Glucose Oxidase from Aspergillus niger

CLONING, GENE SEQUENCE, SECRETION FROM SACCHAROMYCES CEREVISIAE AND KINETIC ANALYSIS OF A YEAST-DERIVED ENZYME*

The gene for Aspergillus niger glucose oxidase (EC 1.1.3.4) has been cloned from both cDNA and genomic libraries using oligonucleotide probes derived from the amino acid sequences of peptide fragments of the enzyme. The mature enzyme consists of 583 amino acids and is preceded by a 22-amino acid presequence. No intervening sequences are found within the coding region. The enzyme contains 3 cysteine residues and 8 potential sites for N-linked glycosylation. The protein shows 26% identity with alcohol oxidase of Hansenula polymorpha, and the N terminus has a sequence homologous with the AMP-binding region of other flavoenzymes such as p-hydroxybenzoate hydroxylase and glutathione reductase. Recombinant yeast expression plasmids have been constructed containing a hybrid yeast alcohol dehydrogenase II-glyceraldehyde-3-phosphate dehydrogenase promoter, either the yeast α-factor pheromone leader or the glucose oxidase presequence, and the mature glucose oxidase coding sequence. When transformed into yeast, those plasmids direct the synthesis and secretion of between 75 and 400 μg/ml of active glucose oxidase. Analysis of the yeast-derived enzymes shows that they are of comparable specific activity and have more extensive N-linked glycosylation than the A. niger protein.

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to glucono-δ-lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide: β-D-glucose + O₂ → glucono-δ-lactone + H₂O. The enzyme activity was first reported by Muller (1928) in extracts of Aspergillus niger, and subsequently, the enzyme has been purified from both Aspergillus (Pazur and Kleppe, 1964; Swoboda and Massey, 1965) and Penicillium species (Kusai et al., 1960). The fungal enzyme consists of a dimer of molecular weight 150,000 containing two tightly bound FAD cofactors (Pazur and Kleppe, 1964). The mechanism of action of the enzyme has been analyzed in some detail (Gibson et al., 1964; Bright and Appleby, 1969).

Glucose oxidase has also been tested as the basis for glucose sensors (Degani and Heller, 1987, 1988), in glucose detection kits, and as a source of hydrogen peroxide in food preservation (Banks et al., 1986). As a first step in trying to elucidate the residues necessary for catalysis and to improve the properties of glucose oxidase by protein engineering techniques, the cloning and expression in yeast of the A. niger enzyme are described.

MATERIALS AND METHODS

Protein Chemistry, Deglycosylation, and Analysis—Glucose oxidase from A. niger (EC 1.1.3.4) was obtained from Sigma (type 5). Sequence analysis was performed on the protein after dialysis into 0.2 M N-ethylmorpholine acetate (pH 8.5) buffer containing 6 M guanidine HCl and 3 mM EDTA followed by reduction with 2-mercaptoethanol and either carboxymethylation with iodoacetic acid (Truett et al., 1985) or pyridylethylolation with 4-vinylpyridine (Freidman et al., 1970). Tryptic fragments of glucose oxidase were prepared by digestion with the enzyme in 2 M urea buffers after blockage of lysine residues with citraconic anhydride (Truett et al., 1985). The peptide mixtures were decitraconylated in acid and resolved by successive chromatography on Vydac C18 and C4 reverse phase columns under acidic conditions at elevated temperature (40 °C) and re-chromatography at room temperature under neutral conditions using gradients of acetonitrile. Cyanogen bromide fragments of the protein were resolved by successive chromatography using Bio-Gel P-10 in 30% formic acid and re-chromatography by reverse phase HPLC using Vydac C18 columns and n-propanol gradients in the presence of trifluoroacetic acid. Edman degradations were performed on the intact protein and peptide fragments using an Applied Biosystems 470A gas phase protein sequencer. The phenylthiohydantoin-derivatives were identified using either the reverse phase resolution system of Hawke et al. (1982) or that employed in the Applied Biosystems 470A phenylthiohydantoin analyzer (Hunkapiller, 1985). N-Linked carbohydrate was removed using either endoglycosidase H or N-glycanase (Boehringer Mannheim). SDS gel electrophoresis was done according to Laemmli (1970).

Glucose Oxidase Assays—Glucose oxidase activity was determined using either A. niger glucose oxidase (Sigma, type 5) or purified yeast derived glucose oxidase as standard. The assay was a modification of the method of Kelley and Reddy (1986) as follows: assays were performed in a volume of 1.0 ml of 0.1 M NaPO₄ (pH 7) containing 0.2 mM o-dianisidine (Sigma), 10 μg of horseradish peroxidase (Boehringer Mannheim), and 9.5 mM D-glucose. Assays were initiated by the addition of glucose oxidase (1–30 ng), incubated at room temperature for 20 min. and quenched by the addition of 0.1 M of 4 N

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H$_2$SO$_4$. The reduced d-oxidiasidase was then measured at 400 nm on a Shimadzu Model UV-160 spectrophotometer or at 405 nm on an enzyme-linked immunosorbent assay reader (Titertek Multiscan). Enzyme activities were calculated as nanograms of glucose oxidase relative to a standard curve of absorbance versus enzyme amount.

