THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 9, Issue of March 1, pp. 6985–6993, 2002

Printed in U.S.A.

Glycine Oxidase from *Bacillus subtilis*

CHARACTERIZATION OF A NEW FLAVOPROTEIN*

Received for publication, November 20, 2001
Published, JBC Papers in Press, December 13, 2001, DOI 10.1074/jbc.M111095200

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Glycine oxidase (GO) is a homotetrameric flavoenzyme that contains one molecule of non-covalently bound flavin adenine dinucleotide per 47 kDa protein monomer. GO is active on various amines (sarcosine, *N*-ethylglycine, glycine) and D-amino acids (D-alanine, D-proline). The products of GO reaction with various substrates have been determined, and it has been clearly shown that GO catalyzes the oxidative deamination of primary and secondary amines, a reaction similar to that of D-amino acid oxidase, although its sequence homology is higher with enzymes such as sarcosine oxidase and *N*-methyltryptophane oxidase. GO shows properties that are characteristic of the oxidase class of flavoproteins: it stabilizes the anionic flavin semiquinone and forms a reversible covalent flavin-sulfite complex. The ~300 mV separation between the two FAD redox potentials is in accordance with the high amount of the anionic semiquinone formed on photoreduction. GO can be distinguished from D-amino acid oxidase by its low catalytic efficiency and high apparent K_m value for D-alanine. A number of active site ligands have been identified; the tightest binding is observed with glycolate, which acts as a competitive inhibitor with respect to sarcosine. The presence of a carboxylic group and an amino group on the substrate molecule is not mandatory for binding and catalysis.

Glycine oxidase (GO)[1] is a new flavoenzyme that was discovered in 1997 following the complete sequencing of the *Bacillus subtilis* genome (1). Two previous investigations reported on the expression of the *yjbR* gene product (up to 3.9% of total soluble proteins in crude extract) and on its purification and partial characterization in *Escherichia coli* (2).[2] The purified protein is a homotetrameric flavoenzyme that catalyzes the oxidation of various amines (*e.g.* sarcosine, *N*-ethylglycine and glycine) and D-amino acids (*e.g.* D-alanine, D-proline, D-valine, etc.). GO seems to partially share substrate specificity with various flavooxidases, such as D-amino-acid oxidase (DAAO) (EC 1.4.3.3) and sarcosine oxidase (SOX), and also appears to be stereospecific in oxidizing the D-isomer of the amino acids tested.[2]

GO exhibits highest sequence homology with the β subunit of TSOX (EC 1.5.3.1), sarcosine dehydrogenase (SDH) (EC 1.5.99.1) and dimethylglycine dehydrogenase (DMGDH) (EC 1.5.99.2) (24–27% identity), in particular with the N-terminal regions corresponding to the flavin-binding domain. Only a modest similarity is observed with the sequences of monomeric sarcosine oxidase (MSOX) (EC 1.5.3.1), pipecolate oxidase (PIPOX) (EC 1.5.99.3) and DAAO or D-aspartate oxidase (DASPO) (EC 1.4.3.1) (18–21% identity).[2] DAAO (containing 1 mole of non-covalently bound FAD per 40 kDa monomer) catalyzes the oxidative deamination of neutral and (with a lower efficiency) basic D-amino acids to give the corresponding α-keto acids, ammonia and hydrogen peroxide (4). Acidic D-amino acids are oxidized by DASPO. *N*-Methyltryptophan oxidase (MTOX) (EC 1.5.3.1) is a monomeric flavoenzyme (42 kDa) from *E. coli* containing FAD covalently linked to a cysteine residue (5). PIPOX is a mammalian enzyme similar in size to MTOX and to the β subunit of MSOX (44 kDa) and also contains a single covalently bound flavin (6). Sarcosine oxidases have been isolated from several bacteria and belong to two different classes of enzymes: heterotetrameric (TSOX) and monomeric (MSOX) enzymes. All bacterial SOXs catalyze the oxidative demethylation of sarcosine to yield glycine, hydrogen peroxide, and formaldehyde (7). The heterotetrameric SOXs contain four different subunits (from 10 to 100 kDa) and also contain non-covalently bound FAD, non-covalently bound NAD^+, and covalently bound FMN, which is linked to the β subunit (42–45 kDa). The monomeric SOXs are similar in size to the β subunit of TSOX and contain covalently bound FAD. Only TSOXs use tetrahydrofolate as a substrate, and, in this regard, they resemble mammalian SDH and DMGDH (7). In mammals, these two enzymes catalyze the oxidative demethylation of sarcosine in the mitochondria. They are monomeric enzymes (97 and 96 kDa, respectively) containing a single, covalently bound flavin and are considered to be the two main folate-containing enzymes in rat liver mitochondria. Furthermore, these enzymes are linked to the electron transport chain and form 5,10-methylenetetrahydrofolate (8).

This paper focuses on characterizing the substrate-binding site and the properties of the prosthetic group in recombinant GO. We identify the products of the reaction catalyzed by GO on a number of substrates, as well as compounds that act as inhibitors and which have proved useful in probing the reaction mechanism of the enzyme. The main goal of this project is to

* This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Fondo di Ateneo per la Ricerca 2000) (to L. P.) and from "Progetto Giovani Ricercatori", University of Insubria (to V. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GO, glycine oxidase; DAAO, D-amino acid oxidase; SOX, sarcosine oxidase; SDH, sarcosine dehydrogenase; DMGDH, dimethylglycine dehydrogenase; MSOX, monomeric sarcosine oxidase; PIPOX, pipecolate oxidase; DASPO, D-aspartate oxidase; MTOX, *N*-methyltryptophan oxidase; TSOX, heterotetrameric sarcosine oxidase; EFl_{ox}, oxidized enzyme; EFl_{seq}, enzyme flavin semiquinone; EFl_{red}, reduced enzyme; APP, 4-aminoantipyrine.

[2] V. Job, G. Molla, M. S. Pilone, and L. Pollegioni, submitted for publication.

elucidate the structure-function relationships in GO, with the ultimate aim of clarifying the modulation of the substrate specificity in enzymes active on similar compounds.

EXPERIMENTAL PROCEDURES

Reagents—Horseradish peroxidase, formaldehyde dehydrogenase from *Pseudomonas putida*, monomeric sarcosine oxidase from *B. subtilis*, tetrameric sarcosine oxidase from *Corynebacterium sp.*, D-amino acids, xanthine, xanthine oxidase, ampicillin, chloramphenicol, and all other compounds were purchased from Sigma. Glutamate dehydrogenase from bovine liver was from Roche Diagnostics. 5-Deazaflavin was a generous gift of Dr. Sandro Ghisla (University of Konstanz, Konstanz, Germany). Kinetic experiments were performed at 25 °C in 75 mM sodium pyrophosphate and 5 μM FAD, pH 8.5, the other experiments at 15 °C in 50 mM sodium pyrophosphate, pH 8.5, or in 50 mM potassium phosphate, pH 7.0, containing 10% glycerol, unless stated otherwise.

Enzyme Expression and Purification—The pT7-HisGO expression plasmid[2] was transferred, for protein production, to the host BL21(DE3)pLysS *E. coli* strains. Recombinant *E. coli* cells were grown at 37 °C, and GO expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside as reported.[2] GO was purified from the crude extract by chromatography on a HiTrap Chelating affinity column using an ÄKTA FPLC system (Amersham Biosciences, Inc.). The fractions containing GO activity were pooled and loaded on a PD10 column equilibrated with 50 mM sodium pyrophosphate buffer, pH 8.5, containing 10% glycerol. Thus, the recombinant enzyme used in these experiments contains an N-terminal His tag sequence.

