Cu(I)-dependent Biogenesis of the Galactose Oxidase

Redox Cofactor*†

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Galactose oxidase is a copper metalloenzyme containing a novel protein-derived redox cofactor in its active site, formed by cross-linking two residues, Cys228 and Tyr272. Previous studies have shown that formation of the tyrosyl-cysteine (Tyr-Cys) cofactor is a self-processing step requiring only copper and dioxygen. We have investigated the biogenesis of cofactor-containing galactose oxidase from pregalactose oxidase lacking the Tyr-Cys cross-link but having a fully processed N-terminal sequence, using both Cu(I) and Cu(II). Mature galactose oxidase forms rapidly following exposure of a pregalactose oxidase-Cu(II) complex to dioxygen (τ1/2 = 8.9 s at pH 7). In contrast, when Cu(II) is used in place of Cu(I) the maturation process requires several hours (τ1/2 = 51 h). EDTA prevents reaction of pregalactose oxidase with Cu(II) but does not interfere with the Cu(I)-dependent biogenesis reaction. The yield of cross-link corresponds to the amount of copper added, although a fraction of the pregalactose oxidase protein is unable to undergo this cross-linking reaction. The latter component, which may have an altered conformation, does not interfere with analysis of cofactor biogenesis at low copper loading. The biogenesis product has been quantitatively characterized, and mechanistic studies have been developed for the Cu(I)-dependent reaction, which forms oxidized, mature galactose oxidase and requires two molecules of O2. Transient kinetics studies of the biogenesis reaction have revealed a pH sensitivity that appears to reflect ionization of a protein group (pKα ≈ 7.3) at intermediate pH resulting in a rate acceleration and protonation of an early oxygenated intermediate at lower pH competing with commitment to cofactor formation. These spectroscopic, kinetic, and biochemical results lead to new insights into the biogenesis mechanism.

Galactose oxidase (GAOX)1 (EC 1.1.3.9) is a secretory fungal copper metalloenzyme that generates hydrogen peroxide in the extracellular space by oxidizing simple alcohols and subsequently reducing dioxygen to H2O2 (1–4). Together with a closely related enzyme, glyoxal oxidase (5, 6), galactose oxidase represents a family of radical-copper oxidases defined by the presence of an unusual free radical-coupled copper active site (comprising a free radical associated with a redox-active metal ion) that functions as a two-electron redox unit in substrate oxidation and O2 reduction (7, 8). These free radical enzymes (9, 10) generate catalytic free radicals by reversible oxidation of a tyrosyl side chain in the protein (11). X-ray crystallography has revealed that the active site of galactose oxidase (Fig. 1) contains a novel post-translational modification, a covalent bond between Cys228 and Tyr272 creating a new, thioether-bridged cross-linked amino acid, tyrosyl-cysteine (Tyr-Cys), without addition of exogenous atoms (12, 13) (Scheme 1). Spectroscopic and chemical modeling studies have demonstrated that this Tyr-Cys side chain is, in fact, the free radical site in the protein (15–19). Characterization of the radical copper catalytic motif in galactose oxidase is complicated by the presence of multiple species in the as-isolated enzyme, including a significant fraction of apoenzyme, distinct forms of the metal- and cofactor-containing holoenzyme differing essentially in the number of electrons in the active site, as well as a portion of holoenzyme that is unable to generate a free radical complex and therefore does not contribute to catalytic activity (2, 7, 8).

X-ray structural studies are revealing cross-linked amino acid side chains in the active sites of a number of other redox metalloenzymes, including cytochrome c oxidase (20–22) (tyrosyl-histidine), catechol oxidase, hemocyanin (23–25) (histidyl-cysteine), and methylamine dehydrogenase (26, 27) (tryptophan-tryptophan). The origins and functions of these specialized elements of protein structure are just beginning to be investigated in detail. Formation of the tryptophanyl-tryptophan catalytic cofactor in methylamine dehydrogenase appears to involve processing by ancillary enzymes (28). In contrast, galactose oxidase cofactor biogenesis appears to be a self-processing event requiring only copper and O2 (29). Galactose oxidase is distinct from the other examples in being a secretory protein, which means that its release in functional form involves at least three processing steps as follows: cleavage of the prepro leader sequence that directs translocation of the nascent polypeptide chain into the secretory pathway, metal binding, and cofactor biogenesis. The structure of a partially processed precursor form of the protein, containing the 17-amino acid N-terminal prosequence leader peptide but lacking both copper and cofactor, has been reported recently (30). The active site region of the unprocessed protein in the crystal exhibits large amplitude displacements of critical active site residues, reflecting a significant degree of conformational flexibility in the incompletely processed protein. Covalent modification of the active site cysteine to a sulfenic acid (Cys228-SOH) observed in the crystal may reflect oxidative damage to the...
Galactose Oxidase Cofactor Biogenesis