An alternate continuous spectrophotometric assay was also employed for kinetic characterization of the purified enzyme. This was a modification of a procedure described by Lockridge et al. (1972) for the assay of H$_2$O$_2$ with lactate oxidase. In 1 ml of total volume was placed 0.8 ml of 0.3 M phosphate buffer (pH 5.6), 0.1 ml of 1 M glucose, 20 µl of horseradish peroxidase (1 mg/ml), and 40 µl of a Triton-stabilized d-oxidiasidase solution. The latter was prepared fresh daily by mixing 0.8 ml of 10 mM d-oxidiasidase with 0.2 ml of 20% Triton X-100. Under equilibration at 37°C, the reaction was started by the addition of glucose oxidase, and the absorbance increase at 460 nm followed with time. Molecular activity was calculated on the basis of 16.6 mU·cm$^{-3}$ for the d-oxidiasidase oxidoreductase product (Worthington and Teller, 1972).

Complete steady state analysis at pH 5.6, 4 °C, was also determined by enzyme-monitored turnover, using a stopped flow spectrophotometer, and analyzed as described earlier (Gibson et al., 1984). The stopped flow instrument was interfaced with a Nova II (Data General) computer and has been described in detail earlier (Beaty and Ballou, 1981). Absorption spectra were determined with a Hewlett-Packard 8452A Diode Array Spectrophotometer equipped with a thermostat cell holder.

**Table 1**

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Units</th>
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<tr>
<td>Glucose Oxidase</td>
<td>mU/ml</td>
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**Growth of A. niger and Isolation of Nucleic Acids—** A. niger (ATCC strain 0092) was grown in YEP medium (1% yeast extract, 2% peptone; Difco) containing 2% glucose at 25 °C. DNA was isolated by the method of Chirgwin et al. (1979) as modified by Turpen and Griffin (1980). Preparative RNA isolation was performed with a modified procedure described by Jeffus et al. (1985) using poly(A)$^+$ RNA which had been isolated by chromatography on oligo-dT-cellulose (Aviv and Leder, 1972). Genomic DNA was isolated from mycelia grown to 40 g/liter in YPD as described (Boel et al., 1984).

**Construction of cDNA and Genomic Libraries—** All recombinant DNA manipulations were done essentially according to Maniatis (1982). A. niger cDNA was constructed by the Agt10 vector system (Boel et al., 1985) using poly(A)$^+$ RNA which had been purified by two cycles on oligo-dT-cellulose as starting material (Aviv and Leder, 1972). A total of 8 × 10$^6$ clones were obtained. The genomic library was prepared as follows: A. niger genomic DNA (50 µg) was digested with 0.5 units of Sau3A (New England Biolabs) for 50 min to maximize DNA in the molecular weight range of 5–20 kb. The digest was resolved on a 1% agarose gel and the region corresponding to the 7–10-kb fragments was excised; the DNA was electroeluted, extracted with phenol/chloroform, and concentrated by ethanol precipitation. This material was ligated with pBR322 which had been electroporated with BamHI, treated with calf intestinal phosphatase (Boehringer Mannheim), and isolated from an agarose gel. The ligated DNAs were used to transform E. coli DH5$\alpha$ (Bertolus and Research Labs, F$^-$, recA1, endA1, hsd R17(k$r^-$, m$k^+$) supE44, $\theta$1, gyrA, relA1) to ampicillin resistance. A total of 7000 transformants were obtained; subsequent analysis showed that >90% of the colonies were recombinant and the average insert size was 8 kb.

**Library Screening and DNA Sequencing—** All oligonucleotides were made using standard phosphoramidite chemistry on an Applied Biosystems Model 380A DNA synthesizer or as described (Warner et al., 1984). Oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase and 32P ATP (Amersham Corp.). DNA fragments were labeled using Amersham Corp. nick translation or pBR322 (Feinberg and Vogelstein, 1983). Oligonucleotide screening of the cDNA library was done using the tetramethylammonium chloride washing method of Wood et al. (1985). Tetramethylammonium chloride was from Aldrich. Nick translated or random primed probes were hybridized to duplicate filters at 42 °C in Wallace mix (Wallace et al., 1979, 1981) containing 50% formamide and 10% dextran sulfate. Potential positives were replated and screened again with the same probe and then were analyzed using Southern blotting as described (Maniatis et al., 1982). Digests of Agt10 clone were directly subcloned into plasmid vectors (either pBR322 or M13) and positives identified by colony hybridization. DNA sequencing was done by subcloning in M13 using the method of Sanger et al. (1977).

**Construction of Yeast Expression Plasmids—** All restriction enzymes and other reagents for recombinant DNA manipulations were obtained from New England Biolabs unless otherwise noted. A GO cDNA clone in Agt10 (2×a) containing the entire coding sequence and the polyA tail was digested with BglII and H3, and the resulting mixture of fragments was subcloned between the BamHI and H35 sites of pBR322 yielding plasmid pBR322. This plasmid contains the entire GO coding sequence, the untranslated region, and approximately 2 kb of flanking 5′ and 3′ sequences. A second cDNA clone (3a) containing most of the GO cDNA, but truncated immediately before the polyA addition site, was digested with EcoRI and a 1100-bp fragment, comprising the 5′ half of the GO coding sequence and the 3′-untranslated, was subcloned into the EcoRI site of pBR322 yielding plasmid pBR322a.