Activity Assay—Glycine oxidase activity was assayed using three different methods: i) polarographically using an oxygen electrode at 25 °C and at air saturation ($[O_2]$ = 0.253 mM)[2]; and ii and iii) spectrophotometrically via determination of H_2O_2 with an enzyme-coupled assay using horseradish peroxidase (6–10 units/ml) and *o*-dianisidine (0.32 mg/ml), assuming $\Delta\epsilon_{440}$ = 13,000 M^{-1} cm^{-1} (9), or 2 mM phenol and 1.5 mM 4-aminoantipyrine (10), assuming $\Delta\epsilon_{505}$ = 6580 M^{-1} cm^{-1}. All assays were performed on a final volume of 1 ml in 75 mM sodium pyrophosphate buffer, pH 8.5, using 10 mM sarcosine as the substrate. The polarographic assay solution also contained 5 μM FAD. One GO unit is defined as the amount of enzyme that converts 1 μmol of substrate (sarcosine or oxygen) or that produces 1 μmol of hydrogen peroxide per minute at 25 °C.

Spectral Experiments—Photoreduction in the presence of EDTA was conducted as described in Ref. 11, using an anaerobic cuvette, made anaerobic by alternative cycles of vacuum and O_2-free argon, containing 12 μM enzyme, 5 mM EDTA, and 0.5 μM 5-deazaflavin. The cuvette with the protein solution was kept in a water bath at 10 °C and ~7 cm from a 150-W quartz halogen light source. The cuvette was removed at specified time intervals, and the progress of the reaction was followed spectrophotometrically (11). The thermodynamic stability of the semiquinone was determined by adding 5 μM benzyl viologen from a side arm of the cuvette after the photoreduction was complete. The disproportionation of the semiquinone was then followed until equilibration was reached (for up to 24 h at 4 °C) (12). Flavin and protein fluorescence measurements were carried out in a Jasco FP750 spectrofluorometer at pH 7.0 and 15 °C. Protein fluorescence emission was monitored at an excitation of 280 nm, and flavin fluorescence emission at an excitation of 450 nm, using an excitation slit = 5 nm and an emission slit = 10 nm.

Flavin Content—Flavin was extracted from GO upon unfolding by heating the enzyme at 95 °C for 5 min and removing denatured protein by centrifugation. The flavin in the supernatant was identified by its absorption spectrum as compared with that of native FAD and by fluorescence spectroscopy before and after treatment with phosphodiesterase, which generates FMN and increases the fluorescence yield (13). The extracted flavin was analyzed by chromatography on an Aquapore RP-300 column, according to the method described in Ref. 14. Peak detection was monitored following the absorbance at 450 nm, and the fluorescence at 530 nm (excitation at 450 nm). The peaks were identified by comparing them with the retention time of chromatographically pure flavins.

Redox Potentials—Redox potentials for the EFl_{ox}/EFl_{seq}, and EFl_{seq}/EFl_{red} couples of GO were determined by dye equilibration (15), using the xanthine/xanthine oxidase reduction system at pH 7.0 and 15 °C (16). The reaction was initiated by adding 10–30 nM xanthine oxidase to an anaerobic cuvette containing ~10 μM enzyme, 0.2 mM xanthine, 5 μM methyl viologen, and 5–10 μM of the appropriate dye (16). The amount of oxidized and reduced dye was determined at a wavelength at which the enzyme shows no absorbance (>550 nm), and the amount of oxidized and reduced enzyme was determined either at an isosbestic point for the dye or by subtracting the dye in the 400–470-nm region. Data were analyzed as described in Ref. 15.

Ligand and Sulfite Binding—Dissociation constants for the ligands were measured spectrophotometrically by adding small volumes (1–10 μl) of concentrated stock solutions to samples containing 800 μl of ~10 μM enzyme at 15 °C. The change in absorbance at the wavelengths at which the modification was more pronounced was plotted as a function of ligand concentration, after correction for any volume change; K_d values were determined according to Ref. 17. For reactions with sulfite, the reagent was prepared just before use as a 500-mM stock solution in 50 mM potassium phosphate, pH 7.0, or in 50 mM sodium pyrophosphate, pH 8.5, containing 10% glycerol, and aliquots were then added to enzyme solution at 15 °C. The rate of decay (k_{off}, dissociation of SO_3^{2-}) for the *N*-(5)-sulfite adduct was kinetically determined spectrophotometrically after removing excess sulfite by gel filtration (application of 1-ml samples to a Sephadex G-25 column, void volume 2.5 ml, at 15 °C). The GO-sulfite reaction was investigated by means of stopped-flow spectrophotometric measurements. The experiments were performed at 25 °C in a BioLogic SFM-300 stopped-flow spectrophotometer equipped with a thermostat, a cell with a path length of 1 cm, and with a J&M diode array detector. The reactions were routinely recorded in the 250–700-nm wavelength range using the buffer already described but containing 2% glycerol (final concentration). Reaction rates were calculated by extracting traces at 456 nm and fitting them to a sum of exponential equations using Kaleidagraph.

Reaction Product Analysis—To study the reaction catalyzed by GO, the products formed during the reaction with 100 mM D-alanine, 10 mM glycine, 50 mM *N*-ethylglycine, and 10 mM sarcosine were analyzed using different methods.

α-Keto Acid Assay—α-Keto acid production was estimated as the production of 2,4-dinitrophenylhydrazone derivatives by a reaction with 2,4-dinitrophenylhydrazine (18). After the addition of GO to a mixture containing the substrate in 75 mM sodium pyrophosphate, pH 8.5, aliquots (300 μl) were withdrawn at different times and mixed with (150 μl) 1 mM 2,4-dinitrophenylhydrazine (dissolved in 1 N HCl). After incubation at 37 °C for 10 min, 1.05 ml of 0.6 N NaOH was added; the absorbance at 445 nm was measured after incubation for another 5 min at room temperature. Calibration curves were achieved using both glyoxylate and pyruvate, since the corresponding α-keto acid derivatives show different absorbance spectra and extinction coefficients.

Glutamate Dehydrogenase-coupled Assay for NH_3 Quantitation—The ammonia produced during the reaction of GO was determined using a coupled assay with glutamate dehydrogenase from beef, according to the manufacturer's instructions and following the oxidation of NADH at 340 nm. GO was added to the assay mixture (1 ml) containing the substrate, 5 mM 2-oxoglutarate, 0.25 mM NADH, and 20 units of glutamate dehydrogenase in 65 mM sodium pyrophosphate, pH 8.5. A calibration curve was achieved using known amounts of ammonium chloride.

Formaldehyde Dehydrogenase-coupled Assay and H_2O_2 Assay—To assess the production of formaldehyde, the GO reaction was coupled to the formaldehyde dehydrogenase NAD-dependent reaction (from *P. putida*) following the production of NADH at 340 nm. GO was added to a mixture reaction (350 μl) containing the substrate, 2 mM NAD^+, and 200 μg of formaldehyde dehydrogenase in 65 mM sodium pyrophosphate, pH 8.5. A calibration curve was determined using formaldehyde; blank reactions contained all the assay components except GO. MSOX (*B. subtilis*) and TSOX (*Corynebacterium sp.*) were used as a positive control, and DAAO (*Rhodotorula gracilis)* as a negative control. H_2O_2 production was determined using the previously described coupled method with horseradish peroxidase.