Quantitation of free sulphydryl groups in pregalactose oxidase, galactose oxidase, and variants was done using the DTNB test on protein in 4 mM guanidine hydrochloride using cysteine as standard (37). Samples were heated in a water bath at 100 °C for 1 min and cooled on ice before addition of DTNB. Deblocking of cysteine sulfenic acid groups was performed as described (38–40). Briefly, protein was incubated with 20 mM t-ascorbate in 50 mM MES buffer, pH 5.3, for 0.5 h, and the reduced form was removed by gel filtration.

Biogenesis of the galactose oxidase cofactor from pregalactose oxidase and Cu(I) was conducted under argon purge in 20 mM MOPS, pH 7. Tetraakis(acetonitrile) Cu(I) hexafluorophosphate [Cu(I)-
(CH3CN)6PF6] was dissolved in anaerobic acetonitrile immediately before addition of an aliquot of this solution to argon-purged pregalactose oxidase. Pregalactose oxidase-Cu(I) was rapidly purged with pure oxygen. Quantitation of the oxygen stoichiometry for cofactor biogenesis was performed by adding aliquots of air-saturated buffer to a mixture containing pregalactose oxidase and a subsistochiometric amount of Cu(I). The pregalactose oxidase-Cu(I) complex was prepared by combining 150 nmol of CuI (CH3CN)6PF6 with 600 nmol of pregalactose oxidase in a 3-mL anaerobic cuvette under argon. The optical absorption of the stirred solution was monitored at 445 nm.

The mutual stability of pre-GAOXCu(I) and fully oxidized mature GAOX in a mixture containing both was determined by sequential preparation of a pre-GAOXCu(I) complex, addition of oxidized GAOX, and O2 in that order. Copper(II) acetonitrile (60 nmol, 0.6 eq) was added to pre-GAOX (100 mM, 1 μl) under anaerobic conditions. An anaerobic solution of fully oxidized mature GAOX (GAOXX) (60 nmol in 50 μl) was added, and the optical absorption spectrum was recorded and monitored at 445 nm. The cuvette was then purged with O2 gas and the optical spectrum recorded.

Copper analyses were performed using a Varian Instruments SpectraAA atomic absorption spectrometer equipped with a GTA 96 furnace. Galactose oxidase activity was measured by oxygen uptake in a Clark-type oxygen electrode. The assay mixture contained 50 mM O-methyl-α-galactopyranoside, 2 mM KFe(CN)6, and 50 mM KH2PO4, pH 7.

EXPERIMENTAL PROCEDURES

Biological Materials—P. pastoris X33 (32) was obtained from Invitrogen. Recombinant galactose oxidase was purified from high density methanol fermentation medium (33) of a P. pastoris transformant prepared by multiplex chromosome integration of an expression cassette comprising the pPICZ Zeocin-selection plasmid (Invitrogen) linearized by digestion with PseI. The coding region of the expression cassette contains a 5′-nucleotide sequence coding for either the Aspergillus niger glucoamylase leader peptide (Gla) or the Brevibacterium fuscum glucoamylase leader peptide (G228C, Y272G) using Stratagene (La Jolla, CA) QuikChange™ site-directed mutagenesis kit, and the protein was expressed and purified as described previously. For secretion of the unprocessed, cofactor-free pregalactose oxidase protein, the transformant was grown as described previously (31) except that the copper content of the medium was reduced to 15% of the amount described for the PTM4 trace metals supplement in the earlier work (33). The other modifications to fermentation conditions reported previously (31) were the elimination of casamino acids from the methanol induction phase and the exclusion of copper from the methanol feedstock during expression. Pregalactose oxidase was purified as described previously (31) except that 2 mM EDTA was present in all buffer solutions. For biogenesis studies, the protein was passed through a gel filtration column to remove EDTA. Protocatechuate 3,4-dioxygenase was isolated from Brettbacterium fuscum as described previously (34).