Plasmid pAGAP (Malcolm et al., 1989) was digested with NcoI and BglII, and the synthetic duplex shown below as a single strand encoding the GO presequence was inserted, yielding plasmid pAGSO-1.

**Table 2**

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Fragment Size</th>
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<tbody>
<tr>
<td>BamHI</td>
<td>2 kb</td>
</tr>
<tr>
<td>BglII</td>
<td>1100 bp</td>
</tr>
</tbody>
</table>

**Construction of Yeast Transformation Plasmids—** All recombinant DNA manipulations were done according to Hinnen et al. (1978). The yeast strain GRF181 (Mato, leu2-3, leu2-212, his3-11, his3-15, ura3-1, CAN, [circ]) was derived from GRF180 (Malcolm et al., 1989) by plasmid-directed deletion of the ura3 gene using 5-fluoro-orotic acid as selection for uracil auxotrophs (Boeke et al., 1984). Transformants were initially obtained on uracil selective plates containing 5% glucose and were maintained on minimal plates lacking leucine to select for the high copy number leu2-D allele (Beggs, 1978; Erhart and Hollenberg, 1983). Innoculae were grown in leucine selective media containing 8% glucose for 144 h at 30 °C and harvested by centrifugation. Secreted GO was determined after dilution media to 0.1 M NaPO$_4$ (pH 7.4), whereas intracellular enzyme activity was measured after lysis of the cells with glass beads (Rosenberg et al., 1984).

**Yeast Transformation and Growth—** Yeast transformations were done according to Hinne et al. (1978). The yeast strain GRF181 (Mato, leu2-3, leu2-212, his3-11, his3-15, ura3-1, CAN, [circ]) was derived from GRF180 (Malcolm et al., 1989) by plasmid-directed deletion of the ura3 gene using 5-fluoro-orotic acid as selection for uracil auxotrophs (Boeke et al., 1984). Transformants were initially obtained on uracil selective plates containing 5% glucose and were maintained on minimal plates lacking leucine to select for the high copy number leu2-D allele (Beggs, 1978; Erhart and Hollenberg, 1983). Innoculae were grown in leucine selective media containing 8% glucose for 14 h and diluted 1:100 into YEP medium containing 4% glucose for expression. Expression cultures (25 ml) were grown for 14 h at 30 °C and harvested by centrifugation. Secreted GO activity was determined after dilution media to 0.1 M NaPO$_4$ (pH 7.4), whereas intracellular enzyme activity was measured after lysis of the cells with glass beads (Rosenberg et al., 1984).

**Purification of Yeast-Derived Glucose Oxidase—** The yeast-derived enzymes were purified from the conditioned medium by a modification of the method of Pazar and Kleepe (1984). Yeast cells were centrifuged, the conditioned medium and the conditioned YEP medium were filtered, and the filtrate was precipitated with 10 fold with 0.1 M sodium acetate (pH 5.4). The protein was applied to a DEAE-Sepharose Fast Flow column (20 ml, Pharmacia LKB Biotechnology Inc.) equilibrated in the same buffer. The column was then washed with 3 volumes of the equilibration buffer and the enzyme eluted with 0.1 M sodium acetate (pH 3.7). Fractions...
Peptide sequences derived from glucose oxidase

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Location</th>
<th>Cleavage method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. SNGIEASLLT</td>
<td>1-10</td>
<td>None</td>
</tr>
<tr>
<td>2. TVDYIAGGGLTGLTTA</td>
<td>10-20</td>
<td>Trypsin</td>
</tr>
<tr>
<td>3. LTENPN'ISVLVIESGSYESD</td>
<td>19-30</td>
<td>Trypsin</td>
</tr>
<tr>
<td>4. GGFHX*TTALLIQYENY</td>
<td>29-40</td>
<td>CNBr</td>
</tr>
<tr>
<td>5. PKE</td>
<td>39-50</td>
<td>Trypsin</td>
</tr>
<tr>
<td>6. SAVEDRGVPTKDFGPQG'GDPHVMS</td>
<td>48-59</td>
<td>None</td>
</tr>
<tr>
<td>7. PFPNTLDIEQV</td>
<td>58-69</td>
<td>Trypsin</td>
</tr>
<tr>
<td>8. ISDALEYASM</td>
<td>68-79</td>
<td>Trypsin</td>
</tr>
</tbody>
</table>

The locations indicated are derived from translation of the cDNA sequence.

These positions are potential N-linked glycosylation sites. The asparagine at position 43 was present in reasonable quantity which suggests that it is not glycosylated; position 5 in peptide 4 yielded a blank cycle in the Edman degradation, suggesting that the asparagine in that position (388) is a site for glycosylation.

The difference at position 206 is due to an error in amino acid identification due to the use of the Applied Biosystems 120A resolution system to identify the PTH-derivative derived from a carboxymethyl peptide; during the course of this study, the PTH-derivative resolution system was changed from that of Hawk et al. (1982) to the on-line 120A PTH-analyzer system of Applied Biosystems (Hunkapiller, 1983).

These sites showed differences between the amino acid sequence shown and that derived from the cDNA: G206 is C206 and L206 is H206.

