \(\text{app}K_d\) value, which due to substrate binding to the enzyme not being in rapid equilibrium equals \((k_2+k_3)/k_1\), increased with increasing pH values. Since the kinetic step of glycolate oxidation defined by the rate constant \(k_3\) is independent of pH above pH 7.0, any pH effect observed in this pH range on the \(\text{app}K_d\) values necessarily arises from binding of the substrate to the active site of the enzyme. Interestingly, the requirement for the same protonated group with an apparent \(pK_a\) value of 8.5 is also seen in the pH profile for the steady-state second-order rate constant for capture of glycolate, \(k_{cat}/K_{glycolate}\). In this case, lack of any \(pK_a\) value above pH 7.0 in the pH profile for the \(k_{cat}\) value establishes the apparent \(pK_a\) of 8.5 seen in the pH profile of the \(k_{cat}/K_{glycolate}\) value as being due to a group participating in the formation of the enzyme-substrate complex but not catalysis (34). In the crystal structure of human liver GOX in complex with glyoxylate (20), the interaction
between the enzyme and glyoxylate is mediated by several hydrogen bond and electrostatic interactions involving ionizable groups of the protein, including Tyr26, Arg167 and Arg263 (Figure 1) (20). While mechanistic data on site-directed mutants are not available on the human liver enzyme, substitution of Tyr24 with phenylalanine in spinach GOX, which is equivalent to Tyr26 of the human enzyme, resulted in a 20-fold increase in the $K_d$ value for glycolate suggesting that the active site tyrosine perhaps participates in substrate binding (19).

An ionizable group with an undefined $pK_a$ value that must be lower than 5.0 needs to be unprotonated in the catalytic step of flavin reduction, as suggested by the pH effects on the rate constant for anaerobic flavin reduction ($k_3$) when human liver GOX is mixed with glycolate in a stopped-flow spectrophotometer. Independent support for this conclusion comes from the pH-independence of the overall turnover of the enzyme under steady-state conditions. These results are in keeping with the hydride ion transfer mechanism that was recently proposed for another member of the $\alpha$-hydroxy acid oxidase superfamily, flavocytochrome $b_2$, based on primary and solvent deuterium kinetic isotope effects on the wild type and selected active site mutant enzymes (35-37). The results are also consistent with the alternative proposal that the initial abstraction of a proton is not occurring on the hydroxyl group of glycolate but instead on the substrate $\alpha$-carbon to generate an activated carbanion species that goes on to form a covalent intermediate with the N(5) atom of the enzyme-bound FMN, as previously proposed by other groups for flavocytochrome $b_2$ and mandelate dehydrogenase (5,19,38,39). Irrespective of the mechanism of substrate activation, i.e., through formation of a substrate alkoxide or a carbanion intermediate, a histidine residue that is highly conserved throughout the $\alpha$-hydroxy acid oxidase superfamily is found in the active site of human liver GOX that could serve as the proton acceptor for the activation of glycolate, namely His260 (Figure 1) (20). In this regard, replacement of the homologous histidine with glutamine in lactate monooxygenase decreased the rate constant for anaerobic flavin reduction by 7 orders of magnitude, consistent with the conserved histidine acting as an active site base (40). Recent mechanistic data on the equivalent histidine residue found in the active site of flavocytochrome $b_2$ (37,41) suggest that in that enzyme the active site histidine plays a dual role in maintaining the integrity of the active site for catalysis and as a proton acceptor in the reaction catalyzed by the enzyme. As shown in this study, the replacement of the conserved histidine in human liver GOX has a major effect on FMN retention, preventing a mechanistic investigation of the role of His260 in catalysis.

Some kinetic step other than the chemical step of catalysis is partially contributing to the overall rate of turnover of the enzyme at pH values lower than 7.0, but not at high pH values. At high pH, the similarity between the $k_3$ and $k_{cat}$ values (i.e., 35 s$^{-1}$ and 29 s$^{-1}$, respectively, at pH 10.0) establishes the oxidation of the glycolate to glyoxylate as being the major rate-limiting kinetic step for the overall turnover of the enzyme. This is clearly not the case at pH 7.0, where the $k_{cat}$ value is significantly lower than the $k_3$ value (i.e., 17 s$^{-1}$ as compared to 52 s$^{-1}$). For the minimal steady-state kinetic mechanism of Scheme 2, the overall turnover of the enzyme at saturating concentrations of the substrates includes all the kinetic steps other than the second-order steps where glycolate binds to the enzyme and oxygen reacts with the reduced enzyme (42). Therefore, it is likely that at low pH the release of glyoxylate from the active site of the enzyme is at least partially rate-determining for the overall turnover of the enzyme. However, it cannot be
ruled out a priori that a first-order kinetic step included in the oxidative half-reaction in which the reduced flavin reacts with oxygen (i.e., $k_7$ in Scheme 2) and that is undetectable when using the steady-state approach may contribute to the overall turnover of the enzyme at pH values lower than 7.0.