Reagents—MOPS, MES, CHES, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 1-cysteine, 1-O-methyl-α-galactopyranoside, and EDTA were obtained from Sigma. Aconitidine, deuterium oxide (99.9 atom % 2H), and tetrakis(acetonitrile) copper(I) hexafluorophosphate [Cu(I)-(CH3CN)6PF6] were purchased from Aldrich. Guanidinium hydrochloride was from Pierce, and potassium ferricyanide was from Fluka (White Plains, NY).

Biochemical Methods—Protein concentrations of purified galactose oxidase and pregalactose oxidase were determined by optical absorption measurements, using the molar extinction coefficient at 280 nm (ε280 = 1.05 × 105 M−1 cm−1) as reported previously (35). Proteins resolved by SDS-PAGE (Bio-Rad ready-gels) were stained with GelCode Blue™ staining solution (Pierce). Gels were digitized using a scanner and analyzed with image Measurement and Analysis program (36). Strip densitometric scan data were further analyzed using the line shape deconvolution routines of the Grams spectral analysis program (Galactic Industries Corp., Salem, NH). N-terminal sequence analysis of purified pregalactose oxidase was performed by Debra A. McMullen at the Biotechnology Laboratory, Institute of Molecular Biology, University of Oregon.

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The reaction was monitored at 25 °C, and the electrode was calibrated using the protocatechuic acid/protocatechuate dioxygenase reaction (41). An aerobic solution of GAOX in methanol was added to the reagent containing mature galactose oxidase. The pH sensitivity of the biogenesis of the galactose oxidase Cofactor was investigated by heating pregalactose oxidase/Cu(I) (prepared anaerobically in buffer-free water) against air-saturated 40 mM buffer solutions in the SFM-300 syringe driver. The...
buffer solutions were used as follows: pH 5.0–5.5, MES; pH 6.0–6.5, MES/MOPS mixture; pH 7.0, MOPS; pH 7.5–8.0, MOPS/CHES mixture; pH 8.5, CHES. Solvent kinetic isotope (SKIE) measurements were performed by shooting pregallactose oxidase/Cu(I) prepared as above in either H2O or D2O (containing 0.5 mM MOPS, pH 7.0) against air-saturated buffers (50 mM before mixing) in H2O or D2O, adjusted to the coordinates (Protein Data Bank code 1GOG) and has been rendered

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thioether cross-link between Cys228 and Tyr272 (Fig. 1) but the transformants produced pregallactose oxidase lacking the induction phase of high density methanol fermentation, glycerol batch growth phase and eliminated completely in the medium (31). When copper supplementation was reduced in the medium (31), the background galactose oxidase activity of the transformants containing pregallactose oxidase/cDNA linked to either A. niger glucoamylase leader peptide or S. cerevisiae aMf leader peptide coding sequences efficiently secrete mature galactose oxidase under methanol-induced expression in complete medium (31). When copper supplementation was reduced in the glycerol batch growth phase and eliminated completely in the induction phase of high density methanol fermentation, the transformants produced pregallactose oxidase lacking the thioether cross-link between Cys228 and Tyr272 (Fig. 1) but exhibiting the N-terminal sequence (ASAPI) of the authentic mature protein. The yield of purified pregallactose oxidase was ~200 mg/liter for a 5-liter fermentation culture harvested 1 day after induction. The background galactose oxidase activity of the purified pregallactose oxidase was about 0.1% (~0.7 μmol of O2/mg of protein at 25 °C) of the recombinant WT galactose oxidase. Analysis of the sulfhydryl content of pregallactose oxidase using the DTNB assay (Table I) shows that two representative preparations of pregallactose oxidase (Table I, pre-GAOX (A) and pre-GAOX (B)) exhibited roughly 0.8 and 0.9 mol SH/mole protein, respectively.

The positions of the pregallactose oxidase (a) and mature galactose oxidase (b) were analyzed using the strip densitometry routine of ImageJ analysis software as described under “Experimental Procedures.” The positions of the pregallactose oxidase (a) and mature galactose oxidase (b) protein standards are indicated.