Peptide 8 is derived from a cleavage with trypsin which was obviously not precluded by citraconylation of the intact protein; R56 was not susceptible to chemical modification under the conditions employed in this study.

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**Analysis of Protein Sequences**

Computer analysis of protein sequences was done initially with the program DFASTP (Lipman and Pearson, 1985) to screen the Dayhoff protein sequence database. Further alignment and analysis was done using the program MA-LIGN (Biomathematics Computation Lab, University of California, San Francisco) and by visual examination.

**RESULTS**

**Determination of Peptide Sequences from A. niger Glucose Oxidase**—Glucose oxidase from A. niger migrates as a protein containing GO activity were pooled and concentrated by ultrafiltration.
Glucose Oxidase, Cloning and Expression

A

FIG. 2. A, southern blot of A. niger DNA probed with a glucose oxidase gene probe. B, map of the A. niger glucose oxidase gene. Aliquots of A. niger DNA (10 μg) were digested with a series of restriction enzymes, electrophoresed on a 1% agarose gel, and transferred to nitrocellulose. A 1.9-kb Bgl2 fragment of the GO gene from plasmid pSG02 was labeled with 32P by random priming, hybridized to the blot, washed at 60 °C in 0.1 × SSC (5.75 g NaCl, 4.4 g sodium citrate/liter, pH 7.0), 0.1% SDS, and exposed to film for 16 h. The map shown was derived by standard methods using restriction analysis and Southern blotting. The solid bar represents the region of the cDNA clones isolated and the arrow the direction of translation. The digests are: lane 1, EcoRI; lane 2, Hind3; lane 3, BarnHI; lane 4, SalI; lane 5, PstI, lane 6, XhoI.

with an apparent molecular mass of 75-80 kDa on SDS-polyacrylamide gel electrophoresis. Treatment with either N-glycanase or endoglycosidase H decreases the apparent molecular mass by 5-10 kDa, suggesting that a few sites for N-linked glycosylation are used, consistent with the observations of others (Pazur et al., 1965, Swoboda and Massey, 1965). When the protein is subjected to automated Edman degradation, the N-terminal sequence: Ser-Asn-Gly-Ile-Glu-Ala-Ser-Leu-Leu-Thr is found. Treatment with cyanogen bromide or digestion with trypsin after treatment with citraconic anhydride, and isolation of individual peptides by HPLC followed by gas phase sequencing, yielded the collection of sequences shown in Table I.

Isolation of cDNA and Genomic Clones Encoding Glucose Oxidase—CNBr peptide 6 was used to design two long unique probes for the glucose oxidase gene. The probes, long7 and long8, were designed with a GC bias in the third position as has been found for other filamentous fungi genes (Corrick et al., 1987).

long7  GCTCACACCGTGGGGATCACCCTGGCCGAAATCTTTCTTGGT
long8  ATCTTTCTTGGTGGGCACGCCGATCCTCCACAGGCCTCAT

These probes were used initially to screen duplicate filters of a cDNA library of 8 × 107 recombinant phage in λgt10. Four double positives were obtained which upon secondary screening remained positive. Subsequent DNA sequence analysis of one of these phage, λ44a, showed 33/42 matches with probe long7 and 36/42 matches with probe long8. The single open reading frame agreed with the peptide sequence in all positions except one, Cys instead of Gln, at position 17. In addition, the sequence of CNBr peptide 7 (Table I) was encoded immediately 3' of the peptide 6 match.

Three cDNA clones were sequenced on both strands in their entirety; all of the overlapping sequences, covering more than three-fourths of the coding sequence, were the same. The longest clone extended 22 bp beyond a likely start codon, whereas a second stopped at that methionine codon. A 600-bp EcoRI-NcoI fragment from one of the cDNA clones was then used to screen a genomic library in pBR322. Several positive clones were obtained and one was analyzed in detail. A combination of DNA sequence and restriction analysis showed that the genomic sequence was contiguous with the cDNA clones, indicating there are no intervening sequences in the coding region. A composite of the coding region from the cDNA clones and the 5'- and 3'-flanking regions from the genomic clone is shown in Fig. 1.

The indicated methionine starts an open reading frame comprised of 605 amino acids, encoding a potential protein of 65,700 molecular weight. The mature N terminus as determined by protein sequencing is found at amino acid 22, whereas a second stop codon was removed. This sequence has some characteristics of a signal peptide, but is likely more complex, since the final processing is at a single arginine residue. The mature protein of 583 amino acids contains 3 cysteine residues and 8 consensus sites for N-linked glycosylation. All of the peptide sequences in Table I are found in the cDNA sequence with only three differences out of 117 residues and one difference due to a known resolution artifact. These differences are likely due to incorrect assignment of amino acids during peptide sequencing.

To determine if there are multiple GO genes and to confirm the genomic clone structure, the entire cDNA was used to probe a genomic blot of A. niger DNA. The results (Fig. 2A) show that GO is most likely encoded by a single gene and is consistent with the map shown of the genomic clone (Fig. 2B).
Expression of the Glucose Oxidase cDNA in S. cerevisiae—We inserted the mature GO coding sequence into two vectors for expression in Saccharomyces cerevisiae. These vectors contain expression cassettes of the yeast alcohol dehydrogenase-II-glyceraldehyde-3-phosphate dehydrogenase promoter, glucose oxidase presequence, GO coding sequence, and glyceraldehyde-3-phosphate dehydrogenase terminator inserted into the BamHI site of the yeast-E. coli shuttle vector pAB24. The second plasmid, p@GO-1, has the yeast α-factor leader substituted for the GO presequence.

