The Soluble α-Glycerophosphate Oxidase from Enterococcus casseliflavus

SEQUENCE HOMOLOGY WITH THE MEMBRANE-ASSOCIATED DEHYDROGENASE AND KINETIC ANALYSIS OF THE RECOMBINANT ENZYME

The Soluble flavoprotein α-glycerophosphate oxidase from Enterococcus casseliflavus catalyzes the oxidation of a “non-activated” secondary alcohol, in contrast to the flavin-dependent α-hydroxy- and α-amino acid oxidases. Surprisingly, the α-glycerophosphate oxidase sequence is 43% identical to that of the membrane-associated α-glycerophosphate dehydrogenase from Bacillus subtilis; only low levels of identity (17–22%) result from comparisons with other FAD-dependent oxidases. The recombinant α-glycerophosphate oxidase is fully active and stabilizes a flavin N(5)-sulfite adduct, but only small amounts of intermediate flavin semiquinone are observed during reductive titrations. Direct determination of the redox potential for the FAD/FADH2 couple yields a value of −118 mV; the protein environment raises the flavin potential by 100 mV in order to provide for a productive interaction with the reducing substrate. Steady-state kinetic analysis, using the enzyme-monitored turnover method, indicates that a ping-pong mechanism applies and also allows the determination of the corresponding kinetic constants. In addition, stopped-flow studies of the reductive half-reaction provide for the measurement of the dissociation constant for the enzyme α-glycerophosphate complex and the rate constant for reduction of the enzyme flavin. These and other results demonstrate that this enzyme offers a very promising paradigm for examining the protein determinants for flavin reactivity and mechanism in the energy-yielding metabolism of α-glycerophosphate.

α-Glycerophosphate (α-GP)1 oxidase is a soluble flavoprotein which catalyzes the oxidation of α-GP → DHAP with concomitant reduction of O2 → H2O2 (1, 2). In the heme- and cytochrome-deficient enterococci (3), the enzyme plays a key role in channeling glycerol into the glycolytic pathway. This FAD-dependent oxidase can be compared with FAD-containing α-amino acid oxidases (e.g. d-amino acid oxidase; Ref. 4) and FMN-dependent α-hydroxy acid oxidases (e.g. lactate oxidase; Ref. 5), as well as with those FAD-dependent oxidases which carry out the oxidations of “non-activated” substrates (e.g. glucose oxidase; Ref. 6). It is firmly established from studies of flavoprotein active-site environments by Massey Ghisla Hemmerich and co-workers (7–9) that, in general, flavoprotein oxidases give substantial stabilization to the blue p-quinoid form of 8-mercaptopflavin and the anionic benzoquinoid forms of other 6- and 8-substituted mercapto- and hydroxyflavins; these proteins also stabilize flavin N(5)-sulfite adducts as well as the anionic semiquinone and fully reduced forms of natural FAD or FMN. All of these properties can be ascribed to the general influence of a positive charge originating either with a specific side chain or helical dipole in the vicinity of the flavin N(1)-C(2)=O locus. In addition, the reaction leading to the formation of the N(5)-sulfite adduct has mechanistic relevance (7, 8) in that it parallels the nucleophilic attack of the substrate carbonyl group on the flavin carbonyl. We have shown that the active site environment of α-GP oxidase closely resembles those of other flavoprotein oxidases; this enzyme also stabilizes the blue p-quinoid form of 8-mercaptop-FAD and the anionic forms of natural FAD in both oxidized (pKα = 8.5 for N(3)-H) and fully reduced states (1).

α-GP oxidase catalyzes the FAD-dependent oxidation of an alcohol, similar to the reactions of glucose oxidase, methanol oxidase, and cholesterol oxidase (11); the pKα for the C(2)-H of α-GP is presumably ~40, reflecting the absence of electron withdrawing or “activating” substituents capable of stabilizing the corresponding substrate carbonyl. We have shown that reduction of α-GP oxidase by nitroethane anion is slow relative to the reduction of d-amino acid oxidase; this observation adds to the conclusion that a carbonyl mechanism is unlikely to apply for α-GP oxidase (1). And, while both radical and hydride transfer mechanisms have been discussed (11) for flavoprotein oxidases with “non-activated” substrates such as α-GP, we have shown that α-GP oxidase reconstituted with 5-deaza-FAD is reduced by α-GP (1); it appears that a hydride transfer mechanism similar to that employed by the cytosolic, pyridine nucleotide-dependent α-GP dehydrogenase (12) is most consistent with the data available for α-GP oxidase. The first direct evidence supporting a hydride transfer mechanism in a flavoprotein oxidase reaction came with the early kinetic studies of glucose oxidase by Bright, Gibson, and co-workers (13–15), and it appears likely that α-GP oxidase and glucose oxidase share similar mechanisms of substrate oxidation.

From the structural perspective, Mattevi et al. (4) have concluded that glucose oxidase and cholesterol oxidase constitute a subfamily of FAD-dependent oxidases distinct from that repre...
sent by d-amino acid oxidase, and this classification has been extended to suggest that other non-activated alcohol oxidases such as methanol oxidase are structurally and mechanistically related to the glucose oxidase/cholesterol oxidase subfamily (16, 17). With these considerations in mind, we present the primary structure of α-GP oxidase along with an analysis of the recombinant enzyme, including its redox properties and reactivity with sulfite. The steady-state kinetic mechanism and the reductive half-reaction have both been analyzed by stopped-flow techniques; in combination these results demonstrate that the rate-limiting step in catalysis is transfer of the hydride equivalent from α-GP to the enzyme flavin.

**EXPERIMENTAL PROCEDURES**

Materials and General Methods—Isopropyl-1-thio-β-D-galactopyranoside was purchased from 5 Prime → 3 Prime, Inc. and agarose was from FMC BioProducts. Both dL-α-GP and the t-isomer were purchased from Sigma, as were di-anisidine-2HCl, horseradish peroxidase (Type VI), and streptomycin sulfate. Indigo carmine (indigo disulfonate) was purchased from Sigma, and sodium sulfite was from Fisher. All other chemicals, as purchased from sources given previously (1, 19, 18), were of the best grades available.

