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Mechanistic and Stereochemical Studies of Glycine Oxidase from *Bacillus subtilis* Strain R5[†]

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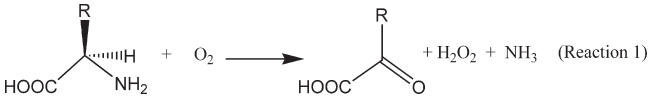
ABSTRACT: Glycine oxidase gene from a strain of *Bacillus subtilis* was cloned and expressed in *Escherichia coli*. The purified enzyme was found, by mass spectrometry, to have a protein M_r of 40763 (value of 40761.6 predicted from DNA sequence) and a FAD prosthetic group M_r of 785.1 (theoretical value of 785.5). Glycine oxidase optimally catalyzes the conversion of glycine and oxygen into glyoxylate, hydrogen peroxide, and ammonia. Using samples of [2-*RS*-$^3H_2$,2-^{14}C]-, [2-*R*-^{3}H,2-^{14}C]-, and [2-*S*-^{3}H,2-^{14}C]glycine, we found that in the overall process H_{Si} is removed. Incubation of the enzyme with [2-*RS*-$^3H_2$,2-^{14}C]glycine under anaerobic conditions, when only the reducing half of the reaction can occur, led to the recovery of 98.5% of the original glycine, which had the same ^{3}H:^{14}C ratio as the starting substrate. The primary isotope effect was studied using [2-$^2H_2$]glycine, and we found that the specificity constants, k_{cat}/K_M, for the protio and deuterio substrates were 1.46×10^3 and 1.05×10^2 M^{-1} s^{-1}, respectively. Two alternative mechanisms for FAD-containing oxidases that involve either the intermediacy of a $FADH_2$−imino acid complex or an amino acid covalently linked to FAD, formed via a carbanion, have been considered. The current knowledge of the mechanisms is reviewed, and we argue that a mechanism involving the $FADH_2$−imino acid complex can be dissected to satisfactorily explain some of puzzling observations for which the carbanion mechanism was originally conceived. Furthermore, our results, together with observations in the literature, suggest that the interaction of glycine with the enzyme occurs within a tight ternary complex, which is protected from the protons of the medium.

D-Amino acid oxidases are flavoproteins and catalyze, optimally, the conversion of D-alanine into pyruvate, releasing ammonia and hydrogen peroxide (*1*), according to reaction 1 (R = CH_3), but also accept other D-amino acids as substrates.



Certain preparations of D-amino acid oxidase, in particular those from sheep kidney, also use glycine as a poor substrate producing glyoxylate (reaction 1, where R = H) (*2*). Whether the reaction with glycine is the property of D-amino acid oxidase itself or a contaminant protein is not known. In recent years, however, genes for glycine oxidases, specific for glycine and its derivatives, have been identified and cloned from *Bacillus subtilis* (*3*−*6*). The genes have also been subjected to site-directed mutagenesis to yield an enzyme with improved activity for serving biotechnological uses (*5*, *7*, *8*).

A *B. subtilis* strain isolated from an oily material in Japan produces several extracellular enzymes (*9*, *10*) and contains a gene for glycine oxidase that was expressed in *Escherichia coli*; the encoded protein was purified, and its physicochemical properties were studied (manuscript submitted for publication). We now describe experiments that elucidate the substrate stereochemistry of the enzyme and shed light on its mechanism of action.

MATERIALS AND METHODS

Materials. [2-$^3H_2$]Glycine, [2-^{14}C]glycine, and scintillation fluid (Fluoro High Performance LSC Cocktail) were obtained from Sigma-Aldrich. [2-*RS*-$^3H_2$,2-^{14}C]Glycine was converted to [2-*R*-^{3}H,2-^{14}C]glycine using serine hydroxymethyl transferase (*11*), whereas [2-*S*-^{3}H,2-^{14}C]glycine was prepared by using the method described in ref *12*. *B. subtilis* strain R5 was isolated by one of us (NR), from whom a culture may be obtained. Glycine oxidase with a specific activity of 0.8 μmol min^{-1} mg^{-1} was purified using the method described in a manuscript submitted for publication. Horseradish peroxidase and catalase were purchased from Calzyme Laboratories, Inc.

Preparation of [2-S-^{3}H,2-^{14}C]Glycine. A reaction mixture (1 mL) containing 1 mM [2-*RS*-$^3H_2$,2-^{14}C]glycine (425200 cpm of ^{14}C; ^{3}H:^{14}C ratio of 11), 2-amino-3-oxobutyrate CoA ligase (1.2 units), and 50 mM Tris-HCl buffer (pH 8) was incubated at 37 °C for 3 h. Aliquots of 50 μL were removed at various time intervals for conversion of glycine into benzyloxycarbonylglycine by the procedure described below. When, after 180 min, the ^{3}H:^{14}C ratio in the glycine derivative reached 5.6, the remaining reaction mixture was stored at −20 °C for stereochemical studies.