Characterization of Yeast-derived Glucose Oxidase and Amino Acid Composition and Sequence Data—In order to begin to examine the GO secreted from yeast, we analyzed samples from both pSG02 and p@GO-1 transformants on 8% polyacrylamide gels. The results (Fig. 4) show that both yeast-derived proteins migrate more slowly than the A. niger enzyme. Treatment with endoglycosidase H, however, results in the yeast and A. niger enzymes migrating as doublets of similar if not identical mobilities. Thus, the yeast proteins have more extensive N-linked glycosylation. The yeast-derived enzymes were concentrated and purified using a modification of the method described for the A. niger protein (Pazur and Kleppe, 1964). They were then subjected to N-terminal sequence and amino acid composition analysis. The enzyme purified from p@GO-1 conditioned medium had the same serine N terminus as the A. niger enzyme. Analysis of the pSG02 derived enzyme showed a majority of the authentic N-terminal serine and a minor sequence: Leu-Pro-X-Tyr-X-Arg-Ser-Asn-Gly, which corresponds to enzyme which has only been processed at position -6. This is the most likely signal peptidase cleavage site as suggested by the analysis of von Hejne (1985). Both enzymes had amino acid compositions in agreement with that predicted from the cDNA and observed previously by other workers (Pazur et al., 1965). The pSG02-derived enzyme was utilized for detailed kinetic analysis.

Spectral Characteristics—The absorption spectrum of the pSG02 yeast-derived enzyme is shown in Fig. 5 and has the same characteristics as those reported for the enzyme isolated from A. niger (Swoboda and Massey, 1965), with 

$$
\text{E}_{452} = 12.83 \text{mM}^{-1} \text{cm}^{-1}
$$

This was determined from the change in absorption spectrum due to release of FAD from denatured protein on diluting native enzyme into guanidinium HCl to give a final concentration of 7.6 M guanidine HCl (pH 7.3) and a determined value of E_{450} of FAD under the same conditions of 12.05 mM^{-1} cm^{-1} (results not shown). As with vector pAB24 (Barr et al., 1987), when grown under derepressing conditions, transformants of these plasmids in yeast strain GRF181 secrete large amounts of active GO into the medium. Typical shake flask data are summarized in Table II. The levels observed (>300 µg/ml) of secreted GO activity from the pSG02 transformants are among the highest observed for secreted proteins from yeast (Pentilla et al., 1988, Tschopp et al., 1987).

Table II

<table>
<thead>
<tr>
<th>Glucose oxidase activitya</th>
<th>pGO01 plasmid</th>
<th>pSG02 plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>1 (16.1)b</td>
<td>2 (17.3)b</td>
</tr>
<tr>
<td>Intracellular</td>
<td>1 (18.1)b</td>
<td>1 (22.1)b</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>85</td>
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</table>

% secreted 83 80 90 90 90

Glcose oxidase activity was determined using a purified sample of yeast derived glucose oxidase as standard.

Transformants are indicated by 1 or 2; the numbers in parentheses are the optical densities at 650 nm.
the wild type enzyme, the pSG02 yeast-derived enzyme is devoid of flavin fluorescence and does not appear to contain the blue fluorophore which has been reported to be present in the enzyme isolated from A. niger (Swoboda and Massey, 1966a, 1966b).

Kinetics Analysis—The pSG02 yeast-derived enzyme, like the wild type, has very high catalytic activity. Under the standard continuous spectrophotometric assay conditions described under "Materials and Methods," at pH 5.6, 25 °C, the yeast-derived enzyme has a turnover number of 17,000-20,000 min⁻¹, compared with a value of 16,200 min⁻¹ reported for wild type enzyme (Gibson et al., 1964). It also gives a series of parallel Lineweaver-Burk plots on systematic variations of both the glucose and O₂ concentrations. The latter results were obtained by stopped flow enzyme-monitored turnover as shown in Fig. 6, where 9.35 µM enzyme in 0.1 M phosphate (pH 5.6) 3 °C was reacted with various concentrations of glucose at an initial O₂ concentration of 456 µM (air saturated at 3 °C). Immediately after mixing, a steady state is established, determined by the relative rates of reduction of the enzyme flavin by glucose and oxidation of reduced flavin by oxygen. The steady state persists until practically all the oxygen is exhausted by catalytic turnover, whereupon the enzyme becomes fully reduced. Analysis of these curves as described by Gibson et al. (1964) yields the set of parallel Lineweaver-Burk plots shown in Fig. 7 (left-hand panel), and a replot of the intercepts versus 1/[oxygen] gives the secondary Lineweaver-Burk plot of Fig. 7 (right-hand panel). These data yield the following kinetic constants: Vₘₐₓ = 500 s⁻¹, Kₘ(glucoae) = 0.14 M, Kₘ(oxygen) = 2.6 × 10⁻⁴ M. Under similar conditions, but at 0 °C, the corresponding values for the A. niger enzyme have been reported: Vₘₐₓ = 235 s⁻¹, Kₘ(glucoae) = 0.12 M, Kₘ(oxygen) = 2.1 × 10⁻⁴ M (Gibson et al., 1964). While the Vₘₐₓ value determined for the yeast-expressed enzyme appears to be significantly higher than the wild type enzyme, it must be emphasized that the steepness of the secondary plots (cf. Fig. 7, right-hand panel) can lead to significant errors in the estimation of the extrapolated 1/Vₘₐₓ value. Similar enzyme-monitored turnover analyses have been performed using 2-deoxyglucose, a much poorer substrate for the enzyme. With this substrate, much closer agreement is obtained with the values reported previously for the wild type enzyme. The following values were obtained at pH 5.6, 3 °C, with values reported for the wild type enzyme (Gibson et al., 1964) being given in brackets: Vₘₐₓ = 20 s⁻¹ (15 s⁻¹), Kₘ(2-deoxyglucose) = 2.4 × 10⁻⁴ M (2.5 × 10⁻³ M). With 2-deoxyglucose, in both cases the Kₘ for O₂ was so low as to be difficult to measure with accuracy (~2 × 10⁻⁶ M). It should be noted that in these enzyme-monitored turnover experiments it was not necessary to add KCN to inhibit catalyzing catalase (as was done with the wild type enzyme; Gibson et al., 1964), since the yeast-expressed enzyme is completely devoid of catalase activity.