**Biological Sources**—E. casseliflavus (ATCC 12755) and Escherichia coli XL1-Blue (Strategene) were maintained and cultured as described previously (20). Streptococcus pneumoniae (ATCC 27336) was grown with shaking at 37 °C in Brain Heart Infusion medium (Difco).

_α-GP_ Oligonucleotide Design and PCR Amplification—A crude lyophilized sample of _E. casseliflavus_ α-GP oxidase was obtained from the Bio-Products Division of Eastman Kodak Co. and purified essentially as described previously (1). The N-terminal sequence determined for the intact protein is: Thr-Phe-Glu-Xxx-Gln-Lys-Asp-Arg-Lys-Glu-Thr-Ile-Gln. One nmol of enzyme was then digested with lysozyme endoproteinase (Wako BioProducts), and an aliquot of the digest was chromatographed on a C8 reverse-phase high performance liquid chromatography column. Ten peptide fractions were collected and submitted for sequence analysis; one peptide (Gpo3) gave the amino acid sequence: Thr-Tyr-Phe-Gly-Thr-Xxx-Asp-Thr-Asp-Tyr-Thr-Gly-Gly-Phe-Ala-His-Pro-Thr-Val-Thr-Gln-Glu-Asp-Val-Asp-Tyr-Leu-Leu-Thr-Ile-Val-Szn. Oligonucleotide primers were then designed from the α-GP oxidase N-terminal (Gln5-Gln32) and Gpo3 peptide (Asp19-Asp38) sequences as follows, using enterococcal codon usage data (21).

**gp1**

5′-CAA AAA CAT GGI AAA GTA ACI ATT CA-3′

C

Gln-lys-Asp-Arg-lys-Glu-Thr-Ile-Gln

**gp3**

5′-TCAATCTTTGIAICCTGACCCTTCTAGAAATGTC-3′

C

Gly-Glu-Asp-Val-Asp-Tyr-Leu-Leu-Thr-Ile-Val-Asn-Glu. Oligonucleotide primers were then designed from the α-GP oxidase N-terminal (Gln5-Gln32) and Gpo3 peptide (Asp19-Asp38) sequences as follows, using enterococcal codon usage data (21), from one of these clones showed that it had a 1.4-kb insert, and this recombinant was designated pGLP01. From the restriction map of the _gplKO_ PCR product (20) and the size of the pGLP01 insert, we estimated that this recombinant would lack at least 650 bp of the _gplO_ coding sequence, corresponding to the C-terminal 40% of the polypeptide in _E. casseliflavus_ _gplO_ (21). XL1-Blue recombinants were screened by hybridization with the labeled _E. casseliflavus_ probe. From approximately 200 recombinant colonies screened, 3 were found to give positive signals. Restriction analysis of DNA isolated

**DNA Sequencing of gplO Clones—**The inserts from the two _E. casseliflavus_ _gplO_ clones, pGLP01 and pGP06, were subcloned into the plasmid pMOB for transposon-facilitated sequencing using TN1000 (20, 25). Overall, the entire _gplO_ locus on the two overlapping clones was sequenced on both strands. The two overlapping PCR products corresponded to the _E. pneumoniae_ _gplO_ locus were gel-purified and submitted for automated analysis by the DNA Sequencing and Gene Analysis Facility, Wake Forest University Medical Center. Contig assemblies, GAP alignments, and data base searches were carried out using the GCG suite of DNA analysis programs (26), in addition to the Fasta, Blast, and AutoAssembler programs (Perkin-Elmer).

**Expression and Purification of Recombinant α-GP Oxidase—**For expression of the _E. casseliflavus_ α-GP oxidase in _E. coli_, the vector used was a chloramphenicol resistance conferring derivative of _pBluescript II KS(−)_. This plasmid was prepared by ligating the 1.63-kb _HinfI_−_Accl_ fragment from pACYC184 with the 1.95-kb _BspHII_ fragment from pBluescript II _KS(+)_. After both fragments had been made blunt-ended by treatment with T4 DNA polymerase and _danTPPs_, the _EcoRI_ site within the _cat_ gene was removed as described by Parsonge et al. (18), and the resultant plasmid was designated pOX10. The sequence upstream of _gplO_ was replaced by a nearly identical copy (GAGGAG) of the ribosome-binding site of the highly expressed gene _10_ from the _T7_ phage (27), using PCR. The template used was the chromosomal clone pGLP01, and the primers were the _T3_ primer and the _gpo3_ primer, which has the sequence: 5′-AAAACCTCGAGGAGGATATACATGGACATCATTCTCTAGAAATGATC-3′. This entire insert was sequenced on both strands to confirm that no spurious mutations had been introduced by _Taq_ polymerase. A ligase reaction was then set up to include the PCR-generated _Xhol_−_PstI_ fragment, the 1.38-kb _PstI-BglII_ fragment from pGP06 containing the 3′-end of _gplO_, and _Xhol−_ _BamHI_ digested _pBluescript II KS(−)_, to generate pGPO15. The entire _gplO_ gene, with the engineered ribosome-binding site, was transferred to _pOX10_ as a _XhoI-SacI_ fragment and the expression plasmid pGPO16 was created.

**Expression of α-GP oxidase was carried out under the control of the pOX10 lac promoter.** XL1-Blue cells transformed with pGPO16 were grown at 30 °C in 10 2.8-liter Fernbach flasks, each containing 600 ml of LB medium, pH 7.4, plus 33 μg/ml chloramphenicol and 50 μ× _isopropyl-1-thio-β-D-galactopyranoside_. After 26 h of growth, cells were harvested and washed with 50 mm potassium phosphate, pH 7.0, plus 0.6 mm EDTA, and frozen at −70 °C. Cells were disrupted for enzyme purification by passage through an SLM/Amino French press. Cell debris was removed by centrifugation and nucleic acids were precipitated with 2.5% (v/v) streptomycin sulfate. The 45% saturated ammonium sulfate supernatant was dialyzed overnight against 0.1 m potassium phosphate, pH 7.0, plus 0.1 m KCl and 0.6 m EDTA, and loaded onto a 25-ml Q-Sepharose Fast Flow column (Pharmacia LKB Biotechnology, Inc.) equilibrated with the same buffer. _α-GP_ oxidase was eluted with a 0.1 to 0.3 m KCl gradient in the pH 7.0 phosphate buffer. Fractions with an _A280/A446_ ratio of less than 14 were pooled and diazylated against 10 m potassium phosphate, pH 7.0. Protein was applied to a 130-ml column of Meso-Prep Ceramic Hydroxyapatite (type 1, 40 μm; Bio-Rad) equilibrated with the dialysis buffer. The fraction corresponding to the 280/280 ratio of less than 7, and which were homogeneous by SDS-PAGE, were pooled, concentrated, and buffer exchanged into 50 m potassium phosphate, pH 7.0, plus 0.6 m EDTA by ultrafiltration. Purified α-GP oxidase was stored in aliquots at −20 °C.