Analysis of free sulfhydryl content of galactose oxidase variants

| Variant            | mol SH/mol protein |
|--------------------|-------------------|
| WT apoGAOX         | 0.76              |
| C228G GAOX         | 0.89              |
| Y272G GAOX         | 1.49              |
| Pre-GAOX (A)       | 1.68              |
| Pre-GAOX (B)       | 1.52              |

*Sulfhydryl content determined by DTNB assay as described under “Experimental Procedures.”

| Variant | lane 1 | lane 2 | lane 3 | lane 4 | lane 5 | lane 6 | lane 7 |
|---------|--------|--------|--------|--------|--------|--------|--------|
| Pre-GAOX (A) | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   |
| Pre-GAOX (B) | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   |

Variation in the free sulfhydryl content between preparations was roughly proportional to Cu(I) added up to about 0.8 eq (Table I, pre-GAOX(A)). Lower conversion was observed in some preparations (Table I, pre-GAOX(B)), in which the amount of cross-linked product was proportional to Cu(I) added only up to about 0.5 eq. Thus, the upper limit to conversion appears to vary somewhat between pre-enzyme preparations. Similar results were observed by addition of Cu(II)SO4 to pregallactose oxidase in the presence of oxygen (Fig. 2, lane 7). Varying the sample concentration (from micromolar to millimolar) did not change the yield, and the protein began to precipitate when more than 1 eq of copper was added. Unmodified pre-GAOX appears to be more sensitive to precipitation in excess copper than the mature GAOX protein. Treating pre-GAOX with ascorbic acid at low pH (method for deblocking cysteine sulfenic acid groups, Cys-SOH) did not increase the

RESULTS

Production of Pregallactose Oxidase—P. pastoris transformants containing galactose oxidase cDNA linked to either A. niger glucoamylase leader peptide or S. cerevisiae aMf leader peptide coding sequences efficiently secrete mature galactose oxidase under methanol-induced expression in complete medium (31). When copper supplementation was reduced in the glycerol batch growth phase and eliminated completely in the induction phase of high density methanol fermentation, the transformants produced pregallactose oxidase lacking the thioether cross-link between Cys228 and Tyr272 (Fig. 1) but exhibiting the N-terminal sequence (ASAPI) of the authentic mature protein. The yield of purified pregallactose oxidase was ~200 mg/liter for a 5-liter fermentation culture harvested 1 day after induction. The background galactose oxidase activity of the purified pregallactose oxidase was about 0.1% (~0.7 μmol of O2/mg of protein at 25 °C) of the recombinant WT galactose oxidase. Analysis of the sulfhydryl content of pregallactose oxidase using the DTNB assay (Table I) shows that two representative preparations of pregallactose oxidase contain approximately twice as many free SH groups as mature WT galactose oxidase, confirming the presence of an additional, predominantly unblocked cysteine residue (Cys228) in these preparations. The amount of free sulfhydryl varies somewhat between preparations.

Detection of Cross-linked Product by SDS-PAGE—Mature, cross-linked cofactor-containing galactose oxidase shows an altered mobility on SDS-PAGE gels (Fig. 2, lane 6) and is resolved from unmodified pregallactose oxidase (Fig. 2, lane 1), as reported previously (29). Mature galactose oxidase appeared following addition of Cu(I)(CH3CN)4PF6 to pregallactose oxidase under anaerobic conditions followed by oxygen gas purging (Fig. 2, lanes 2–5). The amount of mature product formed was roughly proportional to Cu(I) added up to about 0.8 eq (Table I, pre-GAOX(A)). Lower conversion was observed in some preparations (Table I, pre-GAOX(B)), in which the amount of cross-linked product was proportional to Cu(I) added only up to about 0.5 eq. Thus, the upper limit to conversion appears to vary somewhat between pre-enzyme preparations. Similar results were observed by addition of Cu(II)SO4 to pregallactose oxidase in the presence of oxygen (Fig. 2, lane 7). Varying the sample concentration (from micromolar to millimolar) did not change the yield, and the protein began to precipitate when more than 1 eq of copper was added. Unmodified pre-GAOX appears to be more sensitive to precipitation in excess copper than the mature GAOX protein. Treating pre-GAOX with ascorbic acid at low pH (method for deblocking cysteine sulfenic acid groups, Cys-SOH) did not increase the
yield of mature GAOX. Other attempts (varying salt concentration, pH, buffer, addition of superoxide and hydrogen peroxide scavengers, and repeating the Cu(I) treatment on the initially formed biogenesis product) also failed to increase the upper limit of conversion to the mature GAOX product.

Densitometric analysis of the scanned gel (Fig. 2, bottom) provides quantitative information on the yield of mature, cross-linked GAOX in the biogenesis reaction (Table II). Gaussian resolution of the individual protein bands in the stained SDS-PAGE is reported in Table II. The yield of cross-link correlates with the amount of copper added up to about 0.8 eq. The upper band on the gel (a), corresponding to pre-GAOX, is broader than that associated with the mature protein (b), and the broadening increases with the age of the sample.