Formation of Enzyme Flavin N-(5)-Sulfite Complex—One of the characteristics of flavoproteins of the oxidase class, first recognized with glucose oxidase (Swoboda and Massey, 1966a) is the ability to undergo nucleophilic attack by sulfite (or bisulfite) to form a flavin N-(5)-sulfite adduct in a thermodynamically reversible equilibrium (Massey et al., 1969, Muller and Massey, 1969). The yeast-derived glucose oxidase, as expected, also displays the same properties, and the spec-
The free oxidized enzyme.

possible to obtain an estimate of $k_{on}$ by following the spectral change shown presumably involves the dissociation of the enzyme-sulfite complex followed by rapid reduction of the free oxidized enzyme.

$$\text{EF}_{\text{isoO}} \xrightleftharpoons{}^{} k_{\text{off}} k_{\text{on}} \text{glucose} \rightarrow \text{EF}_{\text{red}} + \text{HSO}_3^- \quad (1)$$

$$\text{EF}_{\text{red}} + \text{glucose} \xrightarrow{} k_{\text{off}} k_{\text{on}} \text{EF}_{\text{isoO}}H^+ + \text{glucono-\(\Delta\)-lactone} \quad (2)$$

As $k_{\text{on}}$ is known to be very large (Gibson et al., 1964), it was possible to obtain an estimate of $k_{\text{off}}$ by following the spectral change with time. This was done by adding 20 mM glucose to the enzyme-sulfite complex and following the reaction by taking repetitive scans at 10-20-s intervals with a diode-array spectrophotometer. The spectral change occurred with a $t_{1/2}$ of 90-100 s, independent of the initial bisulfite concentration (5-20 mM), yielding a tentative value for $k_{\text{off}}$ of 7.3 x 10^{-3} s^{-1}. This was confirmed by measuring the rates of formation of the enzyme-sulfite complex at low concentration of bisulfite, again using the diode-array spectrophotometer to record spectra every 3 s until equilibrium was reached. At concentrations of bisulfite of 0.1, 0.2, 0.3, and 0.4 mM, the observed pseudo first order rate constants were $1.41 \times 10^{-2}$ s^{-1}, $2.04 \times 10^{-2}$ s^{-1}, $2.57 \times 10^{-2}$ s^{-1}, and $3.35 \times 10^{-2}$ s^{-1}, respectively. When these $k_{\text{on}}$ values are plotted versus the bisulfite concentration, a linear plot is obtained (cf. Strickland et al., 1975) with slope $k_{\text{off}} = 66$ M^{-1} s^{-1} and intercept $k_{\text{off}} = 7.3 \times 10^{-3}$ s^{-1}. Thus, $K_d = k_{\text{on}}/k_{\text{off}} = 1.1 \times 10^{-4}$ M, in excellent agreement with the values obtained from static equilibrium titration measurements. In addition, it should be noted that the values of $k_{\text{off}}$ determined by the two methods described above are also in remarkable agreement and provide convincing evidence for the validity of Equations 1 and 2.