Preparation of Benzyloxycarbonylglycine. The aliquots, removed from the reaction mixture described above, were mixed

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with 100 mg of unlabeled glycine and added to 100 μL of benzyl chloroformate (95%) in 2 mL of 7% NaOH, and the mixture was stirred vigorously at 4 °C for 3 h (*12*). Unreacted benzyl chloroformate was extracted with 10 mL of a petroleum/diethyl ether mixture (1:1, v/v) thrice, and then the aqueous layer was acidified with HCl and cooled in an ice bath. Crystals of benzyloxycarbonylglycine were filtered, washed with cold water, and dried. A 50 mg portion of the crystals was dissolved in the scintillation fluid and subjected to the determination of radioactivity.

Conversion of Glycine into Glyoxylate, by Glycine Oxidase, under Aerobic Conditions. A 0.2 mL portion of the reaction mixture in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM [2-*RS*-$^3H_2$,2-^{14}C]-, [2-*R*-^{3}H,2-^{14}C]-, or [2-*S*-^{3}H,2-^{14}C]glycine, catalase (16 units), and glycine oxidase (0.96 unit) was incubated at 37 °C for 1 h. A portion of the reaction mixture was applied to silica gel-coated POLYGRAM SIL N-HR/UV_{254} plates (Macherey-Nagel GmbH) which were developed with a chloroform/methanol/formic acid mixture (10:5:0.5 by volume). A strip of the plate was cut; spots of glyoxylate (R_f = 0.87) were visualized by iodine vapors, and that of glycine (R_f = 0.25) was visualized with a ninhydrin solution. Corresponding to these bands, silica was scraped, and radioactivity was counted in the scintillation fluid.

Enrichment of Tritium in Glycine. The reaction mixture (1 mL) containing 50 mM Tris-HCl buffer (pH 8.0), 9 mM unlabeled glycine, 1 mM [2-*RS*-$^3H_2$,2-^{14}C]glycine (288000 cpm of ^{14}C; ^{3}H:^{14}C ratio of 10), catalase (16 units), and glycine oxidase (0.3 unit) was incubated at 37 °C. At 0, 15, 30, 60, and 90 min postincubation, 200 μL aliquots of the reaction mixture were removed, converted into benzyloxycarbonylglycine (as described above), and used for the determination of radioactivity.

Anaerobic Incubation of Glycine with Glycine Oxidase. A reaction mixture with a final volume of 1 mL in 50 mM Tris-HCl buffer (pH 8) containing glycine oxidase (0.8 unit, 0.025 μmol), glucose (30 μmol), glucose oxidase (5.3 units), and catalase (16 units) was incubated at 37 °C for 15 min, in a cuvette. The spectrum of the holoenzyme was recorded and showed peaks at 456 and 378 nm. Another mixture in a final volume of 100 μL of the buffer described above, containing 10 mM [2-*RS*-$^3H_2$,2-^{14}C]glycine (having 70000 ^{14}C cpm; ^{3}H:^{14}C ratio of 3), glucose (30 μmol), glucose oxidase (5.3 units), and catalase (16 units), was incubated at 37 °C for 5 min. A quarter of the latter mixture (25 μL) was added to the cuvette described above, and the spectrum of the reduced glycine oxidase was monitored for 45 min. The reaction mixture after the addition of glycine (50 mg) was processed for conversion into benzyloxycarbonylglycine as mentioned above. An identical reaction was conducted without glucose and glucose oxidase for aerobic conversion.

Assay of Glycine Oxidase under Aerobic Conditions. The enzyme activity was assayed via measurement of hydrogen peroxide produced during the oxidation of the substrate. The reaction mixture (1 mL), in 50 mM Tris-HCl (pH 8.0), contained 10 mM glycine, 1 mM 4-amino-antipyrine, 2 mM phenol, and horseradish peroxidase (5 units). The appearance of quinoneimine was measured at 505 nm. One unit of activity is defined as the formation of 1 μmol of hydrogen peroxide (0.5 μmol of quinoneimine dye) per minute at 37 °C.

Molecular Mass of Glycine Oxidase and of Its Prosthetic Group (*FAD*). The molecular mass of the recombinant protein was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The purified enzyme was desalted with a Sephadex G-25 column (GE Healthcare). Salt free protein (2.5 μg in 1 μL) was mixed with 9 μL of 3,5-dimethoxy-4-hydroxycinnamic acid (10 mg/mL in 0.1% TFA in acetonitrile and 0.1% TFA in water, at a ratio of 1:2), and 1 μL of the mixture was applied to a plate. The sample was allowed to dry at room temperature for 10−15 min. The spectrum was recorded using a Voyager (ABI) in the positive ion mode, by striking 200 shots in the *m*/*z* acquisition range of 20000−80000. The final spectrum was subjected to smoothing, baseline subtraction, and centroiding.