**Static Titrations—**Static titrations and spectral measurements were...
performed using either a Hewlett-Packard Model 8451A or 8452A single-beam diode-array spectrophotometer, as described previously (19). $E_n$ for the $\alpha$-GP oxidase FAD/FADH$_2$ redox couple was determined by dithionite titration (19) in the presence of the reference dye indigo disulfonate ($E_n = -125$ mV; Ref. 28), with benzyl viologen present at low concentration to ensure rapid equilibration of reducing equivalents. 

**Stopped-flow Kinetics**—All rapid-reaction analyses were carried out with the Applied Photophysics DX.17MV stopped-flow spectrophotometer, which includes the Applied Photophysics photodiode-array detector as recently described (29). The reaction of sulfite with $\alpha$-GP oxidase was measured at 446 nm; 28 $\mu$s enzyme (final concentration) was reacted in 0.1 M potassium phosphate, pH 7.0, plus 0.3 mM EDTA, under aerobic conditions at 25 °C. Final sulfite concentrations ([HSO$_3^-$ + SO$_2^-$]) were 0.1, 0.5, 1, 4, and 8 mM; stock solutions were prepared in the same pH 7.0 phosphate buffer. The steady-state kinetic analysis for $\alpha$-GP oxidase was carried out at pH 7.0, 5 °C, using the enzyme-monitored turnover method as described by Gibson et al. (30). These measurements were made with a final concentration of 9 $\mu$m enzyme under [O$_2$]-limited conditions ([O$_2$] = 0.78 mM), where the dissolved [O$_2$] in buffer at 25 °C is taken as 0.26 mM under ambient conditions (31). The range of [L-$\alpha$-GPs] used was 5–50 mM; kinetic data were acquired at 446 nm and were analyzed as recommended by Cornish-Bowden (32). This method was applied previously in the kinetic characterization of the recombinant NADH peroxidase (18), where a detailed description is provided. A stoichiometric reduction of $\alpha$-GP oxidase by L-$\alpha$-GP was investigated in both single wavelength (446 nm) and diode-array detection modes. The stopped-flow system was prepared for anaerobic work following a modified protocol which has recently been described (29).

The contents of the enzyme tomometer and the glass syringes containing L-$\alpha$-GP were prepared for anaerobic work as described previously (33); in addition, both enzyme and substrate solutions contained the protecatechuate dioxygenase/protocteuchic acid oxygen-scruubing system (34). Enzyme (28.9 $\mu$m final concentration) and GPO were mixed at 5 °C in the 0.1 M phosphate, pH 7.0 buffer; the range of [L-$\alpha$-GPs] was 12.5–50 mM. Kinetic data from both the sulfite reaction and the reductive half-reaction were analyzed using the SX.18MV and Pro-Kinetist (Pro-K) packages from Applied Photophysics, as described recently (29).

**Protein Analyses—Electrospray interface-mass spectrometry (ESI-MS) and protein sequence analyses were provided by the Analytical Chemistry and Protein Analysis Core Laboratories, Comprehensive Cancer Center of Wake Forest University.**

**RESULTS**

**Nucleotide Sequence of the E. casseliflavus gplO Gene**—The composite sequence derived from the two overlapping clones (pGLPO1 and pGPO6) includes 2531 bp which accounts for the entire gplO coding sequence (1830 bp) as well as a partial open reading frame of 315 bp preceding gplO, which corresponds to the 3′-end of the gplK gene encoding glycerol kinase (20). An intergenic region of only 3 bp separates the gplK stop codon from the gplO start codon. A third coding sequence begins within the 3′-end of gplO, giving rise to a 387-bp ORF which is interrupted by the end of the pGPO6 insert. The sequence of this third overlapping ORF will be described in a separate article. Fig. 1 gives the 1862-bp sequence, including the gplO gene and the ribosome-binding site (AGAAGG) identified 16 bp upstream of the Met$^1$ codon. On the basis of the N-terminal protein sequence for $\alpha$-GP oxidase given previously (“Experimental Procedures”) the ATG codon corresponding to the start codon was recognized immediately, although the initiating Met is removed post-translationally in E. casseliflavus. gplO encodes a polypeptide of 609 amino acids with a calculated molecular weight of 67,174 (including Met$^1$), similar to the value of 65,000 determined previously by SDS-PAGE for the purified enzyme (1). The amino acid composition is in good agreement with that determined for the protein purified from E. casseliflavus (1), with the significant exception being the low content of cysteine residues, which were consistently under-represented in the chemical analysis (most likely due to incomplete hydrolysis even at 96 h), and Ser, which was overrepresented in the earlier report (36 versus 29 Ser/Thr). The $\alpha$-GP oxidase sequence also corroborates the absence of Cys and the low Trp content. There is perfect agreement with the N-terminal (Thr$^2$-Glu$^{13}$) and Gpo3 peptide sequences given earlier; the latter corresponds to the sequence Thr$^{295}$-Glu$^{327}$ in $\alpha$-GP oxidase. $\alpha$-GP Oxidase Is Homologous with the Membrane-associated $\alpha$-GP Dehydrogenases—Sequence and crystallographic analyses indicate that there are two classes of FAD-dependent oxidases, represented by $\beta$-amino acid oxidase (4) and by glucose oxidase (6) and cholesterol oxidase (16). Frederick et al. (35) initially identified three specific regions of 30–40 residues each which showed identity levels of ~50% between glucose oxidase and methanol oxidase; Vrielink and co-workers (16, 17) have extended the comparison for these two enzymes to include cholesterol oxidase, based on a structural alignment. Initially we anticipated that the $\alpha$-GP oxidase sequence would compare favorably with these proteins. However, a TFASTA search of the GenEMBL Data Bank failed to identify any other FAD-dependent oxidase sequences as related to $\alpha$-GP oxidase; individual GAP alignments with glucose oxidase, cholesterol oxidase, and $\beta$-amino acid oxidase gave identity levels of 17, 17, and 22%, respectively. In contrast, high scores were observed, surprisingly, for the membrane-associated flavoprotein $\alpha$-GP dehydrogenases from several bacterial and mitochondrial sources. GAP alignments reveal 43% identity between $\alpha$-GP oxidase and the $\alpha$-GP dehydrogenase from the Gram-positive Bacillus subtilis (36) and 30–32% for the $\alpha$-GP dehydrogenases from the Gram-negative E. coli (37) and Pseudomonas aeruginosa (38). The CLUSTAL (39) alignment given in Fig. 2 for $\alpha$-GP oxidase and the three bacterial $\alpha$-GP dehydrogenases indicates that conserved regions are generally distributed over the entire oxidase sequence, with the major exception being a 52-residue insert following Gly$^{355}$ (relative to the B. subtilis sequence) which has no counterpart in the dehydrogenases. Also indicated in Fig. 2 is the $\alpha$-GP oxidase segment Asp$^{21}$-Glu$^{49}$, which satisfies eight of the 11 requirements defined by Wierenga et al. (40) for the ADP-binding fold involved in FAD binding, and two short segments (Ser$^{342}$-Ile$^{350}$ and Gly$^{430}$-Ile$^{433}$) which have been suggested by Austin and Larson (37) to be involved in $\alpha$-GP binding with the E. coli dehydrogenase. Individual GAP alignments indicate identity levels of 33–34% for the $\alpha$-GP oxidase and the mitochondrial dehydrogenases from yeast (41, rat (42), and human (43) sources.