RESULTS

Enzyme Expression and Purification—The scheme of the strategy devised to obtain cDNA coding for the chimeric His-tagged GO has been reported previously.[2] His-GO was expressed in *E. coli* BL21(DE3)pLysS cells transformed as a totally soluble protein: about 2.4 units/g wet weight (≈2 mg of GO/g of cell) of enzyme was expressed, the purification yield being ≈100% (>65 mg of pure GO from 6 liters of culture).[2] The recovered His-tagged GO is fully active since the addition of exogenous FAD or FMN to the assay mixture does not increase the GO activity (specific activity on sarcosine as the substrate is 1.06 units/mg protein at 25 °C). The final preparation was homogeneous at 95% (SDS-PAGE reveals the presence of a

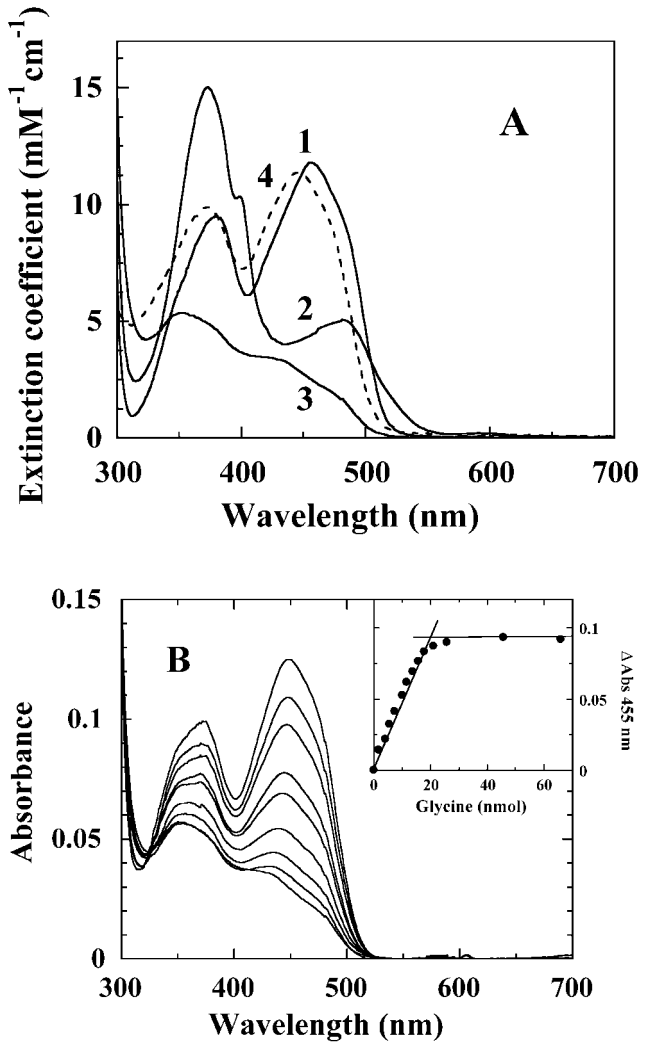


FIG. 1. *A*, absorbance spectra of *B. subtilis* glycine oxidase in the oxidized, half-, and fully reduced states. Spectrum of the oxidized enzyme in 50 mM potassium phosphate, 10% glycerol buffer, pH 7.0, at 15 °C (spectrum 1). The spectrum of the semiquinone form is that generated by anaerobic photoirradiation in the presence of 5 mM EDTA and 0.5 μM 5-deazaflavin (spectrum 2). Spectrum of reduced form of GO after anaerobic addition of 20 mM glycine (spectrum 3). Flavin spectra of the enzyme preparation as in (spectrum 1) after heat treatment (spectrum 4). *B*, anaerobic titration of GO with glycine**.** Oxidized GO (1.8 ml of solution of 11.8 μM GO, corresponding to 21.3 nmol of enzyme in 50 mM potassium phosphate buffer, pH 7.0, 10% glycerol) was anaerobically titrated with small additions of a glycine solution (0.1 mM in the same buffer) and at 15 °C. Selected spectra are shown for the following total concentrations of glycine (from top at 445 nm): 4, 6, 10, 12, 16, 21, 46, and 166 nmol. *Inset*, dependence of the absorbance at 445 nm (upon correction for dilution) on the glycine concentration. The two linear parts of the saturation curve intercept at a ratio enzyme:substrate ≈1.1.

single polypeptide chain corresponding to an apparent molecular mass of ~49.4 ± 1.1 kDa) and was stable when stored at 80 °C for several months.

Coenzyme Determination, Spectral Properties of the Oxidized Form, and Its pH Dependence—The spectrum of GO in the oxidized state is shown in Fig. 1*A*, where it is compared with that of the same sample after heat denaturation and centrifugation (relevant spectral data are summarized in Table I). This latter spectrum is identical to that of free FAD. The protein pellet is colorless, and, after resolubilization using 6 M guanidinium chloride, no absorbance bands are evident in the visible portion of the spectrum, indicating that the flavin cofactor is not covalently bound to the protein moiety. High pressure

TABLE I
Spectral properties of glycine oxidase from B. subtilis

The enzyme solutions used for obtaining the absorbance spectra were ~12 μM in 50 mM potassium phosphate, 10% glycerol, pH 7.0 at 15°C; those for fluorescence spectra were 0.65 μM in the same buffer.

Redox state	Value
EFl_{ox}, λ_{max} (nm)	273, 379, 456
ϵ (mM^{-1} cm^{-1})	103.2, 9.51, 11.81
Absorbance ratios	8.7, 10.9, 1
Fluorescence emission (λ_{max}, nm)	
(λ_{exc} = 280; 450 nm)	342, 525
% of that of free FAD	16.8
EFl_{red}, λ_{max} (nm)	267, 346
ϵ (mM^{-1} cm^{-1})	110, 6.5
Fluorescence emission (λ_{max}, nm)	
(λ_{exc} = 280; 340 nm)	343, 422
EFl_{seq}, λ_{max} (nm)	268, 371, 477
ϵ (mM^{-1} cm^{-1})	113.6, 15.8, 5.3

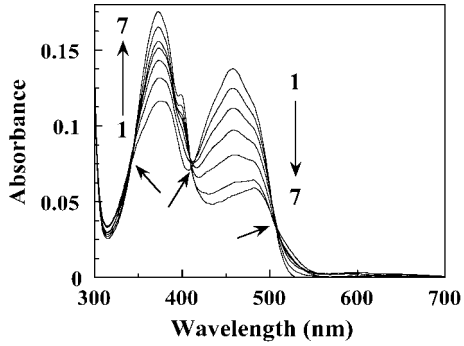


FIG. 2. **Photoreduction of glycine oxidase from *B. subtilis*.** 12.3 μM GO in 0.05 M sodium pyrophosphate and 10% glycerol, pH 8.5, at 15 °C was made anaerobic and subsequently mixed with 5 mM EDTA and 0.5 μM 5-deazaflavin (spectrum 1). The further spectra were recorded after 4 (spectrum 2), 8 (spectrum 3), 14 (spectrum 4), 21 (spectrum 5), 27 (spectrum 6), and 34 (spectrum 7) min of irradiation. The *arrows* indicate the approximate isosbestic points of the conversion.

liquid chromatography of the extracted flavin, using pure FAD and FMN as internal standards (R_t = 4.12 and 4.32 min, respectively), showed that all the extracted flavin was present as FAD (single peak with a R_t = 4.14 min). This result is in agreement with a 7-fold increase in flavin fluorescence and with the slower chromatographic mobility (R_t = 4.33 min) that was observed when phosphodiesterase (0.5 μg/μl) was added to the extracted flavin, as expected due to the conversion of the FAD molecule to FMN. Calculations using the absorption coefficient for free FAD of 11.3 mM^{-1} cm^{-1} resulted in an estimated flavin absorption coefficient of 11.8 mM^{-1} cm^{-1} for the holoenzyme and an A_{274}/A_{456} ratio of ~8.6. According to the amount of FAD extracted and the concentration of the protein in the pellet after heat denaturation (determined from the absorbance at 280 nm of the protein re-dissolved in 6 M guanidinium chloride and using the extinction coefficient theoretically calculated from the amino acid sequence, ϵ = 55190 M^{-1} cm^{-1}), a ratio of 1 mole of FAD:1 mole of enzyme monomer was determined. The 450-nm band exhibited a shoulder of ~470 nm that was similar to the resolved 450-nm band observed for free flavins in non-polar solvents (19) and similar to the spectrum of MSOX (20) and *R. gracilis* DAAO (21).