For the mass spectrum in the negative mode, 1 μL of the desalted protein (2.5 μg/μL) was mixed with 9 μL of α-cyano-4-methoxycinnamic acid (10 mg/mL in 0.1% TFA in acetonitrile and 0.1% TFA in water, at a ratio of 1:2), and 1 μL of the mixture was processed as described above but in the negative mode on an Autoflex Smartbeam III (Bruker Daltonics) by striking 500 × 6 shots in the *m*/*z* acquisition range of 500−1500, for FAD, and 20000−80000 for the apoprotein.

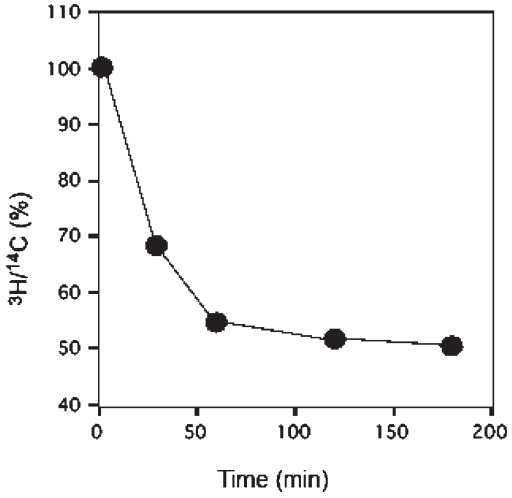


FIGURE 1: Time course of exchange of one of the hydrogen atoms (H_{Re}) of [2-*RS*-$^3H_2$,2-^{14}C]glycine with protons of the medium by 2-amino-3-oxobutyrate CoA ligase. The initial ^{3}H:^{14}C ratio 11 of [2-*RS*-$^3H_2$,2-^{14}C]glycine is taken to be 100%, and other details are given in Materials and Methods.

RESULTS

Synthesis of Stereospecifically Labeled Samples of Glycine. Our previous work has shown that serine hydroxymethytransferase, in the presence of tetrahydrofolate, catalyzes a partial reaction in which H_{Si} of glycine is exchanged with the protons of the medium (*11*). Exploiting this property, we incubated rabbit liver serine hydroxymethytransferase with [2-*RS*-$^3H_2$,2-^{14}C]glycine having a ^{3}H:^{14}C ratio of 9.5. There was a first-order loss of half of the ^{3}H that stabilized after a ^{3}H:^{14}C ratio of 4.6 was reached. Because it is known that in this process H_{Si} is removed, the resulting sample of glycine was formulated as [2-*R*-^{3}H,2-^{14}C]glycine. The enantiomeric counterpart, [2-*S*-^{3}H,2-^{14}C]glycine, was prepared using 2-amino-3-oxobutyrate synthase which in a partial reaction has been interpreted to label the H_{Re} of glycine. Incubation of [2-*RS*-$^3H_2$,2-^{14}C]glycine (^{3}H:^{14}C ratio of 11) with recombinant *E. coli* 2-amino-3-oxobutyrate CoA ligase (*12*) led to the loss of one of the ^{3}H atoms as shown in Figure 1 which ceased at a ^{3}H:^{14}C ratio of 5.6 giving [2-*S*-^{3}H,2-^{14}C]glycine.

Glycine Oxidase. The gene encoding glycine oxidase was isolated from *B. subtilis* strain R5, cloned, and expressed in