The TFASTA search with the E. casseliflavus $\alpha$-GP oxidase also identified the S. pneumoniae exp6 gene, a partial coding sequence described by Pearce et al. (22) in their genetic analysis of exported proteins in pneumococci. Sequence comparisons revealed an optimal 80-residue overlap between Exp6 and the B. subtilis $\alpha$-GP dehydrogenase, with 68% identity. In a subsequent study, Saluja and Weiser (23) cloned a genetic locus which is associated with the expression of colony opacity, and this locus also contains a partial ORF (ORF1) homologous to the B. subtilis dehydrogenase. While both the S. pneumoniae Exp6 and ORF1 sequences compare favorably with the E. casseliflavus $\alpha$-GP oxidase sequence, these analyses suggested that a single base was missing after position 285 of the published ORF1 sequence. We therefore cloned the gene encoding the S. pneumoniae $\alpha$-GP oxidase homolog by PCR, as described under “Experimental Procedures,” and the translated full-length sequence is given in Fig. 3. The pneumococcal sequence is 61% identical to the enterococcal $\alpha$-GP oxidase and includes both the Exp6 and ORF1 partial coding sequences; the full-length pneumococcal gplO sequence also includes an additional guanine base after position 285 of the ORF1 sequence, which changes the translated sequence for the published segment corresponding to amino acid residues 96–154 (23). Fig. 3 also gives the translated sequence for the $\alpha$-GP oxidase homolog identified in the Streptococcus pyogenes genome sequence data base (44); the two
streptococcal oxidase sequences are 76% identical. Recently Erlandson and Batt (45) have reported the sequence of a partial ORF from *Lactococcus lactis* subsp. *lactis* which is 42% identical to the *B. subtilis* α-GP dehydrogenase. The lactococcal ORF corresponds to the N-terminal 250 residues of the enterococcal α-GP oxidase, with 61% identity.

Catalytic, Spectral, and Redox Properties of Recombinant α-GP Oxidase—Since the *E. casseliflavus glpO* locus was cloned in two stages from two subgenomic libraries, we first reconstructed the intact coding sequence as described under "Experimental Procedures." Under standard T7 expression protocols (18), however, the pGPO15 construct gave only modest levels of α-GP oxidase protein (Fig. 4, lane 2). By using a different expression protocol with the pGPO16 plasmid ("Experimental Procedures"), which contains the *glpO* gene under control of the *lac* promoter, up to 11% of the soluble protein in recombinant extracts was shown to be fully active α-GP oxidase. This result is corroborated in the SDS-PAGE analysis given in Fig. 4 (lane 4). The recombinant enzyme was purified from 6 liters of XL1-Blue(pGPO16) cultures; up to 100 mg of the pure, soluble enzyme resulted with an average specific activity of 80.5 units/mg. N-terminal sequence analysis of the recombinant enzyme indicates that the initiating Met is also removed in *E. coli*, resulting in a calculated subunit *m* of 67,085 Da. Electrospray mass spectrometric analysis gives *m* 5 67,082 Da, in excellent agreement. The consistently higher specific activity for the recombinant enzyme is in agreement with the lower *A* 280/*A* 446 ratio of 6.1–6.4 (versus 7.0 for the enzyme from *E. casseliflavus*; specific activity 5 62 units/mg). The visible absorption spectrum of the purified recombinant enzyme (Fig. 5) is identical to that reported originally for the protein purified from *E. casseliflavus*. In the earlier report (1)

**Fig. 1.** DNA sequence of the *E. casseliflavus glpO* gene. The deduced protein sequence is also given. The positions of *PstI*, *HinDIII*, and *BglII* restriction sites are indicated, as are the sequences corresponding to a putative ribosome-binding site (RBS) and the *glpO* initiation codon.