**Monitoring Formation of Fully Oxidized Cofactor in Optical Absorption Spectra**—When freshly prepared Cu(I)-(CH₃CN)₄PF₆ was added to pre-GAOX under strictly anaerobic conditions, the spectroscopic signature of fully oxidized galactose oxidase appears immediately following exposure to oxygen (Fig. 3, spectra 2–5). The reaction is very fast, and rapid mixing stopped-flow techniques were required for more detailed kinetic analysis (see below). Quantitation of the amount of fully oxidized GAOX formed in these reactions, based on the published extinction coefficient for the mature protein-free radical-coupled copper complex, corrected for inactive species (ε₄₄₅ = 9500 M⁻¹ cm⁻¹) (41), shows that the yield is proportional to the amount of Cu(I) added up to about 0.8 eq, similar to the results found for cross-link formation in the SDS-PAGE gel analysis of the reaction products (Fig. 2). No oxidized GAOX was observed prior to addition of O₂ to the pre-GAOX (Fig. 3, spectrum 0) or the pre-GAOXCu(I) mixture (Fig. 3, spectrum 1). The biogenesis products were analyzed for enzymatic activity and copper content before and after gel filtration (Table III). Galactose oxidase activity was essentially unchanged by gel filtration, whereas the copper content decreased slightly, indicating the presence of a fraction of relatively weakly bound metal ions in the biogenesis product that were not associated with functional active sites. The catalytic activity of the biogenesis product, normalized to copper, was similar to that observed for the as-isolated galactose oxidase (Table III). Higher normalized specific activity was observed at lower copper loading, possibly reflecting microheterogeneity within the functional fraction of pre-enzyme, with the fraction that binds Cu(I) most tightly yielding the most efficient processing. Comparison of the fraction of functional active sites formed in the reaction (Table III) with the fraction of sites converted to oxidized mature enzyme based on the absorption spectra (Fig. 3) indicates that every functional site was represented in the 445 nm absorption of oxidized product. Regression of the absorption intensity data for in vitro cofactor biogenesis (from Fig. 3) against the cofactor concentration determined by densitometric analysis of the SDS-PAGE resolved products from these reactions (Fig. 2) yielded an empirical absorption coefficient for cofactor-containing product (ε₄₄₅ = 5200 M(protein)⁻¹ cm⁻¹) which was very similar to the observed absorption coefficient for the as-isolated enzyme (ε₄₄₅ = 5100 M(protein)⁻¹ cm⁻¹) (7)). The maximum optical absorption at 445 nm and catalytic activity of the biogenesis product reached a plateau at 85% of the as-isolated enzyme (Fig. 3; Table III).

In order to evaluate the stability of the pre-GAOXCu(I) complex in the presence of fully oxidized mature GAOX, the optical absorption spectra of anaerobic pre-GAOXCu(I) has been measured following addition of anaerobic fully oxidized AGAOX, as described under “Experimental Procedures.” The resulting mixture exhibited the full absorption intensity of the added AGAOX, which was stable for at least 10 min. Purging this mixture with oxygen gas resulted in a further increase in the intensity of the AGAOX spectrum, yielding an absorption change ΔA₄₄₅ = 0.25, corresponding to 90% of the change observed for reaction of pre-GAOXCu(I) with O₂ in the absence of AGAOX (see above).

The stoichiometry of the O₂ reaction with pre-GAOXCu(I) was investigated at low copper loading (Fig. 4) and the lower range of conversion (spanning the first third of the complete reaction) to ensure the most efficient processing. Aliquot additions of air-saturated buffer produced a linear increase in absorption at 445 nm, corresponding to formation of the oxidized mature AGAOX product, and regression analysis of the data yielded a reaction stoichiometry of 1.8 O₂/AGAOX product under these conditions.