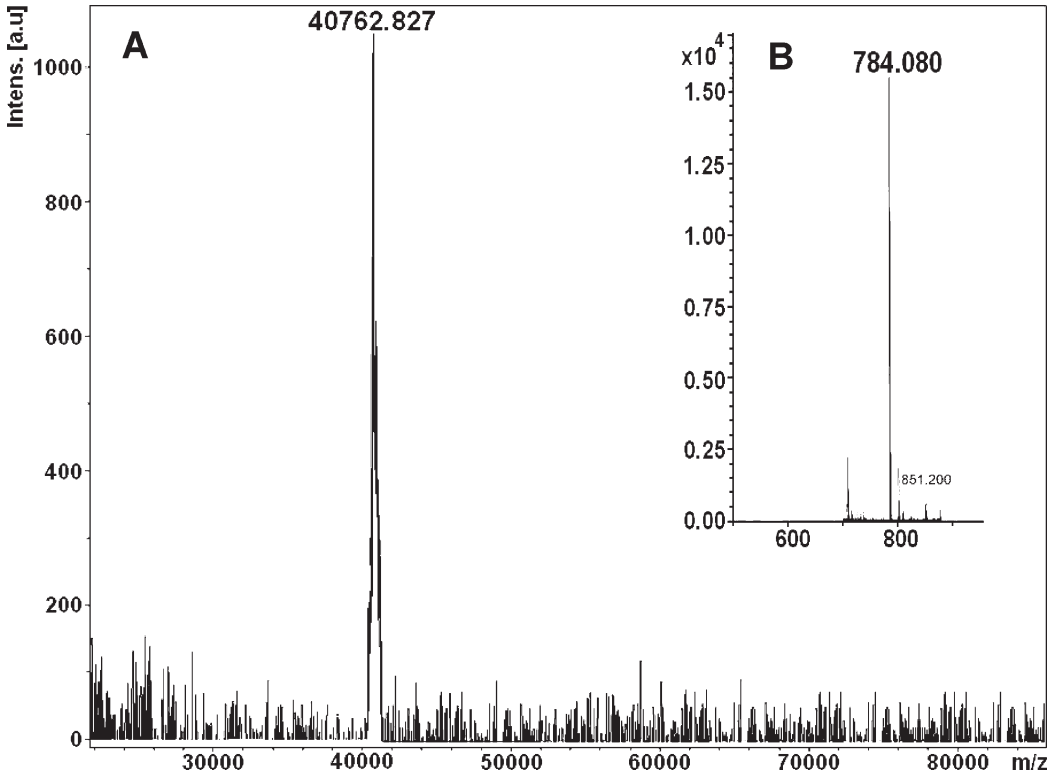


FIGURE 2: MALDI-TOF mass spectrum of glycine oxidase apoenzyme showing the $[M - 1H]^-$ peak for the protein (A) and FAD prosthetic group (B, inset) in the negative mode. The areas under the peaks for the apoprotein and FAD are 7123 and 4846, respectively.

E. coli. The recombinant protein was produced in a soluble form that was purified to give a single band on SDS−PAGE (data not shown). According to the DNA sequence of the gene, the encoded protein should have an M_r of 40761.6 (Figure 1S of the Supporting Information). The MALDI-TOF spectrum of the enzyme in the positive mode gave an $[M + 1H]^+$ peak at m/z 40761.13 (Figure 2S of the Supporting Information), corresponding to an M_r of 40760.6, which is in close agreement with the expected mass for the apoenzyme (40761.6) . The protein was also analyzed in the negative mode (Figure 2) giving an $[M - 1H]^-$ ion at 784.1 corresponding to FAD (theoretical mass of 785.5) which has been shown to be the prosthetic group for glycine oxidases from other organisms. The spectrum in the higher m/z range gave a second $[M - 1H]^-$ ion for the apoprotein at 40762.8 (M_r = 40763.8) The areas under the two peaks for the protein and FAD were in the ratio of 1:0.68, consistent with the expectation that the prosthetic group in the holoenzyme enzyme is present at a stoichiometry of 1 mol/subunit (Figure 2).

Preparation and Analysis of Glyoxylate Using Variously Tritiated Glycine Samples. The incubation of the enzyme with a sample of [2-*RS*-$^3H_2$,2-^{14}C]glycine (^{3}H:^{14}C ratio of 11.6) followed by the separation of the products by thin layer chromatography and measurement of radioactivity showed that the product, glyoxylate, had a ^{3}H:^{14}C ratio of 5.4, corresponding to the loss of 53% of the ^{3}H from the precursor glycine. The fact that the ^{3}H lost from the prochiral C-2 of glycine was in the *pro-S* position (H_{Si}) was shown by the conversion of [2-*S*-^{3}H,2-^{14}C]glycine (^{3}H:^{14}C ratio of 5.2) into glyoxylate (^{3}H:^{14}C ratio of 0.34) which was attended by the loss of 93.5% of the tritium. Complementary experiments with [2-*R*-^{3}H,2-^{14}C]glycine (^{3}H:^{14}C ratio of 4.5) showed that the tritium in this position, H_{Re}, was retained in glyoxylate to the extent of 98% (Table 1).

Table 1: Stereospecificity of the Glycine Oxidase Reaction[a]

compound	^{14}C (cpm)	^{3}H (cpm)	^{3}H:^{14}C	loss (%)
[2-*RS*-$^3H_2$,2^{14}C]glycine	10349	120048	11.6	0
derived glyoxylate	3754	20353	5.4	53
[2-*S*-^{3}H,2-^{14}C]glycine	15545	81775	5.2	0
derived glyoxylate	3576	1229	0.34	93.5
[2-*R*-^{3}H,2-^{14}C]glycine	12166	54747	4.5	0
derived glyoxylate	3593	16370	4.6	2

[a]Details are given in Materials and Methods.

Anaerobic Incubation of Glycine Oxidase with [2-*RS*-$^3H_2$,2-^{14}*C*]*Glycine*. To separate the overall process catalyzed by the enzyme into partial reactions, 25 μM glycine oxidase was incubated with 250 μM [2-*RS*-$^3H_2$,2-^{14}C]glycine under anaerobic conditions, generated from glucose, glucose oxidase, and catalase. The resting state of the enzyme, like other flavoproteins, exists in a state in which the flavin is present in an oxidized form, having absorption bands at 456 and 378 nm as shown by curve 1 in Figure 3. The addition of labeled glycine to the mixture led to a progressive loss of the peak at 456 nm which stabilized after 5 min and remained so for a further 45 min. The reaction was then terminated by the addition of unlabeled glycine, and the glycine was converted into benzyloxycarbonylglycine for the determination of radioactivity. The data in Table 2 show that, within the accuracy of our measurements, the ^{3}H:^{14}C ratio of the recovered sample of glycine was similar to that of the initial sample. This indicated that if an initial adduct is formed between glycine and the enzyme, it is irreversible, in the absence of O_2, or if reversal occurs the hydrogen atom of glycine removed in formation of the adduct is returned to glycine without exchange with the protons of the medium.