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**Analysis of Recombinant Enterococcal α-Glycerophosphate Oxidase**

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we also described an intense blue fluorescence which developed on storing the pure enzyme at $-20$ °C for a few weeks. The fluorescent species was not identified, however, and the recombinant protein exhibits no such blue fluorescence, even after storage at $-20$ °C for up to 2 1⁄2 months. Analysis of the very weak flavin fluorescence indicates that the recombinant enzyme is only 3% as fluorescent as free FAD at the same concentration.

In parallel with their fundamentally different reactivities toward oxygen, flavoprotein oxidases and dehydrogenases also offer striking contrasts in their reactivities with sulfite (46). All oxidases stabilize flavin N(5)-sulfite adducts, but no sulfite reaction is observed with the dehydrogenases. Given the clear homology between $\alpha$-GP oxidase and the $\alpha$-GP dehydrogenases, how does the sulfite reactivity of the oxidase compare with that of other flavoprotein oxidases? As also shown in Fig. 5, titration of the recombinant oxidase with sulfite does lead to progressive bleaching of the oxidized flavin absorbance, attributed to the formation of a flavin N(5)-sulfite adduct with $K_d$ ($\text{HSO}_3^-$) of 0.81 mM at 25 °C. Still, completion of the absorbance changes on each addition was only observed after 10–12 min, indicating a relatively slow sulfite reaction. In order to extend this observation, and in order to confirm the $K_d$ value independently, the kinetics of the sulfite reaction with $\alpha$-GP oxidase were studied by stopped-flow spectrophotometry at pH 7.0, 25 °C. These results are also given in Fig. 5 and yield a second-order rate constant of $11.5 \text{ M}^{-1} \text{s}^{-1}$ for the reversible reaction; the $y$-axis intercept from the direct plot of $k_{\text{obs}}$ versus $[\text{HSO}_3^-]$ yields $k_{\text{off}} = 5.0 \text{ s}^{-1}$. Together these values give $K_d = 0.87$ mM for the $\alpha$-GP oxidase sulfite complex, in very good agreement with the determination from the static titration. These values can also be compared with $k_{\text{on}} = 6.2 \text{ M}^{-1} \text{s}^{-1}$ and $66 \text{ M}^{-1} \text{s}^{-1}$, respectively, for the sulfite reactions of cholesterol oxidase from Brevibacterium sterolicum ($K_d = 0.14$ mM at pH 7.5, 25 °C; Ref. 47) and glucose oxidase ($K_d = 0.11$ mM at pH 5.6, 25 °C; Ref. 35) but contrast sharply with $k_{\text{on}} = 1.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for lactate oxidase ($K_d = 0.5$ mM at pH 7.0, 25 °C; Ref. 5). While the formation of the sulfite adduct in each case is taken to reflect the presence of a positively charged locus of the respective protein which interacts with the pyrimidine ring of the flavin (46, 48), there is a substantial difference in the free energies of activation ($\Delta G^\ddagger$) of 4.6–6.0 kcal/mol favoring the reaction with lactate oxidase over those with $\alpha$-GP oxidase, glucose oxidase, and cholesterol oxidase.

A second general feature of flavoprotein oxidases, which has been taken as a hallmark for those oxidases operating via...
carbanion mechanisms (8), involves stabilization of the red anionic flavin radical on one-electron reduction. Fig. 6 gives the spectral courses for dithionite titrations of the recombinant α-GP oxidase at pH 7.0 (phosphate buffer) and pH 9.0 (Tris sulfate buffer). At pH 7.0, the titration proceeds in two distinct phases; the first phase is characterized by isosbestic points at 327, 404, and 495 nm and leads to the formation of a mixture of anionic and neutral semiquinones, as evidenced by increases in absorbance at 370 and 600 nm, respectively. Absorbance changes plotted versus equivalents of dithionite per FAD are linear at 362, 446, and 580 nm over the range of 0–0.5 mol of dithionite/FAD. Using the value of $\varepsilon_{610}$ for the blue semiquinone of glucose oxidase (49), we can estimate that about 17% neutral radical is present at $0.8 \text{ mol of dithionite/FAD}$ in the α-GP oxidase titration. Similarly, since $D\varepsilon_{366} = 5,200 \text{ M}^{-1} \text{cm}^{-1}$ for conversion of oxidized glucose oxidase to the red semiquinone form (49), we can estimate that perhaps 30% of the anionic α-GP oxidase radical is present in the mixture. The second phase of the titration at pH 7.0 leads to full reduction of the semiquinone and residual oxidized enzyme forms; the overall titration requires 1.5–1.6 equivalents of dithionite/FAD. This stoichiometry is identical to that reported earlier (1) for the enzyme purified from *E. casseliflavus*. It is also important to point out that under our experimental conditions at pH 7.0, complications could have potentially arisen due to the formation of the N(5)-sulfite complex, as documented in Fig. 5. However, when the dithionite-reduced enzyme at pH 7.0 (Fig. 6) was opened to air, 95% of the original oxidized enzyme spectrum returned rapidly, indicating that a maximum of 5% of α-GP oxidase could possibly have been present in the form of the sulfite

**Fig. 3.** CLUSTAL alignment comparing the α-GP oxidase sequences from *E. casseliflavus*, *S. pyogenes*, and *S. pneumoniae*.

**Fig. 4.** SDS-PAGE analysis of α-GP oxidase-expressing clones and the purified recombinant enzyme. A 10% SDS-polyacrylamide gel was run with samples treated as described previously (18). Lanes 1–4 contain crude extracts of *E. coli* JM109DE3(pBluescript), JM109DE3(pGPO15), XL1-Blue(pGPO16), and XL1-Blue(pGPO10). Lane 5 contains 1 μg of the recombinant enzyme, purified as described in the text. Lane M contains low range protein standards (Bio-Rad); the corresponding molecular masses (in kDa) are indicated.