The reaction of the reduced flavin of human liver GOX with molecular oxygen is an order of magnitude faster when a group with apparent $pK_a$ of 6.8 is unprotonated. This conclusion is supported by the pH profile of the second-order rate constant $k_{cat}/K_{oxygen}$ determined under steady-state kinetics conditions, which increased from a limiting value of $\sim 7000 \text{ M}^{-1}\text{s}^{-1}$ at low pH to a limiting value of $\sim 60000 \text{ M}^{-1}\text{s}^{-1}$ at high pH values. The limiting value in the upper $10^4 \text{ M}^{-1}\text{s}^{-1}$ range seen at high pH is in keeping with values previously reported for other flavoprotein oxidases, such as for example spinach glycolate oxidase (17), choline oxidase (43,44), monoamine oxidase (45,46), nitroalkane oxidase (47), lactate oxidase (48), sarcosine oxidase (49), and glucose oxidase (50), and it is $\sim 2.5$ orders of magnitude larger than the value of $2.5\times10^2 \text{ M}^{-1}\text{s}^{-1}$ that was reported for the non-enzymatic oxidation of flavins in solutions (48,51). However, the pH profile for the $k_{cat}/K_{oxygen}$ value for GOX is significantly different from the pH profiles of the $k_{cat}/K_{oxygen}$ values that were previously reported for glucose oxidase and choline oxidase. In glucose oxidase, effective oxidation of the reduced flavin was proposed to require an active site histidine with $pK_a$ of 7.9 to be protonated (50,52). In choline oxidase, oxidation of the reduced flavin is facilitated by the non-dissociable, positively charged trimethylammonium moiety of the choline substrate but not by ionizable groups in the active site of the enzyme (43,53-55). In GOX, one can immediately rule out the ionization of the flavin hydroquinone species as being responsible for the $pK_a$ value of 6.8, since the UV-visible absorbance spectra of the reduced enzyme after anaerobic reaction with glyoxylate, with well-resolved maxima at $\sim 340 \text{ nm}$, strongly support the notion that the hydroquinone species is in the anionic state between pH 6.0 and 10.0 (56). At this stage of the investigation, we can only speculate that the $pK_a$ of 6.8 may reflect a kinetic $pK_a$ value defining a change in the rate-limiting step that governs reactivity of the reduced flavin with oxygen. Alternatively, the $pK_a$ of 6.8 may reflect a thermodynamic equilibrium for the interconversion of two enzyme forms that react differently with oxygen. Future studies will be required to further elucidate the interesting reactivity of human glycolate oxidase with molecular oxygen.

In conclusion, in this study the pH profiles of the steady-state and pre steady-state kinetic parameters of human liver GOX have been examined. The enzyme showed a Ping-Pong Bi-Bi mechanism throughout the pH range from 6.0 to 10.0. Formation of the enzyme-substrate complex is favored at pH values lower than 8.5, whereas the chemical step in which the flavin is reduced by glycolate is faster at pH values above 7.0. Finally, the reaction of the reduced flavin with molecular oxygen showed a pH profile that is unprecedented in flavoprotein oxidases, and that complements the cases previously reported for glucose oxidase and choline oxidase. The results of the mechanistic investigation presented here thereby define a platform for future mechanistic studies of human liver GOX and raise important questions that will require attention, such as for example what is the mechanism for the activation of the organic substrate and of molecular oxygen for reaction with the enzyme-bound flavin catalyzed by GOX.

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**Supplemental Information Available**
Figure S1 shows the anaerobic reduction of human liver GOX with glycolate as substrate at pH 5.0 (panels A-B), 6.0 (panels C-D), 8.0 (panels E-F), 9.0 (panels G-H) and 10.0 (panels I-L).
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Footnotes
1 Abbreviations used: GOX, glycolate oxidase.

2 Although chloride ions at high concentrations inhibit GOX (IC50 value of 280 mM at pH 7.0, 25 °C; see ref. 24), the final concentration of NaCl that was carried over to the assay reaction mixtures for the measurement of steady-state kinetic parameters was typically ≤0.5 mM, thereby resulting in negligible effects on the determination of the initial rates of reaction. As described in the Experimental Procedures, the enzyme used for anaerobic flavin reduction experiments with a stopped-flow spectrophotometer was freshly prepared prior to use by gel filtration through PD10 columns equilibrated with the proper buffers devoid of NaCl.