**Discussion**

The gene for *A. niger* glucose oxidase has been cloned from both cDNA and genomic libraries in this study. This conclusion is based on the following: first, the sequences of several peptides isolated from the protein are found encoded in the same translational reading frame in the gene sequence. Second, the single open reading frame encodes a protein of 605 amino acids, consistent with the mass of glucose oxidase of ~75 kDa, as determined by SDS gel electrophoresis and sedimentation analysis (Kelley and Reddy, 1986; Jones et al., 1982). The native molecular weight of 150,000-185,000 determined by others (Swoboda and Massey, 1965; Pazur and Kleppe, 1964) is consistent with the protein being a dimer of identical subunits, each containing a FAD cofactor. Third, these clones have been used to engineer yeast strains to secrete proteins having glucose oxidase activity which react with antibodies to the *Aspergillus* protein on Western blotting (this work).2

We have sequenced a total of 2821 bp of *Aspergillus* DNA including 485 bp of $5'$-untranslated and 517 bp of $3'$-untranslated sequence. Two cDNA clones covering the coding region were sequenced in their entirety. The 463-bp $5'$ of the longest cDNA and 365-bp $3'$ of the polyA addition site were determined from a genomic clone. The coding and flanking sequences show substantially different base compositions; the coding region is 56% G:C in keeping with other fungal genes (Innis et al., 1985), whereas the $5'$-untranslated is 52% G:C and the $3'$-untranslated is 60% A:T. The putative promoter sequence contains a TATAA sequence, at -59 with respect to the longest cDNA clone isolated, and at +81 with respect to the initiation codon. The sequence between the TATAA and the likely ATG is highly pyrimidine rich (74%). Similar regions are seen in the promoter regions of highly expressed yeast genes (Dobson et al., 1982). The assignment of the initiating ATG is based upon the A at +3 (Kozak, 1984) and the fact that the subsequent 15 amino acids comprise a likely signal peptide sequence (von Heine, 1985). In addition, there are no other in-frame ATGs within 60 bp. Two sets of direct repeats are found in the $5'$-untranslated region: GGATTAT at -125 and -232 and GGAGGATG at -193 and -152.

The $3'$-flanking region does not contain a consensus polyA addition site (AATAAA). A comparison of the sequence of a cDNA and genomic clone shows that the GO mRNA can be polyadenylated at the sequence AAACAA at +151. Other fungal genes such as *ulcR* of Aspergillus nidulans (Felenbok et al., 1988) do not contain a canonical polyA site. The sequence CTACCAA (+81 and 147) is of possible interest because it overlaps the polyA addition site at +151. The possible role

\[2 \text{K. Frederick, unpublished data.}\]
these repeats in mRNA stability or polyA site selection awaits further work.

The amino acid composition of glucose oxidase derived from the cDNA sequence is in close agreement with that measured by other workers (Pazur et al., 1965; Jones et al., 1982) and with that determined on the proteins expressed in yeast (this work). This and other observations require that glucose oxidase be a dimer of identical subunits. The presequence is somewhat similar to that found for A. niger glucoamylase, except that a single Arg residue is found prior to the mature sequence as opposed to a dibasic Lys-Arg sequence (Innis et al., 1985). The enzyme which processes the GO presequence in yeast is unknown. Several groups have studied glucose oxidase based upon its properties as an acidic (polyanionic) protein. These include the effect of ionic strength on catalysis (Voet et al., 1981), inhibition by putrescine (Voet and Andersen, 1984), and resistance to inactivation by SDS at neutral pH (Jones et al., 1982). From the cDNA sequence a charge of -58 on the glucose oxidase dimer at neutral pH is predicted, ignoring any ionic contributions from histidines, tyrosines, and the carbohydrate. Previous work measured a charge of -77 by titration using an isoelectric point of 4.05 (Voet et al., 1981). The difference between this result and that predicted from the cDNA sequence is unknown, but may be due to a slightly higher isoelectric point of 4.2 reported by others (Swoboda and Massey, 1965) or to contributions from the FAD cofactor and covalent non-cofactor phosphate (James et al., 1981).

The mature enzyme contains eight potential N-linked glycosylation sites and 3 cysteine residues. Pazur and co-workers (Pazur et al., 1964) isolated a glycopeptide from GO by Pronase digestion and determined its amino acid composition. The closest correspondence to their results in the cDNA sequence is at positions 88-92 (Asn-Asn-Gln-Thr-Ala). From sequencing of peptide fragments of GO (Table I), we found that Asn-43 is not glycosylated, whereas Asn-388 likely is modified. Thus, at least two of the eight potential sites are utilized in Aspergillus. Of the potential sites, five contain the sequence Asn-X-Thr and three the alternative Asn-X-Ser.

Although the data are very limited, the former sequence may underestimate as a highly homologous region near the C-terminus of GO contains two disulfide bonds/dimer and they suggested that only a single free cysteine was present, making a dimer of identical subunits. The results presented here show that if there are two disulfide bonds then there are 2 free cysteines/dimer, since a single gene encodes the two subunits.

A comparison of the mature GO sequence with the Dayhoff protein sequence database yielded a single homologous protein, alcohol oxidase of Hansenula polymorpha. The initial scan showed 25% identity over almost the entire GO sequence (122/593 amino acids). Subsequent work showed this to be an underestimate as a highly homologous region near the C-terminus of the proteins was missed, due to a large insertion of about 70 amino acids in the alcohol oxidase as compared to the GO sequence. The best alignment of the sequences is shown in Fig. 8. The three overlaid regions of the proteins show relatively high degrees of sequence identity: amino acids 21-52 versus 9-41 (48%); 294-328 versus 274-308 (53%); and 519-556 versus 571-614 (51%), where the former numbers indicate the GO sequence and the latter those of alcohol oxidase. Using this alignment, the proteins are 26% identical (148/583).

Subsequent analysis of these regions shows that two are recognizably related to motifs found in other flavoenzymes.