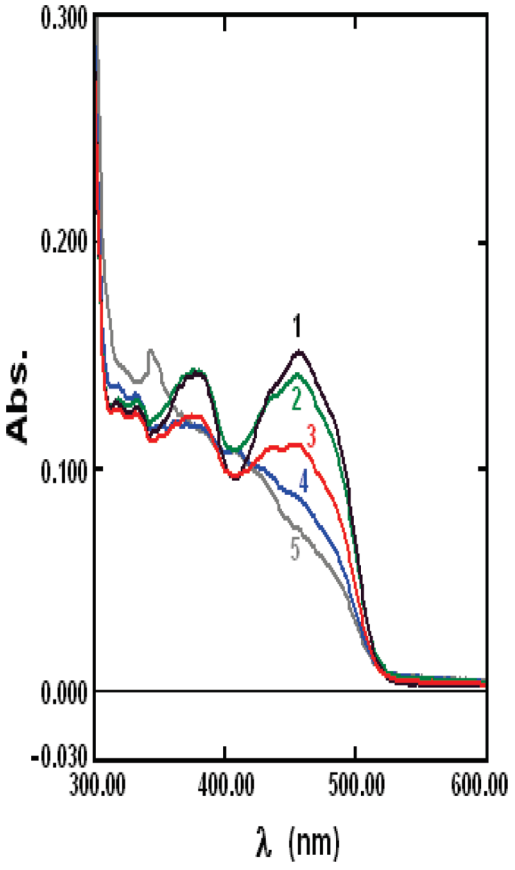


FIGURE 3: Anaerobic reduction of glycine oxidase. The enzyme was incubated with glucose, glucose oxidase, and catalase and reduced with [2-*RS*-$^3H_2$,2-^{14}C]glycine: (1) spectrum of the FAD prosthetic group with absorption maxima at 456 and 378 nm, before the addition of glycine, and (2−5) spectra 1, 2, 3, and 5 min, respectively, after the addition of glycine. Other details are given in Materials and Methods.

Table 2: ^{3}H:^{14}C Ratios of Glycine Recovered from Anaerobic and Aerobic Incubation with [2-*RS*-$^3H_2$]Glycine[a]

time postincubation	^{14}C (cpm)	^{3}H (cpm)	^{3}H:^{14}C
zero time (anaerobic)	4857	15681	3.2
45 min (anaerobic)	4783	17090	3.6
zero time (aerobic)	4857	15681	3.2
45 min (aerobic)	710	7490	10.5

[a]Details are given in Materials and Methods.

Isotope Effect in the Removal of the Cα Hydrogen Atom of Glycine. In the conversion of [2-*RS*-$^3H_2$,2-^{14}C]glycine into glyoxylate, by glycine oxidase, when the ^{3}H:^{14}C ratio of unreacted glycine was monitored, during the course of reaction, there was a progressive increase in the tritium content of the recovered glycine with time (Figure 4). This finding indicated the operation of an isotope effect in the removal of the α-hydrogen atom of glycine. This feature was quantified using deuteriated glycine, when kinetic data Table 3 (Figure 3S of the Supporting Information) showed that the k_{cat} values for the protio and deuterio substrates were 0.54 and 0.21 s^{-1}, respectively, giving a k_H/k_D of 2.5. When the data were analyzed as k_{cat}/K_M, the specificity ratio of the protio species was 1 order of magnitude greater than that of its deuterio counterpart, the k_{cat}/K_M values for [2-$^1H_2$]glycine and [2-$^2H_2$]glycine being 1.46×10^3 and 1.05×10^2 M^{-1} s^{-1}, respectively.