**Cu(II) Reactions**—In the presence of oxygen the addition of Cu(II)SO₄ to pre-GAOX led to the slow formation of mature cross-linked protein (Fig. 2, lane 7) exhibiting absorption features resembling those characteristic of the oxidized enzyme (Fig. 5). The slow conversion allowed the progress of the reac-
Table III

| Reaction/sample | Before gel filtration | After gel filtration |
|----------------|----------------------|----------------------|
|                | Copper added | Activity | Copper retained | Activity | Activity |
|                | mol copper/mmol protein | µmol O₂/mg protein at 25 °C | mol copper/mmol protein | µmol O₂/mg protein at 25 °C | µmol O₂/mg protein/copper at 25 °C |
| 1 Pre-GAOX + Cu(I) | 0.215 ± 0.013 | 155 ± 1 | 0.175 ± 0.002 | 167 ± 4 | 954 ± 25 |
| 2 Pre-GAOX + Cu(I) | 0.364 ± 0.002 | 307 ± 5 | 0.335 ± 0.015 | 307 ± 1 | 916 ± 41 |
| 3 Pre-GAOX + Cu(I) | 0.539 ± 0.008 | 400 ± 9 | 0.457 ± 0.004 | 385 ± 20 | 842 ± 44 |
| 4 Pre-GAOX + Cu(I) | 0.790 ± 0.004 | 422 ± 31 | 0.582 ± 0.001 | 453 ± 20 | 778 ± 34 |
| 5 Pre-GAOX + Cu(I) | 0.711 ± 0.013 | 288 ± 4 | 0.494 ± 0.004 | 301 ± 3 | 609 ± 8 |
| GAOX (as isolated) | 0.002 | 144 | 0.013 | 258 |
| GAOX + Cu(I) (0.4 eq) | 0.699 ± 0.033 | 520 ± 23 | 0.652 ± 0.015 | 532 ± 14 | 850 ± 30 |

Fig. 4. Oxygen stoichiometry of the cofactor biogenesis reaction. Air-saturated 20 mM MOPS, pH 7 (265 µM dissolved O₂, 23 °C), was added incrementally to a stirred solution of pre-GAOX (0.2 mM in 20 mM MOPS, pH 7) plus 0.25 eq Cu(II) (CH₃CN)₂PF₆, and absorption changes were monitored at 445 nm. Regression of the total amount of O₂ added (ordinate) versus the yield of oxidized, mature GAOX product (evaluated from the A₁₇₀, using the extinction coefficient for GAOX, \( \epsilon_{A_{170}} = 1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1} \) (absissa) yields an O₂ stoichiometry of 1.8 O₂/GAOX product. The regression line (\( R = 0.998 \)) is shown.

Fig. 5. Formation of oxidized galactose oxidase by Cu(II)-dependent cofactor biogenesis. The reaction mixture contained 0.2 mM pre-GAOX + 0.6 µl of 20 mM CuSO₄ in 20 mM MOPS, pH 7, under aerobic conditions. Inset, analysis of the absorption data in terms of single-exponential relaxation. The theoretical curve (solid line) is calculated for \( k_{\text{Cu(I)oxid}} = 3.8 \times 10^{-5} \text{ s}^{-1} \).

Fig. 6. Anaerobic transformation of the pregalactose oxidase Cu(II) complex. The reaction mixture contained 0.25 mM pre-GAOX in 20 mM MOPS, pH 7, + 0.8 eq CuSO₄ under argon. Progress of the reaction was followed by repetitive scanning (10 min intervals displayed). Inset, analysis of the absorption data in terms of single-exponential relaxation. The theoretical curve (solid line) is calculated for \( k_{\text{Cu(II)oxid}} = 3.2 \times 10^{-4} \text{ s}^{-1} \).
Resonance Raman (RR) spectroscopy has been used to characterize further the 406 nm species. The RR spectrum of the pregalactose oxidase Cu(II) complex (20 mM MOPS, pH 7, buffer) showed strong resonance enhancement of a fundamental characteristic feature of the oxidized mature GAOX (Fig. 8). The RR spectrum is sharp (15 cm$^{-1}$ full-width at half-height) corresponding to a well defined structure from a discrete copper complex. No other RR signals were observed at higher frequency. When this pre-formed 406 nm species (Fig. 6B) is exposed to oxygen, the spectra progressively develop the characteristic features of the oxidized mature GAOX (Fig. 8). The kinetics of this transformation are associated with a first order rate constant $k_{406,ox} = 3.9 \times 10^{-5}$ s$^{-1}$ (Fig. 8, inset).

Biogenesis of the Tyr-Cys cofactor with Cu(II) was blocked in the presence of EDTA (Fig. 9, spectrum B; inset, lane 3). Weak absorption is observed near 750 nm, consistent with formation of a Cu(II)-EDTA complex under these conditions. This spectrum did not change for up to 6 h, indicating that the EDTA chelate competes very effectively with pre-GAOX for the Cu(II) metal ion. In contrast, the Cu(I)-dependent biogenesis reaction was not affected by the presence of EDTA (Fig. 9, spectrum C; inset, lane 2). There was no evidence for the formation of Cu(II)-EDTA in this sample (Fig. 9, spectrum B), demonstrating that the copper remained in the Cu(I) oxidation state.