**Fig. 5.** Sulfite reaction with recombinant α-GP oxidase: A, the enzyme (23 μM, in 50 mM potassium phosphate, pH 6.8, plus 0.6 mM EDTA) was titrated with sodium sulfite solutions of 0.1 and 1.0 mM. Spectra shown, in order of decreasing absorbance at 446 nm, correspond to the addition of 0 (—), 0.24 mM (—), 0.65 mM (—), 1.5 mM (—), and 23.9 mM (—) total sulfite (HSO$_3^-$ + SO$_3^{2-}$). B, the enzyme (28 μM final concentration) was reacted in the stopped-flow spectrophotometer at pH 7.0, 25°C, with sulfite; the range of [HSO$_3^-$ + SO$_3^{2-}$] was 0.1–8 mM, as indicated under “Experimental Procedures.”
The redox potential \( E' \) for the \( \alpha\)-GP/DHAP couple is \(-190 \) mV (28), and we initially attempted to determine the \( \alpha\)-GP oxidase FAD/FADH\(_2\) redox potential by dithionite titration in the presence of the reference dye anthraquinone-2,6-disulfonate \( E' \) \(-184 \) mV; Ref. 28. Enzyme reduction in this case was essentially complete before significant dye reduction was observed, however, indicating that the enzyme potential was much higher than \(-184 \) mV. This experiment was then repeated in the presence of indigo disulfonate \( E' \) \(-125 \) mV; Ref. 28. Reduction of the reference dye was monitored at 610 nm, where the enzyme has no absorbance. Enzyme reduction was followed at 452 nm, an isosbestic wavelength for oxidized and reduced forms of the dye. By calculating the concentrations of oxidized and reduced dye and enzyme at each titration point, the midpoint potential can be determined from the Nernst equation, given the known potential of the reference dye and the ratio of the concentrations of oxidized and reduced dye at the midpoint of enzyme reduction (50, 51). Following this procedure a redox potential of \(-118 \) mV was determined for the FAD of recombinant \( \alpha\)-GP oxidase; the plot of \( \log [E' \text{FAD}] / [E' \text{FADH}_2] \) versus \( \log [\text{dye}_{\text{red}}] / [\text{dye}_{\text{oxd}}] \) has a slope of 0.85.

**Enzyme-monitored Turnover**—The enzyme-monitored turnover method (30) was applied in order to determine the steady-state kinetic mechanism for recombinant \( \alpha\)-GP oxidase and the associated kinetic parameters. The stopped-flow traces given in Fig. 7 represent enzyme absorbance at 446 nm as \( 9 \) \( \mu \)m oxidase is reacted at \( 5 \) °C in \( 0.1 \) mM phosphate, pH 7.0, plus \( 0.6 \) mM EDTA with \( 0.78 \) mM O\(_2\) and varying concentrations of L-\( \alpha\)-GP (5–50 mM). Immediately after mixing, a steady state is established as determined by the relative rates of enzyme reduction and oxidation (30, 52); even at 50 mM L-\( \alpha\)-GP it appears that \( k_{\text{red}} \) at the steady state. The steady state persists until nearly all O\(_2\) is consumed, and the enzyme is rapidly reduced at this point. Analysis of these traces as described by Gibson et al. (30) gives rise to the plots of \([O_2]_0 / [O_2]_k\) shown in Fig. 8; as discussed by Cornish-Bowden (32), the observed intersection of these lines representing fixed concentrations of L-\( \alpha\)-GP on the y axis is consistent with a ping-pong mechanism. A secondary plot of \([l-\alpha\)-GP]/\( V_{\text{app}} \) versus \([l-\alpha\)-GP] gives \( V_{\text{max}} \) and \( K_m(l-\alpha\)-GP); at pH 7.0, \( 5 \) °C, \( K_m(l-\alpha\)-GP) = 24 mM, \( K_m(O_2) = 35 \) \( \mu \)M, and \( k_{\text{cat}} = 37 \) s\(^{-1}\).

**Reductive Half-reaction with L-\( \alpha\)-GP**—In order to determine the kinetic mechanism for reduction of \( \alpha\)-GP oxidase by substrate, the reaction was examined under anaerobic conditions at a series of [L-\( \alpha\)-GP],. Stopped-flow diode array analysis at 12.5 mM L-\( \alpha\)-GP failed to provide evidence for any spectral intermediates in the reaction, and direct reduction was observed over the wavelength range 320–500 nm. No charge transfer species was detected over the wavelength range 400–1000 nm, as might be expected for a reduced \( \alpha\)-GP oxidase-DHAP complex (5). When analyzed in single-wavelength mode
at 446 nm, however, reaction traces were clearly biphasic; the faster phase accounted for 65% of the total ΔA446 at 12.5 mM L-α-GP, but this increased to 87% of the total ΔA at 50 mM substrate (Fig. 9). Over this L-α-GP range the rate constant for the slow phase was essentially unchanged (3.6 s⁻¹ to 5.4 s⁻¹ at 5 °C); this rate constant is also significantly lower than the turnover number of 37 s⁻¹. In contrast, when the values for $k_{fast}$ are analyzed versus [L-α-GP] in a double-reciprocal plot (53), these data are consistent with the following two-step mechanism for enzyme reduction,

$$K_i \ E\text{-FAD} \ + \ L\text{-α-GP} \rightleftharpoons \ E\text{-FAD} \cdot \ L\text{-α-GP} \rightleftharpoons \ E\text{-FADH}_2 + \text{DHAP} \quad \text{(Eq. 1)}$$

where $K_i$([L-α-GP]) = 25 mM and $k_{red} = 48$ s⁻¹. These values are in very good agreement with $K_i$([L-α-GP]) = 24 mM and $k_{cat} = 37$ s⁻¹, respectively, as determined above.