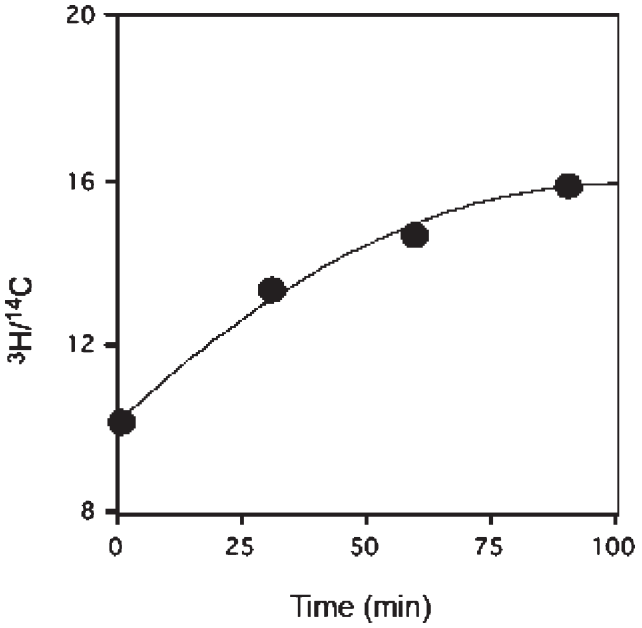


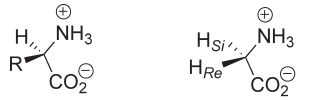
FIGURE 4: Time course of the enrichment of ^{3}H in recovered glycine. [2-*RS*-$^3H_2$,2-^{14}C]Glycine was incubated with glycine oxidase, and aliquots were removed at various time intervals for the analysis of radioactivity in benzyloxycarbonylglycine.

Table 3: Kinetic Parameters of Glycine Oxidase[a]

substrate	K_M (M)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
[2-$^1H_2$]glycine	0.37×10^{-3}	0.54	1.46×10^3
[2-$^2H_2$]glycine	2.0×10^{-3}	0.21	1.05×10^2

[a]Assay of the glycine oxidase with [2-$^1H_2$]glycine and [2-$^2H_2$]glycine. The details are in Materials and Methods section.

Scheme 1: H_{Si} of Glycine Corresponds to the α-Hydrogen Atom of D-Alanine



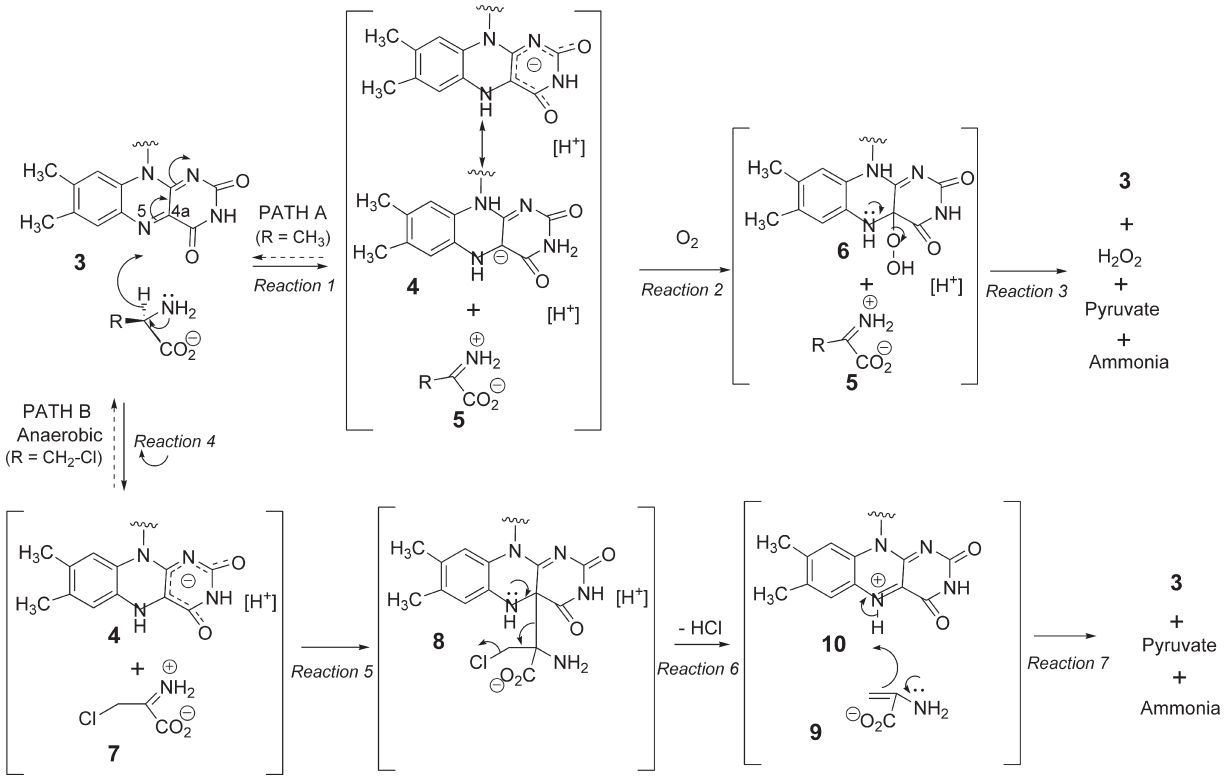
D-Alanine **1** Glycine **2**

DISCUSSION

The fact that the glycine oxidase used in this work, and in other studies, can use D-alanine as a substrate, albeit a poor one, would suggest that the prochiral hydrogen at C-2 of glycine, involved in the oxidative process, may be the one that corresponds to the α-hydrogen of D-alanine. The comparison of structures in Scheme 1 shows that it is the H_{Si} of glycine that satisfies this requirement. This feature, however, needed to be proved unambiguously as has been achieved here using *RS*-, *R*-, and *S*-tritiated samples of glycine. That H_{Si} is exclusively removed in the process suggests that in the glycine oxidase−glycine complex the substrate is rigidly bound to the active site, allowing discrimination between the two prochiral hydrogen atoms.