GO shows a marked dependence of the absorption spectrum of the oxidized form on the pH value. When the pH value of a solution is shifted from 8.0 to high values (>11) a hypochromic shift is observed for the near-UV band (from 376 to 350 nm), as well as a bathochromic shift of the 455-nm peak (data not shown). The spectral changes resemble those observed with free FAD upon ionization of the N(3)-H position (22). This is consistent with N(3)-H of bound FAD not being ionized at

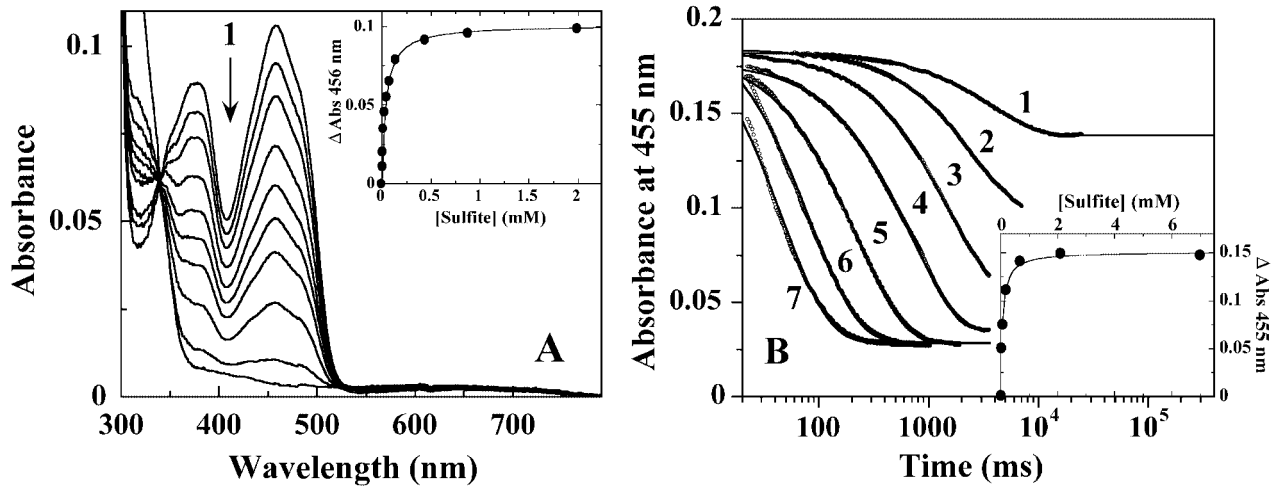


FIG. 3. **Binding of sulfite to glycine oxidase from *B. subtilis*.** *A*, spectrophotometric titration of GO. Curve (spectrum 1), 9.1 μM of GO in 50 mM sodium pyrophosphate, 10% glycerol, 2 mM EDTA, and 5 mM mercaptoethanol, pH 8.5, at 15 °C, with incremental concentrations of sulfite. Selected spectra (from top at 455 nm), obtained upon addition of 0.0045, 0.0094, 0.021, 0.033, 0052, 0.075, 0.132, 0.88, and 20.3 mM sulfite are shown. *Inset*, the observed decrease in absorbance at 456 nm is plotted as a function of the sulfite concentration. The *solid line* is a fit of the data to a theoretical binding curve (17) (K_d = 0.04 mM). *B*, time courses of stopped-flow absorbance traces at 456 nm observed upon mixing GO (14.3 μM in 50 mM potassium phosphate, pH 7.0, 2% glycerol) with (spectrum 1) 0.03, (spectrum 2) 0.07, (spectrum 3) 0.21, (spectrum 4) 0.7, (spectrum 5) 2.1, (spectrum 6) 7.0, and (spectrum 7) 21 mM sulfite (all final concentrations). The *solid lines* are a fit of the data to a single exponential expression. *Inset*, plot of the total change in absorbance at 455 nm as a function of the sulfite concentration.

physiological pH. The pK_a value of this flavin position was determined by plotting the absorbance at 385 nm against pH; it is increased compared with free FAD (10.6 ± 0.1 *versus* ~10.4) (22). GO undergoes denaturation at high pH values; it is fully denatured when brought to pH 12 and then changing the buffer by desalting on a PD10 column and bringing the pH to 7. Worthy of note is that a change in the absorbance spectrum is also evident when the pH value is shifted from 8.0 to a more acidic value. Unluckily, due to the low stability of GO at pH ≤6.5,[2] this ionization can not be followed over a pH range that is wide enough to determine the corresponding pK_a.

The flavin fluorescence emission of GO is rather weak in the oxidized state with $\lambda_{max(emiss)}$ ≈525 nm (Table I). Compared with flavin standards the relative fluorescence quantum yield of protein-bound FAD is less than 17% of free FAD.

Photoreduction and Stabilization of the Anionic Flavin Semiquinone—Typically, the anionic semiquinone stabilizes in this class of flavoprotein oxidases (23). GO is photoreduced in the presence of EDTA and 5-deazaflavin at 15 °C and pH 8.5 forming a red anionic semiquinone. Three isosbestic points are observed at 340, 411, and 506 nm while the semiquinone is being formed (Fig. 2). The amount of semiquinone form stabilized by GO at pH 7.0 and 8.5 (as determined by anaerobic photoreduction (11) until the spectrum of the flavin semiquinone (EFl_{seq}) reached a maximum) represents near-complete formation of EFl_{seq} (≈95%). When oxygen is added, essentially, complete re-oxidation is observed.

The anionic semiquinone species of GO is fully stable when kept in the dark but slowly disproportionates to the oxidized and reduced forms upon anaerobic addition of benzyl viologen, the end-product containing the final amount of thermodynamically stabilized semiquinone (11). After 24 h, when equilibrium was finally reached, ~40 and 24% of the photoreduced GO remained in the semiquinone form at pH 7.0 and 8.5, respectively. These results are compatible with a kinetic stabilization of the (red) anionic flavin semiquinone as also observed with other flavoprotein oxidases (23).

Spectral Properties of the Reduced GO—Anaerobic addition of an excess of glycine resulted in instantaneous flavin enzyme reduction, yielding a spectrum like that of the reduced flavin, with a maximum at 346 nm (line 3 in Fig. 1*A*). The anaerobic reduction demonstrates that GO is competent in catalysis. An anaerobic titration of GO with glycine is depicted in Fig. 1*B*. The intercept of the initial slope of the titration curve with the maximal observed change at 455 nm (*inset* of Fig. 1*B*) indicates a stoichiometry ~1 for the reaction with the substrate (19.0 nmol of enzyme and 19.5 nmol of substrate). In the reduced state, the fluorescence emission at 530 nm (excitation at 450 nm) is essentially lost.

The Reaction with Sulfite—The ability to bind sulfite and to form reversible covalent N(5) adducts distinguishes oxidases from other classes of flavoproteins (23, 24). It is generally assumed that in this class of flavoenzymes a positive (partial) charge near the flavin N(1)-C(2)=O locus inductively promotes the process (23). GO reversibly forms flavin N(5)-sulfite adducts, which essentially bleaches the oxidized flavin spectrum (Fig. 3*A*). The GO complex is quite stable; the K_d values obtained for GO at pH 7.0 and 8.5 (13 and 41 μM, see *inset* of Fig. 3*A*) are in the micromolar range, analogously to many oxidases (24).

The reversibility of sulfite binding was assessed by measuring the time-dependent recovery of the flavin absorbance spectrum of the oxidized enzyme upon filtration over Sephadex G-25 (k_{off} ≈0.04 s^{-1} at pH 8.5). At pH 7.0, the reaction is very fast, and thus the value determined ($k_{off} \geq 0.1$ s^{-1}) gives the lower limit. The reaction with sulfite was thus followed by means of stopped-flow spectroscopy by mixing a fixed amount of GO with different concentrations of sulfite. As shown in Fig. 3*B*, with GO the adduct formation depends monophasically on the sulfite concentration and yields a spectrum that is typical of the flavin N(5)-sulfite adduct only when a high, saturating concentration of sulfite is used (Fig. 3) (24). Binding is a second-order process, and the plot of k_{obs} *versus* sulfite concentration is linear (not shown); values for the rates of complex formation (k_{on} = 1660 M^{-1} s^{-1}) and dissociation (k_{off} = 0.20 s^{-1}) were estimated from the slope and *y*-intercept, respectively. The latter value is compatible with that obtained by following the reappearance of the oxidized flavin spectrum upon removal of excess sulfite by gel filtration. The K_d value calculated for the GO complex based on the rate constants observed ($K_d = k_{off}/k_{on}$ = 0.12 mM) is in fairly good agreement with that (K_d = 67 μM) determined from the 455 nm absorbance change as a function