**Transient Kinetics**—The relatively rapid biogenesis reaction supported by Cu(I) and dioxygen, which occurs efficiently on a physiologically relevant time scale, has been developed for a more detailed kinetic and mechanistic analysis. Rapid kinetics analysis of the biogenesis reaction takes advantage of the intense optical spectrum of the oxidized GAOX product to monitor the formation of the protein cross-link and potentially detect other absorbing species involved in the reaction. Because the pre-GAOXCu(I) complex was extremely sensitive to O$_2$, special precautions are required in its preparation and handling to exclude air and ensure anaerobiosis without compromising the stability of the oxidized GAOX product. We have found that preparation of the pre-GAOXCu(I) complex in an air-free tonometer allowed storage for the duration of the kinetics experiment and that argon purging of the luer inlet ports for the stopped-flow syringe head substantially improved the anaerobic transfer of the sample into the firing syringe. Nitrogen purging of the syringe chamber and scrubbing dioxygen from internal Teflon surfaces in the flow circuit by preincubation with a protocatechuic acid/protocatechuate dioxygenase mixture (41, 42) also contributed to the anaerobic performance of the stopped-flow system.

The reaction of the preformed pre-GAOXCu(I) complex with air-saturated buffer at 20°C is shown in Fig. 10. The raw spectra (Fig. 10, top left) were recorded with an OLIS RSM-1000 rapid scan monochromator, collecting the complete 325–800 nm visible spectrum (3 nm resolution for acquisition) every 1 ms and averaging to generate 31 scans/s. Data were collected for 10 s or as required to reach an end point for the absorption transients. Raw binary data were subsequently analyzed using global fitting routines (Specfit/32, Spectrum Software Assoc i-
Fig. 9. Biogenesis of the galactose oxidase Tyr-Cys cofactor with Cu(I) or Cu(II) in the presence of EDTA. A, 50 μM pre-GAOX prepared anaerobically in 20 mM MOPS, pH 7, containing 0.25 mM EDTA. B, following addition of 0.75 eq of Cu(I)(CH$_3$CN)$_4$PF$_6$ to sample (A). C, following O$_2$ purging of sample (B). D, 50 μM pre-GAOX prepared anaerobically in 20 mM MOPS, pH 7, containing 0.25 mM EDTA. E, following addition of 0.92 eq of CuSO$_4$ to sample (D). Copper quantitation for each sample was determined by atomic absorption spectrometry as described under “Experimental Procedures.” Inset, SDS-PAGE of reaction products. Lane 1, pre-galactose oxidase; lane 2, sample C; lane 3, sample E; lane 4, mature GAOX. Each lane was loaded with 0.5 μg of protein.

ates, Marlborough, MA). The noise-factored spectra (Fig. 10, left middle) illustrate the quality of spectroscopic information that may be evaluated in the large data sets resulting from rapid scan spectroscopy. The formation of the oxidized GAOX product occurs in a single exponential phase with a unique set of kinetic parameters giving excellent fits to the data over the entire spectral region (Fig. 10, right) and leaving uniform excursions for the residuals over the entire time course of the data. Allowing both starting and final species to contribute to the optical absorption spectrum leads to a resolution of spectral vectors as shown in Fig. 10 (lower left).

Varying the pH of the air-saturated buffer solution used to initiate the biogenesis reaction allowed the pH profile for the reaction to be systematically explored. In the higher range of pH (7 and above), the simple exponential relaxation kinetics described above provided an excellent description of the data, although the rate of the transformation showed a strong pH sensitivity. At lower pH, a very different product spectrum was observed lacking the NIR absorption characteristic of oxidized GAOX and exhibiting a blue-shifted absorption maximum (Fig. 11, top). The kinetics associated with this low pH transformation is clearly multiphasic, and the process has been analyzed in terms of competing first order reactions yielding oxidized GAOX and the 406-nm species of pre-GAOXCu(II) as the respective products (Fig. 11, bottom). The model is justified in terms of the following mechanistic interpretation shown,

$$\begin{align*}
\text{(A)} & \quad \text{pre-GAOXCu(II)} + O_2 \rightarrow \text{oxidized GAOX} \\
\text{(B)} & \\
\text{(C)} & \quad \text{pre-GAOXCu(II)} + O_2 + H^+ \rightarrow \text{pre-GAOXCu(II)} \\
\text{(D)} & \quad k_4 \quad \text{HO}_2 \rightarrow \text{406 nm species} \\
n & \quad k_1 \quad \text{Reaction 1} \\
n & \quad k_2 \\
n & \quad k_3 \quad \text{Reaction 2}
\end{align*}$$