**DISCUSSION**

The proteins involved in glycerol uptake and catabolism in the Gram-positive *Enterococcus* represent a system for salvaging the glycerol moiety derived from the breakdown of phospholipids and triglycerides (54); glycerol kinase and the α-GP oxidase act in concert to catalyze the net ATP-dependent conversion of glycerol to DHAP (20, 55). O₂ is reduced to H₂O₂ in the process, and the DHAP produced enters the glycolytic pathway. Functionally α-GP oxidase is similar to the membrane-associated α-GP dehydrogenases; in the latter case additional ATP is produced through oxidative phosphorylation as electrons are transferred from the reduced dehydrogenase to O₂ via the respiratory chain (56). The enterococci lack the ability to synthesize heme and thus lack the respiratory cytochromes (3); the soluble α-GP oxidase therefore allows for the aerobic growth of these organisms when glycerol is the sole carbon and energy source. The present work shows that the glpK and glpO genes in *E. casseliflavus* are very tightly linked as in an operon, which would allow for coordinate transcriptional regulation of these genes in the presence of different carbon sources. The capacity for aerobic conversion of glycerol to lactate is one of the distinguishing metabolic features of *Enterococcus*, when compared with the related genera *Streptococcus* and *Lactococcus* (57). Although *S. pneumoniae* is capable of slow acid production from glycerol under aerobic conditions, this organism has complex growth requirements and cannot grow with glycerol as its sole carbon and energy source (23). Similarly, the lactococci have not been reported to metabolize glycerol, either as a sole or cofermentable carbon source (45). This work demonstrates that *S. pneumoniae*, *S. pyogenes*, and *L. lactis* all contain α-GP oxidase homologs; in the former case the presence of the glpO (and other glp) gene products provides the enzymological basis for the slow acid production observed from glycerol. Furthermore, Saluja and Weiser (23) have shown that the 2-kb colonial opacity locus from *S. pneumoniae* overlaps the glp gene cluster, but there is no evidence linking these genes directly with the opacity phenotype. Given the inability of either *S. pyogenes* or...
Lactobacillus lactis to produce acid from glycerol, the significance and/or function of the glp genes in these organisms is at present unknown.

The primary structure of α-GP oxidase, apart from the N-terminal FAD-binding region, is distinct from those of other FAD-dependent oxidases. It should be emphasized, however, that the cholesterol oxidase and glucose oxidase sequences exhibit only 21% identity; yet these proteins clearly show structural homology (17). Nonetheless, on the basis of sequence analyses alone it is not possible to make deductions regarding specific residues and their catalytic roles in α-GP oxidase. More significant at this point is the striking homology between the oxidase and the membrane-associated α-GP dehydrogenases; this observation suggests that both genes originally diverged from a common ancestor, perhaps in coordination with the advent of cytochrome biosynthesis during the evolution of the prokaryotes. Primarily because of the requirement for detergent and/or phospholipid in the respective preparations, and in some cases the very limited amounts of pure enzyme available, only a few kinetic and redox properties have been described for the membrane-associated dehydrogenases (56, 58, 59). In the absence of detailed structural information, we can identify the βαβ supersecondary structural elements involved in FAD binding (40), and two of the postulated “α-GP-binding” segments (37) can also be identified by comparing α-GP dehydrogenase and oxidase sequences. One particular region which is strongly conserved in the α-GP oxidase and the six known α-GP dehydrogenases that we have analyzed corresponds to Thr57-Leu72 in the oxidase; 11 of the 16 residues are absolutely conserved, but their functional significance is unknown. It is also tempting to speculate that the 52-residue insert (Ser356-Val407) that distinguishes α-GP oxidase from the dehydrogenases may contribute to the soluble versus membrane-associated properties of the two enzymes. It is important to note that in at least one other case, a membrane-associated flavoprotein dehydrogenase has been identified as a homolog of a soluble oxidase, as in the present case. Mandelate dehydrogenase is therefore closely related to the FMN-dependent α-hydroxy acid oxidases, and Mitra et al. (60) have demonstrated that a 50-residue segment in the middle of the protein sequence accounts for the tight membrane association of the dehydrogenase. In comparing α-GP oxidase with the homologous α-GP dehydrogenases, however, it is the soluble protein which exhibits the extra internal segment, unlike its counterparts among the membrane-associated enzymes. Adding further to the difficulty in clarifying the factors responsible for membrane association with the α-GP dehydrogenases is the fact that, although the E. coli enzyme has been described as an integral membrane protein (58), hydrophobicity plots indicate that the polypeptide is sufficiently hydrophilic to exist as a soluble protein (37). The possibility of membrane-anchoring subunit(s) interacting with the E. coli dehydrogenase has also been considered.

By applying enzyme-monitored turnover analysis (30) under \([O_2]\)-limited conditions, we have shown that the recombinant α-GP oxidase follows a ping-pong kinetic mechanism,

\[
E\text{-FAD} + α\text{-GP} \rightleftharpoons E\text{-FAD} \cdot α\text{-GP} \rightleftharpoons E\text{-FADH}_2 \cdot α\text{-GP} \rightleftharpoons E\text{-FADH}_2 + α\text{-GP}
\]

This scheme is similar to those which have been shown to apply for glucose oxidase, with 2-deoxyglucose as substrate (30), and for lactate oxidase (5); as given, this kinetic pathway differs from the classical ping-pong mechanism in the absence of a Michaelis complex between \(E\text{-FADH}_2\) and \(O_2\). As derived for lactate oxidase (5), the corresponding kinetic constants are as follows,

\[
K_{\text{cat}} = \frac{k_{\text{cat}}}{k_3 + k_4 + k_5}
\]

\[
K_{\alpha(α-GP)} = \frac{k_{\text{cat}} + k_2(\alpha_2 + k_3)}{2k_2(\alpha_2 + k_4 + k_5)}
\]

\[
K_{\text{(O}_2\text{)}} = \frac{k_{\text{cat}}}{k_3 + k_4 + k_5}
\]

Our analysis of the reductive half-reaction requires that \(k_2 \gg k_3\) and that \(k_4 = 0\) (53); the latter condition is also supported experimentally by the fact that no reoxidation is observed when 10 mM DHAP is mixed anaerobically with the reduced enzyme (1). In addition the linear plots of \([O_2] / v\) versus \([O_2]\) from the turnover analysis, as [DHAP] approaches 0.7–0.8 mM, confirm that this product binds very weakly, if at all. Furthermore, the reduction kinetics indicate that the rate constant for DHAP release is much greater than the rate constant for reduction (i.e. \(k_5 \gg k_3\)). Under these conditions the steady-state data yield,

\[
k_{\text{cat}} = 3.7 \text{ s}^{-1} (48 \text{ s}^{-1})
\]

\[
K_{\alpha(α-GP)} = \frac{1}{k_1} = 24 \text{ mM} (25 \text{ mM})
\]

\[
K_{\text{(O}_2\text{)}} = \frac{5}{k_{\text{cat}}} = 35 \mu\text{M} (44 \mu\text{M})
\]

The numbers in parentheses represent the values calculated from the corresponding rate constants. Although we have not directly determined \(k_7\), the rate constant for reoxidation of reduced enzyme, the \(k_{\text{cat}} / K_{\alpha(α-GP)}\) value of \(1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) at pH 7.0, 5 °C, does provide a lower limit and this value is comparable to the second-order rate constants of \(1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) and \(1.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) determined for the oxygen reactions of reduced glucose oxidase (pH 5.6, 25 °C; Ref. 30) and lactate oxidase (pH 7.0, 25 °C; Ref. 5). With this in mind, there is very good agreement between the experimentally determined and calculated values for these kinetic constants, further supporting the α-GP oxidase mechanism presented above.