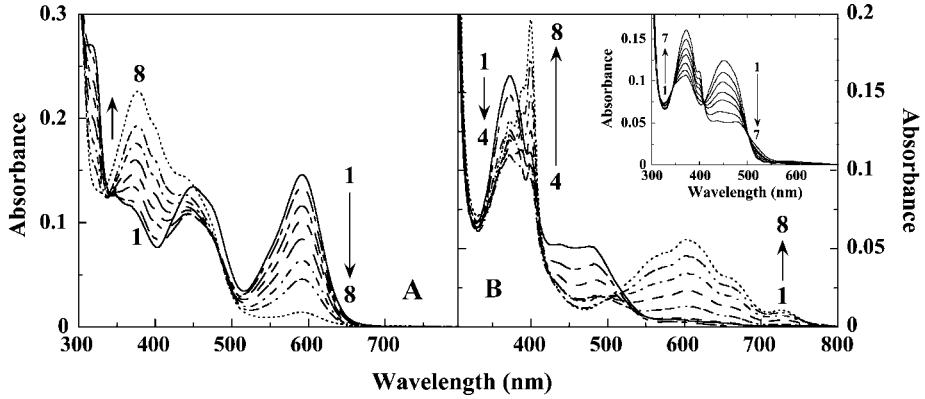


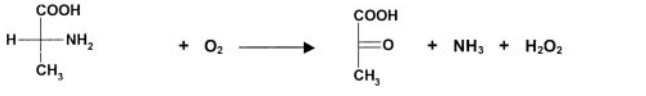
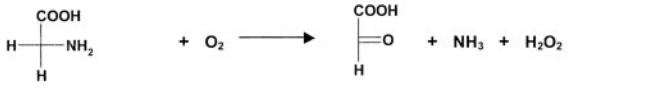
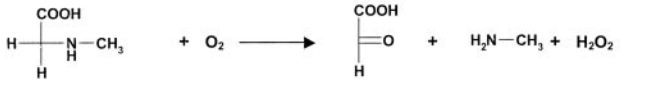
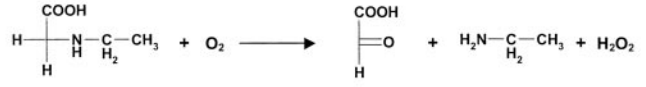
FIG. 4. **Redox potential determination of glycine oxidase from *B. subtilis* at pH 7.0.** *A*, selected spectra obtained during the course of the anaerobic reduction of 9.8 μM GO in 50 mM potassium phosphate and 10% glycerol, pH 7.0, using 200 μM xanthine, 5 μM indigotetrasulphonate (E_m = −24.3 mV), 10 μM methyl viologen and 30 nM xanthine oxidase. The spectra were recorded before (—) and 1 h (– – –), 2 h (– – –), 3 h (- - -), 3 h, 40 min (- -), 4 h, 20 min (– · –), 5 h (– · · –), and 18 h (· · · ·) after mixing with xanthine oxidase. *B*, selected spectra obtained during the course of the anaerobic reduction of 9.8 μM GO in 50 mM potassium phosphate and 10% glycerol, pH 7.0, using 200 μM xanthine, 5 μM benzyl viologen (E_m = −359 mV), 10 μM methyl viologen and 30 nM xanthine oxidase. *Main graph*, conversion of EFl_{seq} of GO to fully reduced form. The spectra were recorded 43 min (—), 55 min (– - –), 65 min (– – –), 80 min (- - -), 90 min (5), 105 min (– · –), 120 min (– · · –), and 150 min (· · · ·) after mixing with xanthine oxidase. *Inset*, time course of the first half of reaction: absorbance spectra of oxidized enzyme recorded before (spectrum 1) and 12 min (spectrum 2), 20 min (spectrum 3), 25 min (spectrum 4), 30 min (spectrum 5), 35 min (spectrum 6), and 43 min (spectrum 7) after mixing with xanthine oxidase.

of sulfite concentration (Fig. 3*B*, *inset*). In contrast, a ~10-fold difference between the K_d values determined by the static spectrophotometric titration and the kinetic measurements is evident (this discrepancy may be ascribed to the 10 °C difference in temperature used in the two determinations).

Determination of the Redox Potentials—The method described in Ref. 16 was used to determine the midpoint redox potentials for the transfer of electrons to the flavin at 15 °C and pH 7.0. The separation of the two redox potentials was determined from the maximal percentage of the semiquinone reached during the reduction using methyl viologen (E_m = −440 mV) as dye. When benzyl viologen was used instead of methyl viologen, the broad absorbance band at ~600 nm, corresponding to the conversion of oxidized benzyl viologen to the reduced form, appeared before the semiquinone form of the enzyme was completely reduced (see below), *i.e.* the potential for transfer of the second electron to the enzyme is close to that of the dye. For this reason, all the subsequent experiments were carried out using methyl viologen as the final acceptor. Thus, when the xanthine oxidase-mediated reduction of GO was monitored in the absence of a reference dye, the percentage of semiquinone formed during the reduction is ≈95%, *i.e.* the potentials for transfer of each single electron are quite far from each other. The separation between the potentials (25) is ≥200 mV.

The redox potential for the transfer of each single electron was determined using various dyes with different redox potentials. In Fig. 4*A* the reduction of GO at pH 7.0 in the presence of 5 μM indigotetrasulphonate as mediator is shown. The absorbance decrease at 590 nm (corresponding to the wavelength of maximal change during the conversion from the oxidized to the reduced form of the dye) parallels the oxidized-semiquinone conversion of the enzyme (decrease at 455 nm and increase at 366 nm, an absorbance maximum for the EFl_{seq} form). From these data and from the known extinction coefficients of the oxidized, semiquinone, and reduced forms of GO (Table I), log (EFl_{ox}/EFl_{seq}) for GO was plotted against log (ox/red) for the dye according to (15). From this the ΔE_m between the dye and the enzyme was calculated and a E^o of −41.9 mV was estimated (25). The redox potential for the transfer of the second electron to GO at pH 7.0, E_2^o, was similarly determined using benzyl viologen (E_m = −359 mV) as dye. During the first half of the reaction (0–50 min) the oxidized enzyme was fully converted to the semiquinone form, with no reduction of the dye (Fig. 4*B*, *inset*). During this conversion three isosbestic points at 504, 405, and 350 nm were evident. Subsequently, the EFl_{seq} converted to the reduced enzyme form, and the absorbance band centered around 600 nm, corresponding to the formation of the reduced form of the dye, appeared (50–110 min) (Fig. 4*B*). From the plot of the log (EFl_{seq}/EFl_{red}) for GO *versus* log (ox/red) for the dye, an E_2^o = −340 mV was determined. GO possesses at pH 7.0 a midpoint redox potential, E_m, of −298.1 mV. The ~300 mV separation between the two 1-electron potentials is in accordance with the high percentage of the anionic semiquinone enzyme form stabilized by photoreduction.