The N-terminal homology is clearly a representative of the β-α-β motif involved in AMP binding in p-hydroxybenzoate hydroxylase and human glutathione reductase (Hofsteenge et al., 1980; Wierenga et al., 1979; Thieme et al., 1981; Krauth-Siegel et al., 1982). An alignment of eight flavoproteins in this region is shown in Fig. 9. The glycines at positions 26, 28, and 31 in GO are analogous to contributions from the AMP portion of the FAD cofactor bonded to the 2'-OH of ribose as in p-hydroxybenzoate hydroyxylase. The substantial degree of homology of GO with glutathione reductase and p-hydroxybenzoate hydroxylase in this region suggests that the AMP portion of the FAD cofactor binds near the N terminus of the GO subunits. The second
This work describes the first example of the secretion of an active flavoenzyme from *S. cerevisiae*. Glucose oxidase is both one of the largest and most efficiently secreted glycoproteins engineered to be secreted from yeast, as the native molecular weight is ~150,000 and more than 100 mg/liter of active enzyme is obtained. Both yeast derived enzymes show substantially more N-linked carbohydrate than the *A. niger* protein, and the degree of carbohydrate is dependent upon the secretion signal used (Fig. 4). A detailed kinetic comparison of the various enzymes shows no effect of the additional carbohydrate on enzyme activity, although the most hyperglycosylated material synthesized with the GO leader appears to be more thermostable than the *A. niger* protein.4 For some other heterologous proteins which are hyperglycosylated by yeast, such as tissue plasminogen activator, the extra carbohydrate substantially reduces the activity of the enzyme (MacKay, 1985). The GO proteins from both yeast and *A. niger* migrate as doublets on SDS gel electrophoresis after EndoH treatment. The cause of this is unknown but may be due to O-linked glycosylation, the anomalous SDS binding properties of the protein (Jones et al., 1982), or the absence of phosphate found at least in the *A. niger* enzyme (Swoloda and Massey, 1966b; James et al., 1981).

At present, the reason for the differential glycosylation of glucose oxidase between yeast and *A. niger* and in yeast using the two different leaders is unknown. Yeast is known to hyperglycosylate some secreted foreign proteins (Schultz et al., 1985; MacKay, 1987), especially those using the α-factor leader for secretion. This is most likely due to increased transfer of outer chain mannose to the same sites used by other organisms (*e.g.* *Aspergillus*) and, less likely, to the glycosylation of additional sites. Recent experiments suggest that KEX2 and mannosyltransferase I reside in different post-endoplasmic reticulum compartments in yeast (Cunningham and Wickner, 1989). The intermediate level of glycosylation seen in the pcrGO-1 transformants could be due to more rapid transit through a compartment or an alternate route through the secretory pathway for the α-factor-GO fusion rather than the GO protein. Alternatively, it is known that the three sites for N-linked glycosylation in the α-factor leader are utilized very efficiently (Julius et al., 1984). Thus, in the case of the α-factor-GO fusion protein, the α-factor sites may compete with the GO sites as exogenously added acceptors for N-linked glycosylation. This could either occur before or after cleavage of the α-factor-GO fusion protein, as exogenously added acceptors for N-linked glycosylation as small as tripeptides have been shown to compete efficiently for glycosylation sites in yeast (Rothblatt et al., 1987).

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REFERENCES


3 S. Rosenberg and K. Frederick, unpublished results.

4 K. Frederick and S. Rosenberg, unpublished data.
Additions and Corrections

Vol. 265 (1990) 908-913

Electrophysiological characterization of contact sites in brain mitochondria.

Oscar Moran, Gabriella Sandri, Enrico Panfili, Walter Stuhmer, and M. Catia Sorgato

Page 910: Fig. 3A should be replaced with the following figure:

Page 911. Fig. 5A: The correct unit of current amplitude is 140 pA.
Fig. 6A: The correct unit of current amplitude is 15 pA.
Fig. 7A: The correct unit of current amplitude is 20 pA.

Vol. 265 (1990) 3793-3802

Glucose oxidase from Aspergillus niger. Cloning, gene sequence, secretion from Saccharomyces cerevisiae, and kinetic analysis of a yeast-derived enzyme.


Dr. Schopfer's name was misspelled. The correct spelling is shown above.

Vol. 265 (1990) 6961-6966

Molecular cloning and amino acid sequence of peptide-N\textsuperscript{4}-(N-acetyl-\beta-D-glucosaminyl)asparagine amidase from Flavobacterium meningosepticum.

Anthony L. Tarentino, Geraldine Quinones, Anne Trumble, Li-Ming Chiang, Barry Duceman, Frank Maley, and Thomas H. Plummer, Jr.

Page 6963, Fig. 3: The nucleotide sequence contains two typographical errors: 1) At nucleotide position 97, the codon for arginine should read CGC, not GGC; and 2) at nucleotide position 444, the codon for serine should be TCC and not TCG.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Glucose oxidase from Aspergillus niger. Cloning, gene sequence, secretion from Saccharomyces cerevisiae and kinetic analysis of a yeast-derived enzyme.
K R Frederick, J Tung, R S Emerick, F R Masiarz, S H Chamberlain, A Vasavada, S Rosenberg, S Chakraborty, L M Schopfer and L M Schopter


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