Our analysis of the reductive half-reaction with L-α-GP also revealed a second, slower phase in the \(A_{646}\) traces which was independent of \([\alpha\text{-GP}]\), with \(k_{\text{slow}} = 3.6–5.4 \text{ s}^{-1}\) at 5 °C. Clearly \(k_{\text{slow}} \ll k_{\text{cat}} = 37 \text{ s}^{-1}\), and no comparable behavior was observed in the steady-state analysis. Although not tested rigorously, we presently interpret this result to indicate that the resting oxidized enzyme exists in two states which are distinct in their abilities to bind α-GP productively for reduction (53),

\[
E\text{-FAD} \rightleftharpoons E\text{-FAD} \cdot α\text{-GP} \rightleftharpoons E\text{-FADH}_2 \cdot α\text{-GP} \rightleftharpoons E\text{-FADH}_2 + α\text{-GP}
\]

where reduction of \(E\text{-FAD} \cdot α\text{-GP}\) is limited by \(k_{10} = 3.6–5.4 \text{ s}^{-1}\). The ratio of the amplitudes (\(ΔA_{446}\)) corresponding to \(k_{\text{cat}}\) and \(k_{\text{slow}}\) at saturating \([\alpha\text{-GP}]\) should equal \(k_{12} / k_{11}\) at 50 mM substrate this gives \(k_{12} / k_{11} \sim 6.7\).

The clear homology between α-GP oxidase and the dehydrogenases raises an interesting question regarding the active site environment of the oxidase, since flavoprotein oxidases and dehydrogenases have historically been contrasted on the basis of their differences rather than compared in terms of their similarities. The reduced α-GP oxidase reacts rapidly with oxygen; we have also shown in this work that the recombinant enzyme stabilizes the flavin N(5)-sulfite adduct but does not provide for strong stabilization of the anionic semiquinone. With regard to the latter property it should be pointed out that
although glucose oxidase yields a maximum of 90% anionic semiquinone during dithionite titration at pH 9.3, determination of the midpoint potentials for the two one-electron transfers indicated that kinetic factors account for a substantial component of the observed radical stabilization (61). At pH 5.3, where $k_{\text{eq}}$ is maximal with glucose oxidase, there is essentially no separation in the corresponding midpoint potentials. Recently Gadda et al. (47) have reported only a modest extent of kinetic stabilization for the anionic semiquinone of cholesterol oxidase (Streptomyces hygroscopicus) on photoreduction. Given the relatively weak semiquinone stabilization observed with α-GP oxidase, it appears that these three enzymes are designed to oppose thermodynamic stabilization of the respective flavin radicals and thus favor simultaneous two-electron transfer mechanisms.

α-GP oxidase and glucose oxidase stabilize the respective flavin N(5)-sulfite adducts, although the corresponding $\Delta G^\circ$ values for sulfite addition at 25 °C are 4.6–5.6 kcal/mole more positive than that for lactate oxidase (5, 35). α-GP oxidase and glucose oxidase also stabilize the anionic p-quinoim form of 8-mercapto-FAD, and in general the properties of α-GP oxidase contribute to a view of the active-site environment in which the flavin N(1)-C(2)-O locus is strongly influenced by either a positively charged side chain or helix dipole, as is also the case for α-amino acid oxidase and other flavoprotein oxidases (7). In addition, the present work demonstrates that the α-GP oxidase apoprotein raises the redox potential of the bound FAD/FADH$_2$ couple by 100 mV relative to the free coenzyme (−118 mV versus −219 mV; Ref. 28); this effect is necessary in order to accommodate the redox potential of −190 mV for the α-GP/DHAP couple and may involve some of the same structural factors which, for example, facilitate adduct formation with sulfite (62). As has been stated (8), one of the hallmarks of those flavoenzymes operating via carbanion mechanisms is the ability to form a flavin N(5)-sulfite adduct; mechanistic parallels have been drawn between the nucleophilic addition of sulfite to the flavin N(5)-position and the postulated attack of a substrate carbanion (e.g. with α-amino acid oxidase; Ref. 11) in the catalytic process of flavin reduction. While the mechanisms of flavin reduction for α-amino acid oxidase and lactate oxidase are presently the subjects of intensive experimental study (63–66), it is nonetheless clear that α-GP oxidase and glucose oxidase, both of which operate via hydride transfer mechanisms, also stabilize sulfite adducts. To our knowledge the point has not been emphasized that the reaction sequence leading to the nucleophilic addition of sulfite to the flavin N(5)-position also nicely parallels the addition of a hydride ion (H$:−$) to the α-GP oxidase flavin (Scheme 1).

The positive charge contributed by the apoprotein allows the stabilization of anionic FAD derivatives (e.g. N(1)- and N(3)-anionic forms of FADH$_2$ and FAD, respectively, N(5)-sulfite complex, and anionic p-quinoim form of 8-mercapto-FAD) by the enzyme; the active site environment also provides for both a 100 mV increase in the bound FAD redox potential and the facile addition of the hydride ion to the flavin N(5)-position. Given the homology between α-GP oxidase and the membrane-associated α-GP dehydrogenases, our present deductions regarding the structural and mechanistic parameters for α-GP oxidase should also prove useful in analyses of this aspect of membrane-associated electron transport in both bacterial and mitochondrial systems.
Analysis of Recombinant Enterococcal α-Glycerophosphate Oxidase

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