TABLE II
Summary of the reactions catalyzed by glycine oxidase on D-alanine, glycine, sarcosine, and N-ethylglycine

Substrates	Products
 D-alanine	Pyruvate
 Glycine	Glyoxylate
 Sarcosine	Glyoxylate
 N-ethylglycine	Glyoxylate

Products of the Reaction Catalyzed by GO—GO is a new flavoenzyme that can use various compounds as substrate (2)[2]; its reactions have not yet been studied in detail. Following the addition of GO to a solution containing D-alanine, glycine, sarcosine, or *N*-ethylglycine in the chamber of a Rank type oxymeter, a rapid decrease in the oxygen concentration was

observed. The rate of O_2 consumption is the function of substrate and GO concentration in the assay mixture. Addition of catalase halved the slope of O_2 consumption, indicating that H_2O_2 is the product of oxygen reduction. This was also confirmed by the spectrophotometric assay coupled with horseradish peroxidase and using both *o*-dianisidine or 4-aminoantipyrine as acceptor. The production of α-keto acids during the reaction of GO was determined by a reaction with 2,4-dinitrophenylhydrazine. With all the substrates tested, the production of α-keto acids was evident, although comparison of the absorbance spectra of the phenylhydrazone derivatives with those obtained using pure pyruvate and glyoxylate shows that pyruvate is the product of the reaction of GO with D-alanine (its spectrum shows a shoulder at ~500–520 nm), while glyoxylate is produced when sarcosine, glycine, and *N*-ethylglycine are used as substrates (data not shown). Using the glutamate dehydrogenase-coupled assay, a reduction of NADH was evident only when D-alanine and glycine were used as substrate, whereas no change in absorbance was observed using sarcosine and *N*-ethylglycine, *i.e.* ammonia was a product of the GO reaction only when the former two amino acids were used as substrates. Conversely, the oxidation of sarcosine and *N*-ethylglycine yields a primary amine, which is not a substrate of the coupling enzyme glutamate dehydrogenase. Finally, the formaldehyde dehydrogenase-coupled assay did not reveal any formaldehyde production for any of the substrates tested (different to that observed in control experiments in which the formaldehyde dehydrogenase reaction was coupled to monomeric or tetrameric sarcosine oxidase).

TABLE III

Comparison of the activity values determined for glycine oxidase using different substrates and different assays

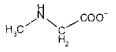
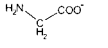
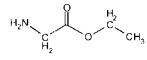
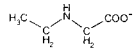
The activity, expressed as units/ml, was measured using the O_2 consumption (oxymeter assay), the α-keto acid production (phenylhydrazine assay), and the hydrogen peroxide production (H_2O_2) spectrophotometric assay. The activities were measured at 25°C in 75 mM potassium pyrophosphate, pH 8.5.

Substrate	Oxymeter assay	Phenylhydrazine assay	H_2O_2 spectrophotometric assay	
			o-DNS	4-AAP
Sarcosine	0.564	0.526	0.538	0.519
Glycine	0.490	0.433	0.428	0.490
D-Alanine	0.085	0.074	0.078	0.101
n-Ethyglycine	0.467	0.458	0.549	0.564

TABLE IV

Comparison of the apparent kinetic parameters of glycine oxidase using different substrates and assays

The kinetic parameters were determined at 25°C, air saturation, and pH 8.5, using the following assays: (1) O_2 consumption (oxymeter assay); (2) spectrophotometric assay using *o*-DNS; (3) spectophotometric assay using 4-AP.

Substrate		Assay	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m ($mM^{-1}\ min^{-1}$)
Sarcosine		(1)	35.8 ± 0.7	0.7 ± 0.1	
Sarcosine		(2)	49.7 ± 5.7	0.8 ± 0.1	
Sarcosine		(3)	41.3 ± 1.9	0.6 ± 0.1	
			42.3 ± 7.4	0.7 ± 0.1	60.4
Glycine		(1)	36.4 ± 0.2	0.7 ± 0.1	52.0
Ethylglycinate		(1)	12.8 ± 0.7	2.5 ± 0.6	5.1
N-ethylglycine		(1)	15.1 ± 0.5	2.1 ± 0.3	7.2
D-alanine		(1)	54.8 ± 1.5	490 ± 40	0.11
D-proline		(1)	29.7 ± 0.3	16.9 ± 0.8	1.8

A summary of the reaction catalyzed by GO on the different substrate tested is reported in Table II. From this we conclude that GO is a deaminating oxidase, analogous to DAAO and DASPO, and differs from the sarcosine oxidases that catalyze demethylation of the amino acids (such as sarcosine and proline) used as substrates (7). According to the determined reaction catalyzed by GO, we propose classifying it as a member of the EC 1.4.3 class (enzymes acting on the CH-NH_2 group of the donor and with a oxygen as acceptor) instead of the EC 1.5.3 class, as currently reported in the Swiss-Prot Database.

A fairly good agreement in the estimated activity values is observed with all the various assays and using different substrates (Table III). A comparison of the kinetic parameters obtained with sarcosine as substrate using the different assays is reported in Table IV; the data obtained agree reasonably well with all assays used. The apparent kinetic parameters V_{max} and K_m for GO on a number of compounds are also listed in Table IV. The preference of GO for glycine and sarcosine (a similar V_{max}/K_m ratio is shown by GO with these two substrates) is related not only to the rate of substrate conversion but also to substrate affinity, *e.g.* GO shows high K_m values on D-alanine and D-proline, which are also substrates of D-amino acid oxidase. As also confirmed by the binding data (see below), the presence of a carboxylic group in the substrate is not mandatory for it to be converted by GO (the V_{max} and K_m catalytic parameters on ethylglycinate and *N*-ethylglycine are

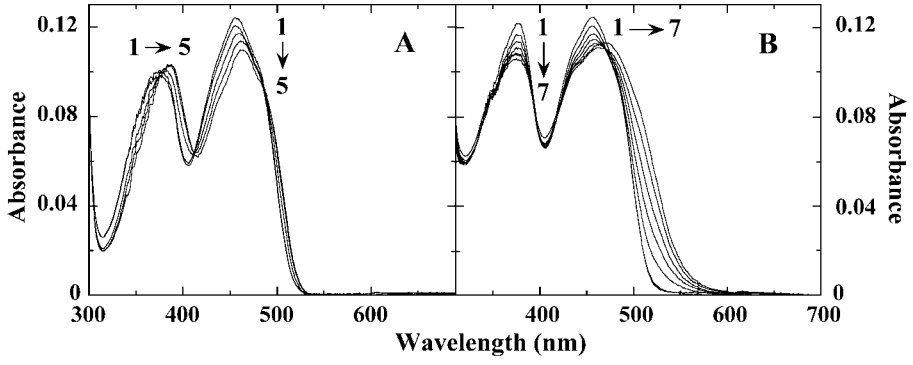
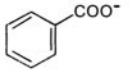
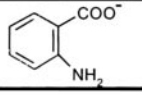
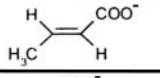
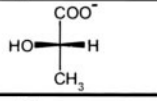
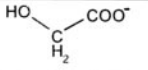
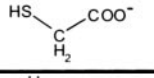
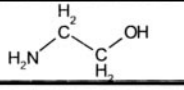
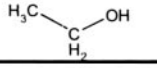
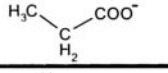
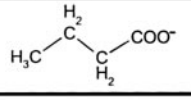
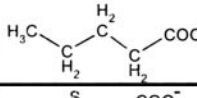
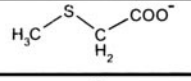
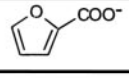
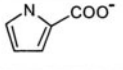
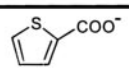
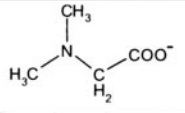


FIG. 5. *A*, effect of sodium butyrate binding on the absorbance spectrum of GO at 15 °C. 10.5 μM GO in 50 mM sodium pyrophosphate buffer, pH 8.5, containing 10% glycerol before (spectrum 1) and after the addition of 0.03 mM (spectrum 2), 62 mM (spectrum 3), and 100 mM (spectrum 4), and 148 mM (spectrum 5) butyrate. *B*, effect of sodium methylthioacetate binding on the absorbance spectrum of GO at 15 °C. 10.5 μM GO in 50 mM sodium pyrophosphate buffer, pH 8.5, containing 10% glycerol before (spectrum 1) and after the addition of 1 mM (spectrum 2), 7.8 mM (spectrum 3), 21.6 mM (spectrum 4), 44 mM (spectrum 5), 78 mM (spectrum 6), and 141 mM (spectrum 7) methylthioacetate.

TABLE V

Binding of various aromatic and aliphatic compounds to glycine oxidase

All measurements were made in 50 mM sodium pyrophosphate buffer, 10% glycerol, 2 mM EDTA and 5 mM 2-mercaptoethanol, pH 8.5 at 15°C. Complex formation was estimated on the basis of the perturbation of the visible absorption spectrum of the enzyme. The dissociation constant was calculated according to the method described in Ref. 17. The compounds indicated as "substrate" bind to GO and gives the reduction of the flavin coenzyme under anaerobic conditions. The values determined for the tetrameric sarcosine oxidase are in parentheses (28).