Figure Legends
Figure 1. Active site of human liver GOX (PDB: 2RDU). Dotted lines represent proposed hydrogen bonding and electrostatic interactions of selected amino acid residues in the active site of the enzyme with glyoxylate.

Figure 2. Purification of recombinant human liver GOX heterologously expressed in E. coli Rosetta(DE3)PLysS as analyzed by SDS-PAGE. Lane 1: Molecular mass marker choline oxidase from A. globiformis (60 kDa) and 2-nitropropane dioxygenase from Neurospora crassa (40 kDa). Lane 2: cell-free extract. Lane 3: purified GOX.

Figure 3. Double reciprocal plots of the oxidation of glycolate catalyzed by human liver GOX at pH 7.0 and 30 °C. Initial rates of reaction were measured at varying concentrations of both glycolate and oxygen in 100 mM sodium phosphate, pH 7.0, at 30 °C. Panel A, e/v as a function of the inverse glycolate concentration determined at fixed concentration of oxygen: (○) 0.054 mM (●) 0.181 mM, (□) 0.352 mM, and (■) 0.858 mM oxygen. Panel B, e/v as a function of the inverse oxygen concentration determined at several fixed concentrations of glycolate: (○) 0.05 mM (●) 0.1 mM, (□) 0.25 mM, (■) 0.5 mM, (Δ) 1 mM glycolate. The lines in panels A and B are from the global fit of the kinetic data to eq. 1.

Figure 4. Glyoxylate inhibition of human liver GOX with glycolate as substrate. Double reciprocal plot of the steady-state kinetic data determined in 100 mM sodium phosphate, pH 7.0, at 30 °C and atmospheric oxygen concentration. Concentrations of glyoxylate were: (●) 0 mM (○) 0.5 mM, (■) 1 mM and (□) 2.5 mM. Data derived by steady-state kinetic were fit to eq. 2.

Figure 5. Anaerobic reduction of human liver GOX with glycolate as substrate. Reactions were carried out in a stopped-flow spectrophotometer in 100 mM sodium phosphate, pH 7.0, at 30 °C. Panel A, time courses of the reaction at 450 nm obtained by anaerobic mixing of ~15 μM glycolate oxidase and (from left to right) 4.0 mM, 1.5 mM, 0.5 mM, 0.25 mM and 0.13 mM glycolate, respectively. All traces were fitted to eq. 5. Time indicated is after the end of flow, i.e., ~2 ms. For clarity, one experimental point out of every ten is shown (vertical lines). The inset shows the hyperbolic dependence of the observed rates for the fast phase of flavin reduction (kobs) on the concentration of glycolate. Data were fit to eq. 7. Panel B, the thick curve represents the UV-visible absorbance spectrum recorded ~2 ms after mixing anaerobically GOX with glycolate, i.e., oxidized enzyme before flavin reduction; the thin curve is the UV-visible absorbance spectrum recorded ~2 ms after mixing GOX and glycolate under anaerobic conditions.
absorbance spectrum recorded at the end of the reaction between GOX with glycolate, i.e., reduced enzyme.

Figure 6. Effect of solvent viscosity on the $k_{\text{cat}}/K_{\text{glycolate}}$ values for human liver GOX. The dashed line with slope of 1 describes a case in which the reaction is diffusion-controlled. GOX activity was measured at varying concentration of glycolate and atmospheric oxygen at 30 °C in 100 mM sodium phosphate, pH 7.0, in the absence and presence of glycerol as viscosigen. The solid line represents a fit of the data to eq. 9.

Figure 7. pH profiles for the steady-state and pre steady-state kinetic parameters of human liver GOX with glycolate as substrate. Panel A, pH-dependences of the $k_{\text{cat}}$ (◇), $k_{\text{cat}}/K_{\text{glycolate}}$ (○) and $k_{\text{cat}}/K_{\text{oxygen}}$ (●) values determined by using the steady-state approach with glycolate as substrate for GOX. Enzymatic activity assays were measured at varying concentration of both glycolate and oxygen between pH 6.0 and 10.0, at 30 °C. The curves were obtained by fitting the kinetic data to eq. 3 for the $k_{\text{cat}}/K_{\text{glycolate}}$ values and eq. 4 for the $k_{\text{cat}}/K_{\text{oxygen}}$ values. Panel B, pH-dependence of the $k_3$ (○) and $K_{d,\text{app}}$ (●) values determined by using stopped-flow spectrophotometry. The curve represents the fit of the kinetic data to eq. 8.
Table 1. Steady-state kinetic parameters for recombinant GOX with glycolate as a substrate between pH 6.0 and 10.0.\(^a\)