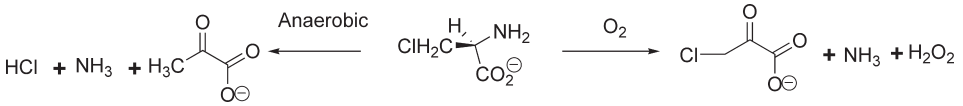
In the experiments in which glycine oxidase was incubated with [2-*RS*-$^3H_2$,2-^{14}C]glycine, under anaerobic conditions, the spectrum of the reduced enzyme was maintained for 45 min, and from the ^{14}C radioactivity recovered in glycine, it could be deduced that ~98.5% of the latter was untransformed (Table 2). The ^{3}H:^{14}C ratio of the recovered glycine, within the limits of

Scheme 2: Mechanisms for the Reactions Catalyzed by D-Amino Acid and Glycine Oxidases[a]



[a]Path A shows aerobic reactions with physiological substrates. In reactions 1 and 4, hydrogen transfer may occur via a hydrogen atom or hydride transfer (*14*). Reaction 2 is oxidation of **4** via **6**, and reaction 3 is the release of products with the hydrolysis of **5**, presumably, nonenzymically. Path B shows the reaction of D-chloroalanine with D-amino acid oxidase, or of glycine (CH_2-Cl = H), with glycine oxidase under anaerobic conditions. Reaction 5 is the nucleophilic attack of the C-4a anion on the imino derivative **4**. Reaction 6 is the fragmentation with the expulsion of a chloride ion. Reaction 7 is the conversion of the enamine **9** to the corresponding imino compound via donation of a hydrogen from the protonated flavin **10**, followed by the release of products and hydrolysis of the imine.

Scheme 3: Aerobic and Anaerobic Reactions Catalyzed by D-Amino Acid Oxidase using D-Chloroalanine as a Substrate



experimental error, was the same as that of the original glycine. The enzyme:substrate ratio of 1:10 (25 and 250 μM, respectively) used in the preceding anaerobic incubation in the presence of O_2 would have led to the oxidation of 36 μmol of glycine. In other words, the capacity of the enzyme for catalytic turnover was 144-fold greater than the amount of substrate present in the system. Despite such a favorable opportunity, the fact that no significant ^{3}H from glycine was removed suggests that during catalysis, the adduct forming reaction is essentially irreversible or that reversal occurs but the hydrogen atom of glycine removed in the formation of the reduced form of the enzyme is faithfully returned to the substrate during the reversal process. A shielding from exchange of the α-hydrogen atom has been described for reactions catalyzed by lactate and D-amino acid oxidases (*13*).

The earliest view, on the mechanism of action of FAD/FMN-dependent amino acid oxidases, influenced by the mechanism of L-glutamate dehydrogenase, was that removal of a hydrogen from the amino group of D-amino acid and the other from its α-position directly produces a flavin-H_2 moiety [**4** (Scheme 2)] and an imino acid (**5**) (for detailed coverage of the mechanistic aspects of oxidases, see ref *14*). The former (**4**) is reoxidized by reaction with O_2, and the latter undergoes hydrolysis to produce the oxoacid and ammonia (Scheme 2, path A). This, apparently, straightforward view, however, was challenged by two types of seminal discoveries made in the early 1970s. The first was the observation by Bright and co-workers that D-amino acid oxidases are inactivated by the anion of nitroethane which, by comprehensive chemical studies, was shown to add to the N-5 atom of the flavin moiety (*15*). In the oxidized flavin moiety, N-5, thus, is an electrophilic center. The second was the finding that β-chlorolactate and D-chloroalanine were used as substrates by lactate oxidase and D-amino acid oxidase, respectively, giving the expected chloropyruvate, in the presence of O_2, but producing dehalogenated products under anaerobic conditions (Scheme 3). Features emphasized in the original studies that need to be kept in mind are the fact that the flux between the two pathways of Scheme 3 is entirely dependent on the presence or absence of O_2 and the fact that the turnover numbers for the two processes are very similar (*16*). These observations were rationalized by assuming that a common, carbanion intermediate is formed between the flavoprotein and the halogenated substrate, which in the presence of O_2 follows the physiological reaction course but in its absence can promote an elimination reaction (*14*, *16*).