Compound		K_d (mM)			
		GO	DAAO[a]	MSOX[b]	MTOX[c]
Benzoate		15.8	0.9	61.5	37.2
Anthranilate		no effect	1.9		11.8
Crotonate		18.9	0.4		
L-lactate		No effect	16.2		
Glycolate		0.6		183	
Thioglycolate		substrate		7.7	
Ethanolamine		substrate			
Ethanol		no effect			
Acetate		3.7 and 58		(0.72)	
Propionate		135			> 500
Butyrate		170	no effect	118	
Valerate		235			
Methylthioacetate		6.9		2.6	32.3
2- Furoate		55		29 (0.34)	
Pyrrole-2-carboxylate		17		1.4 (0.38)	25.1
Thiophene-2-carboxylate		11		20.2 (3.3)	
N,N-Dimethylglycine		no effect		17.4	32.4

[a] See Ref. 26.
[b] See Ref. 3.
[c] See Ref. 32.

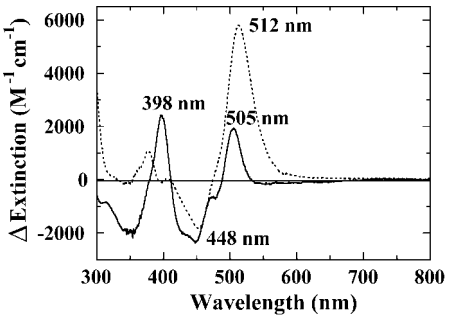


FIG. 6. **Difference spectra for binding of butyrate (—) and methylthioacetate (· · · ·) to glycine oxidase.** The difference spectra were obtained by subtraction of the absorbance spectrum of the free oxidized form of GO to the spectrum of the same enzyme after addition of 270 mM and 200 mM sodium butyrate and methylthioacetate, respectively at 15 °C (as determined in Fig. 5).

quite similar, see Table IV).

Identification of Active Site Ligands—To gain insight in the binding/recognition ability of this new flavoenzyme, we determined the dissociation constants of GO for a variety of aromatic and aliphatic compounds. These ligands were based on their ability to perturb the visible spectrum of the FAD upon formation of the bound complex. Thus, we investigated the binding of benzoate, anthranilate, crotonate, and L-lactate, which are known to be competitive inhibitors of yeast DAAO (26, 27). The binding of benzoate and crotonate is 1–2 orders of magnitude weaker than in yeast DAAO (the complex dissociation constant, K_d, was determined by fitting absorbance changes at the wavelength at which the change is maximal to a theoretical binding curve (17), see Table V). With anthranilate no evidence of binding was obtained up to a 800-mM concentration. Other compounds reported to bind sarcosine oxidase were also tested (28); glycolate binds GO most tightly (K_d <1 mM). Steady-state kinetic studies with glycolate indicate that this compound is a competitive inhibitor with respect to sarcosine; the double reciprocal plots of the reaction rate as the function of substrate concentration obtained at different inhibitor concentrations gave a pattern of lines converging at the same value on the ordinate (data not shown). The inhibition constant of K_i = 0.37 mM observed is in good agreement with the complex dissociation constant determined by spectral titration under the same conditions (Table V). Interestingly, the analogue thioglycolate, in which the OH-function has been substituted by a SH group, reduces the FAD coenzyme under similar experimental conditions, *i.e.* it is a substrate of GO. Thioglycolate is also able to reduce MSOX, in that a case yielding a 1-electron-reduced form, however (29). A similar result was obtained using ethanolamine; GO is fully reduced 4 days after the anaerobic addition of 250 mM of ethanolamine (data not shown). It is noteworthy that the tightest binding is observed with glycolate, a compound similar to the substrate glycine, in which the α-amino group is substituted by the α-OH group. The substitution of the α-amino group with a –SH group (*e.g.* thioglycolate, Table V) still maintains the activity. Following the loss of the –COOH group in ethanol, the ability to bind GO is abolished in comparison to glycolate. In contrast, acetate is still able to bind GO even if two binding constants (3.7 mM and 58 mM) are evident. A decrease in binding affinity is observed as the number of methylene groups increase in the aliphatic carboxylic acids used (compare the K_d values determined using C-2, C-3, C-4, and C-5 carboxylic acids, Table V). All the aromatic compounds tested show K_d values in the same order of magnitude (11–55 mM).

Concerning the spectral changes accompanying the binding, all the compounds reported in Table V, the only exception being methylthioacetate, show the spectral perturbations resembling those observed for DAAO with benzoate, yielding the classical resolution of the flavin spectrum; a shoulder at 500 nm appears, and the 456-nm peak shifts to 460 nm (Fig. 5*A* with butyrate). All the difference spectra show a prominent positive peak at ~500 nm and at ~400 nm, accompanied by two negative peaks in the ~450 and ~340 nm regions (see Fig. 6 with butyrate). On the other hand, with methylthioacetic acid completely different spectral changes are evident; the bathochromic shift of the ~460 nm peak to 472 nm is not accompanied by the bathochromic shift of the flavin peak at ~380 nm (Fig. 5*B*). The spectrum obtained at a saturating concentration of methylthioacetate significantly differs from that obtained with butyrate (compare spectra in Fig. 6); a prominent, positive peak at 512 nm is evident, accompanied by a less intense, positive peak at 380 nm and by a negative peak at 455 nm.

Charge-transfer complexes are formed by DAAO with anthranilate (30) and by MTOX with sarcosine analogues where the amino group is replaced by various group VI elements (31), as demonstrated by the appearance of a broad absorbance band centered at ~650 nm. Using GO and the compounds we tested, a true charge-transfer band was never well resolved.

DISCUSSION

The results reported in the present work indicate that the recombinant GO from *B. subtilis* is a flavoenzyme containing 1 mol of a non-covalently bound FAD/45-kDa protein monomer. The observation that adding exogenous FAD does not modify the activity of GO indicates a strong binding of the flavin to the apoprotein. It also shares some of the typical properties of the class of flavoprotein oxidases, namely the ability to form a reversible flavin-adduct with sulfite and the (partial) thermodynamic stabilization of the red anionic semiquinone upon photoreduction (23). The amount of flavin anionic semiquinone stabilized by GO during photoreduction represents near-complete formation of this form (~95%). Its (in)stability in the presence of benzyl viologen points to a kinetic stabilization of the EFl_{seq} form. The K_d for the binding of sulfite to GO is in the range usually observed with flavoprotein oxidases (≈10–40 μM). The midpoint redox potential determined with GO (−298 mV) is close to that of free FAD (−207 mV FAD), and similar to that of other flavoenzymes in the oxidase family (32). This result confirms a strict correspondence between a higher stability of the red, anionic flavin semiquinone and a tighter binding of sulfite (as a general rule, the nucleophilic attack to the N(5) position of FAD by sulfite is facilitated by the localization and stabilization of a negative charge on the flavin N(1)-C(2)=O locus) (24). Recently, Khanna and Schuman Jorns (31) reported that, using lactate oxidase, glucose oxidase, cholesterol oxidase, α-glycerophosphate oxidase, and FAD a linear relationship is observed when log k_{on} is plotted against log K_d (slope −0.95). The values determined for GO match this relationship nicely and can be similarly explained by the fact that the observed variation in K_d is largely due to changes in k_{on} (in all the flavoproteins tested, the only exception being MTOX, a similar rate of complex dissociation, k_{off} = 0.2–0.02 s^{-1}, was determined).

The pK_a value for the deprotonation of the flavin N(3)-H position was determined from the pH dependence of the visible spectrum of oxidized enzyme. For GO, a pK_a ≈10.6 was determined, a value higher than the corresponding value for free FAD (10.0) and for mammalian DAAO (9.4) (22) but similar to that determined for yeast DAAO (33). This change can be interpreted by assuming that different microenvironments affect the properties of the N(1)-C(2)=O and N(3)-H flavin positions, and which could be proven if the structure of GO (currently under solution) was compared with that of the known

crystal structure of *R. gracilis* (27) and mammalian DAAO (34, 35). In fact, the three-dimensional structure of RgDAAO demonstrates that a net negative charge close to the N(3)-H position is not present (27), as otherwise observed for many other flavooxidases (36).