| pH   | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(K_{\text{glycolate}}\) (mM) | \(K_{\text{oxygen}}\) (mM) | \(k_{\text{cat}}/K_{\text{glycolate}}\) (M\(^{-1}\) s\(^{-1}\)) | \(k_{\text{cat}}/K_{\text{oxygen}}\) (M\(^{-1}\) s\(^{-1}\)) |
|------|--------------------------------|-------------------------------|--------------------------|-------------------------------------------------|----------------------------------|
| 6.0  | 0.28 ± 0.01                   | 54300 ± 200                   | 9700 ± 100               |                                                 |                                  |
| 6.5  | 0.25 ± 0.03                   | 78500 ± 12100                 | 13000 ± 2100             |                                                 |                                  |
| 7.0  | 15.7 ± 0.4                    | 0.23 ± 0.01                   | 0.64 ± 0.03              | 68300 ± 3500                                     | 24500 ± 1300                     |
| 7.5  | 20.0 ± 0.4                    | 0.20 ± 0.01                   | 0.44 ± 0.02              | 99800 ± 5400                                     | 45400 ± 2300                     |
| 8.0  | 24.3 ± 1.0                    | 0.50 ± 0.03                   | 0.60 ± 0.05              | 48600 ± 3500                                     | 40500 ± 3800                     |
| 8.5  | 24.1 ± 1.2                    | 0.54 ± 0.05                   | 0.40 ± 0.04              | 44700 ± 4700                                     | 60300 ± 6700                     |
| 9.0  | 31.2 ± 0.1                    | 2.00 ± 0.01                   | 0.59 ± 0.01              | 15600 ± 100                                      | 52900 ± 100                      |
| 9.5  | 28.5 ± 0.1                    | 3.10 ± 0.01                   | 0.39 ± 0.01              | 9200 ± 100                                       | 73200 ± 200                      |
| 10.0 | 29 ± 2                        | 13.7 ± 1.3                    | 0.61 ± 0.07              | 2100 ± 300                                       | 48000 ± 6400                     |

\(^a\) Steady-state parameters were measured at varying concentrations of both organic substrate and oxygen at 30 °C. Rates are expressed per active site oxidized flavin content.
Table 2. Reductive half-reaction of recombinant GOX with glycolate as a substrate between pH 5.0 and 10.0.  

| pH  | $k_3$ (s$^{-1}$) | $^{\text{app}}K_d$ (mM) |
|-----|-----------------|-------------------------|
| 5.0 | 26.8 ± 0.2      | 0.66 ± 0.02             |
| 6.0 | 45.9 ± 0.3      | 0.41 ± 0.01             |
| 7.0 | 52.0 ± 0.4      | 0.32 ± 0.01             |
| 8.0 | 43.8 ± 0.2      | 0.39 ± 0.01             |
| 9.0 | 39.9 ± 0.2      | 1.64 ± 0.03             |
| 10.0| 34.6 ± 0.5      | 9.35 ± 0.38             |

$^a$ Flavin reduction was carried out in a stopped-flow spectrophotometer at 30 °C. Pre steady-state kinetic parameters were determined by fitting the data acquired upon mixing anaerobically the enzyme with glycolate to eq. 7.
Scheme 1. Oxidation of glycolate catalyzed by human liver GOX

\[
\begin{align*}
\text{H}_2\text{O}_2 & \quad \text{O}_2 \\
\text{FMN} & \quad \text{FMNH}^- \\
\text{H} & \quad \text{H}
\end{align*}
\]

Scheme 2. Minimal steady-state kinetic mechanism of human liver GOX (E-FMN\text{ox}, oxidized enzyme; E-FMN\text{red}, reduced enzyme, S, glycolate, and P glyoxylate)

\[
\begin{align*}
\text{E-FMN}_{\text{ox}} + \text{S} & \quad \text{E-FMN}_{\text{ox}}\text{S} \quad \text{E-FMN}_{\text{red}}\text{P} \\
\text{H}_2\text{O}_2 & \quad \text{O}_2 \\
\text{E-FMN}_{\text{red}} & \quad \text{P}
\end{align*}
\]
Figure 1
Figure 3
Figure 3
Figure 5
Figure 6

\[ \frac{k_{cat} \text{glycolate}}{K_{cat} \text{glycolate}} \times \frac{k_{cat}}{K_{cat}} \]

Relative Viscosity (\( \eta_{rel} \))

\(0\) \quad \(1.2\) \quad \(2.4\) \quad \(3.6\) \quad \(4.8\) \quad \(6\)

Values:
- (1, 1.5)
- (2, 3)
- (3, 4.5)
- (4, 6)

Figure 6
Figure 7
Figure 7
Involvement of ionizable groups in catalysis of human liver glycolate oxidase
Andrea Pennati and Giovanni Gadda

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