However, subsequently, the X-ray structure of pig kidney D-amino acid oxidase, in complex with a competitive inhibitor (benzoate), was determined and used to model a D-alanine molecule at the active site that had its α-hydrogen atom pointing toward N-5 of the flavin (*17*, *18*). This feature together with the absence of a suitable base, at the active site, which could abstract a hydrogen atom, to produce a carbanion, led to the revival of the earliest mechanism, involving the direct transfer of the substrate α-hydrogen to N-5 of the prosthetic group producing flavin-H_2 (Scheme 2, path A). At the same time as the preceding study was being conducted, Miura et al. (*19*) described the crystal structure of pig kidney D amino acid oxidase in a complex with another inhibitor, *o*-aminobenzoate, using molecular modeling of the D-amino acid oxidase D-leucine, which confirmed the active site organization reported by Mattevi et al. (*17*, *18*), including the fact that the α-hydrogen atom of the D-leucine was directed toward N-5 of the flavin ring. Miura et al. (*19*), however, considering it mandatory that a credible mechanism of D-amino acid oxidase should rationalize not only the physiological oxidative reaction catalyzed by the enzyme but also the anaerobic dehalogenation process, observed with chloroalanine and cholorolactate, retained a carbanion mechanism and used N-5 as a proton acceptor. Their suggested mechanism is outlined in Scheme 1S of the Supporting Information, in which two alternative courses are driven by the same carbanion species.

D-Amino acid oxidase and D-lactate oxidase, for which the anaerobic dehalogenation reactions were discovered, belong to a large class of flavin-dependent enzymes that catalyze the oxidation of substrates and transfer the resulting reducing equivalent to oxygen or another electron acceptor (*14*, *20*, *21*). A broad consensus regarding their mechanism is the involvement of the transfer of hydrogen to N-5 producing a reduced flavin moiety. Indeed, for D-amino acid oxidase, indirect evidence of such a hydrogen transfer was provided using the enzyme reconstituted with 5-deaza-FAD when the α-hydrogen from [2-^{3}H]D-alanine was transferred to the "artificial" prosthetic group (*22*). Let us attempt to explain the physiological aerobic reaction along with the anaerobic dehalogenation process of retaining the flavin-linked oxidation of the substrate by transfer of hydrogen to N-5 as a key event to produce dihydroflavin, as its anion (reaction 1, Scheme 2). Special attention, then, needs to be paid to the fact that it is the reoxidation of the anion (**4**), the second half-reaction (reactions 2 and 3), that provides the main energetic driver for the overall process. Here, while the imino intermediate is still bound to the active site, the negative charge on the anion, localized at C-4a, is dissipated by two single-electron transfers to oxygen producing hydrogen peroxide, presumably via the hydroperoxide (**6**) (*23*, *24*). It could be argued that under anaerobic conditions, when the anion at C-4a cannot be quenched by reaction with oxygen, it adds to enzyme-bound imino species to produce an adduct of type **8** (path B, reaction 5). The latter when formed from a halogenated substrate will regenerate a protonated form of the oxidized flavin moiety (**10**) by an elimination process (reaction 6). The proton from the α-position of the substrate removed in reaction 4 can now be used to convert the enamine **9** into an imine (of type **5**) ready for hydrolysis to yield the products of reaction 7. The proton transfer reaction involved in reaction 7 would explain the observation that in the anaerobic dehalogenation reactions, involving D-amino acid and lactate oxidase, the α-hydrogen atom of the substrates is found in the methyl group of the products (*13*, *16*). If this argument is extended, when an amino acid is a substrate, like glycine in this paper, the imino acid, in the absence of oxygen, is trapped by the C-4a anion to produce a dead-end complex, which may explain the retention of tritium when [2-*RS*-$^3H_2$,2-^{14}C]glycine is incubated with glycine oxidase under anaerobic conditions. The mechanism of path B (Scheme 2) proposed here may be regarded as a modification of that suggested on page 715 of ref *14*, except that in the latter proposal, a halide ion is directly displaced by a hydride, from the N-5−H bond of the reduced flavin, whereas we have used an addition−elimination sequence for the dehalogenation process.

In light of the current situation, then, the shielding of the hydrogen atom of glycine, from exchange with the protons of the medium may be ascribed to one or a combination of any of the following factors. (i) The adduct forming reaction 1 is essentially irreversible. (ii) The reversal occurs but within a tight cavity, protected from medium water. (iii) The reversal is prevented by the formation of a dead-end complex of type **8** (CH_2-Cl = H). The isotope effect shown in this work with glycine oxidase and previously reported for amino acid oxidase (*16*, *25*) would be consistent with both mechanisms, involving the transfer of hydride to FAD or deprotonation to give a carbanion intermediate (for example, Scheme 1S of the Supporting Information), though our preference is for a hydride transfer mechanism, for the reasons mentioned above and well elaborated by Frey and Hegeman (*14*), also see (*26*).

SUPPORTING INFORMATION AVAILABLE

Flow sequence of a carbanion mechanism adapted from ref *19* (Scheme 1S), amino acid sequence and composition of glycine oxidase (Figure 1S), mass spectrum of the apoenzyme in the positive mode (Figure 2S), and kinetic plots for the enzymic reaction (Figure 3S). This material is available free of charge via the Internet at http://pubs.acs.org.

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