GO catalyzes the deaminative oxidation of a wide range of amino acids (see also Ref. 2)[2] to form the corresponding α-keto acid. GO distinguishes from MSOX and MTOX since it catalyzes the deamination of amino acids (MSOX and MTOX catalyze the oxidative demethylation), shows a high pK_a for flavin N(3)-H ionization, and reacts easily with sulfite (both MSOX and MTOX show an apparent kinetic barrier against sulfite complex formation). For all these properties GO resembles DAAO, although the sequence homology is lower with DAAO and DASPO than with SOX and MTOX.[2] The DAAO active site must clearly be larger than that of GO to accommodate the bulky side chains of its substrates (all the neutral and basic D-amino acids). The key role of bulky side chains of the active site residues of GO may be to promote the proper positioning of small-sized substrates in the active site with respect to the N(5) flavin position. Even the non-covalent link of the FAD co-factor is a characteristic that combines GO, DAAO, and DASPO and represents a main difference to MSOX, MTOX, PIPOX, SDH, and DMGDH. In fact, the conserved histidine residue (His-45) in MSOX, MTOX, and PIPOX aligns with the histidine that serves as the covalent FAD attachment site in SDH and DMGDH, although it is not the site of flavinylation (which is Cys-308 and Cys-319 in MSOX and PIPOX, respectively) (37). In GO such a histidine is not conserved (Ala-46 is present).

In this study we have also identified a number of active site ligands that are useful in ongoing kinetic and crystallographic studies. The tightest binding is observed with glycolate (K_d = 0.6 mM), a compound closely similar to the substrate glycine, which also acts as a competitive inhibitor with respect to sarcosine. These results are consistent with the proposal that the enzyme has a single binding site for substrate and ligand near the non-covalently bound FAD coenzyme (ligand binding causes the perturbation of the visible absorption spectrum of the flavin). Analysis of the binding data (Table V) and the kinetic parameters (Table IV) suggests that the GO active site preferentially accommodates amines of small size such as glycine and sarcosine. The high K_m values determined for D-alanine and the inability to bind dimethylglycine indicates a steric hindrance in the binding of compounds with a tetrahedric center or with three substituents larger than an H atom. The presence of a carboxylic group is not mandatory for binding and catalysis, as demonstrated by comparison of the V_{max} and K_m values determined with ethylglycinate and *N*-ethylglycine. Analogously, the amino group is not mandatory for the binding and for the activity (in thioglycolate the amino group of glycolate is replaced by a –SH group). Analysis of the binding data for linear aliphatic acids suggests that each methylene group contributes very little to binding energy (0.2–0.4 kcal/mol). Interestingly, no compounds have been found that bind GO and give spectral perturbations resembling those of charge-transfer complexes.

Expression and purification of relatively large amounts of recombinant GO from *E. coli* cells and the characterization of its flavin and substrate specificity will enable us to address the catalytic mechanism and structure-function relationships of this novel oxidase in further detail in the near future. Finally, the similarities in kinetic properties of GO, DAAO, and DASPO paired with the differences in their primary structures and overall biochemical behavior reported here suggest a case of converging evolution.

REFERENCES

1. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G. *et al. (1997) Nature* **390,** 249–256
2. Nishiya, Y., and Imanaka, T. (1998) *FEBS Lett.* **438,** 263–266
3. Wagner, M. A., Trickey, P., Chen, Z., Mathews, F. S., and Schuman Jorns, M. (2000) *Biochemistry* **39,** 8813–8824
4. Pilone, M. S. (2000) *Cell. Mol. Life Sci.* **57,** 1732–1747
5. Wagner, M. A., Kanna, P., and Schuman Jorns, M. (1999) *Biochemistry* **38,** 5588–5595
6. Mihalik, S., J., McGuinnes, M., and Watkins, P. A. (1991) *J. Biol. Chem.* **266,** 4822–4830
7. Wagner, M. A., and Schuman Jorns, M. (1997) *Arch. Biochem. Biophys.* **342,** 176–181
8. Cook, R. J., Misono, K. S., and Wagner, C. (1985) *J. Biol. Chem.* **260,** 12998–13002
9. Chlumsky, L. J., Zhang, L., Ramsey, A. J., and Schuman Jorns, M. (1993) *Biochemistry* **32,** 11132–11142
10. Mori, N., Sano, Y., Tani, Y., and Yamada, H. (1980) *Agric. Biol. Chem.* **44,** 1391–1397
11. Massey, V., and Hemmerich, P. (1978) *Biochemistry* **17,** 9–16
12. Sander, S. A., Williams, C. H., and Massey, V. (1999) *J. Biol. Chem.* **274,** 22289–22295
13. Whitby, L. G. (1953) *Biochem. J.* **54,** 437–442
14. Light, D. R., Walsh, C., and Marletta, A. (1980) *Anal. Biochem.* **109,** 87–93
15. Minnaert, K. (1965) *Biochim. Biophys. Acta* **110,** 42–56
16. Massey, V. (1991) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., and Zanetti, G., eds) pp. 59–66, Walter de Gruyter and Co., Berlin
17. Strickland, S., Palmer, G., and Massey, V. (1975) *J. Biol. Chem.* **250,** 4048–4052
18. Nagata, Y., Shimojo, T., and Akino, T. (1988) *Int. J. Biochem.* **20,** 1235–1238
19. Harbury, H. A., LaNoue, K. F., Loach, P. A., and Amick, R. (1959) *Proc. Natl. Acad. Sci. U. S. A.* **45,** 1708–1717
20. Willie, A., Edmondson, D. E., and Schuman Jorns, M. (1996) *Biochemistry* **35,** 5292–5299
21. Pilone Simonetta, M., Pollegioni, L., Casalin, P., Curti, B., and Ronchi, S. (1989) *Eur. J. Biochem.* **180,** 199–204
22. Massey, V., and Ganther, H. (1965) *Biochemistry* **4,** 1161–1173
23. Massey, V., and Hemmerich., P. (1980) *Biochem. Soc. Trans.* **8,** 246–257
24. Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., and Foust, G. P. (1969) *J. Biol. Chem.* **244,** 3999–4006
25. Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Compounds,* pp. 184–203**,** Williams and Wilkins, New York
26. Pollegioni, L., Falbo, A., and Pilone, M. S. (1992) *Biochim. Biophys. Acta* **1120,** 11–16
27. Umhau, S., Pollegioni, L., Molla, G., Diederichs, K., Welte, W., Pilone, M. S., and Ghisla, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12463–12468
28. Zeller, H. D., Hille, R., and Schuman Jorns, M. (1989) *Biochemistry* **28,** 5145–5154
29. Wagner, M. A, and Schuman Jorns, M. (2000) *Biochemistry* **39,** 8825–8829
30. Curti, B., Ronchi, S., and Pilone Simonetta, M. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) pp. 69–94, CRC Press, Boca Raton, FL
31. Khanna, P., and Schuman Jorns, M. (2001) *Biochemistry* **40,** 1441–1450
32. Stankovich, M. T. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) pp. 401–422, CRC Press, Boca Raton, FL
33. Pollegioni, L., Ghisla, S., and Pilone, M. S. (1992) *Biochem. J.* **286,** 389–394
34. Mattevi, A., Vanoni, M. A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., and Curti, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7496–7501
35. Mizutani, H., Miyahara, I., Hirotsu, K., Nishina, Y., Shiga, K., Setoyama, C., and Miura, R. (1996) *J. Biochem.* (*Tokyo*) **120,** 14–17
36. Fraaije, M. W., and Mattevi, A. (2000) *Trends Biochem. Sci.* **25,** 126–132
37. Trickey, P., Wagner, M. A., Schuman Jorns, M., and Mathews, F. S. (1999) *Structure* **7,** 331–345