The two alternative pathways compete for the same reactive pre-GAOXCu(II) starting complex (A), and the relative rates determine the commitment to either cofactor biogenesis and formation of oxidized GAOX (B) (Reaction 1) or an abortive process in which the initially formed oxygenated complex breaks down to a pre-GAOXCu(II) complex (C) without forming the Tyr-Cys cross-link, and subsequently converts to the more strongly absorbing 406-nm species (D) (Reaction 2). In this scheme, only species B and D contribute significantly to the optical absorption spectrum. In order to explore this scheme, the full data set was resolved onto target spectra corresponding to pure oxidized GAOX and the 406 nm-absorbing (relaxed) form of the pre-GAOXCu(II) complex (Fig. 11, middle), providing estimates for the rate constants $k_1$ and $k_2$ for the two processes competing for the pre-GAOXCu(II) initial complex. As expected from previous characterization of the pre-GAOXCu(II) complex (Fig. 6), the rate constant for emergence of the 406-nm species ($k_4$) is orders of magnitude slower and not well determined by measurements on the seconds time scale. The spectroscopic resolution of the kinetic processes predicts a spectrum associated with C and defined by a weak absorption maximum above 500 nm ($\varepsilon = 800$ M$^{-1}$ cm$^{-1}$), similar to that observed for the initial pre-GAOXCu(II) complex (Fig. 6A). The pH dependence of the rates is plotted in Fig. 12, which shows that above pH 7, Reaction 1 dominates, whereas at lower pH Reaction 2 becomes significant. SDS-PAGE analysis of the pH 5 reaction mixture (data not shown) shows a dramatic decrease in the yield of cross-linked product.
The sensitivity of the biogenesis reaction to the isotopic composition of the solvent (\(H_2O\) or \(D_2O\)) has also been investigated, comparing reactions based on Cu(I) and Cu(II) in native sequences and pre-GAOCu(I) complexes (29). The observation that chimeric proteins based on Gla or GMF and the mature galactose oxidase protein sequence are efficiently expressed as functional, cofactor-containing enzyme by \(P.\) pastoris (31) implies that the native signal sequence is not specifically required for cofactor formation. As demonstrated in the present work, the purified recombinant pre-enzyme free of any signal sequence is also competent for cofactor biogenesis.

\(P.\) pastoris secretes pregalactose oxidase (rather than the mature enzyme) under copper-limiting conditions even without stringent exclusion of copper from the culture media, apparently through regulation of the metal ion distribution within the cell. All of the copper present in the medium can be accounted for in the \(Pichia\) cell mass following high density fermentation, implying efficient absorption of the trace metal. Intracellular copper may be specifically targeted to the mitochondria and peroxisomes in methylotrophic yeast, particularly under methanol induction (45), effectively restricting the amount of copper reaching the secretory pathway without impairing the robust respiratory proficiency required for methanol fermentation. In contrast, pre-GAOCX expression in \(Aspergillus\) culture is reported to require strict exclusion of copper from the culture media (29), resulting in copper starvation and pleiotropic stress effects on the expression host that may account for the incomplete leader sequence processing reported under these conditions.

The metal oxidation state dependence for cofactor biogenesis (29), Little information is presently available on the in vivo maturation of galactose oxidase, which likely occurs as a post-translational event in the trans-Golgi compartment of the secretory pathway. Copper is delivered to this compartment by a Cu(I)-specific transporter (for example, the CCC2 copper transporter in \(S.\) cerevisiae) (44). Previous work has demonstrated that the GAOX biogenesis reaction can occur in vitro when purified prosequence-containing precursor protein is treated with Cu(II) and \(O_2\) (29). However, the observation that chimeric proteins based on a fusion between heterologous leader sequences (Gla or GMF) and the mature galactose oxidase protein sequence are efficiently expressed as functional, cofactor-containing enzyme by \(P.\) pastoris (31) implies that the native signal sequence is not specifically required for cofactor formation. As demonstrated in the present work, the purified recombinant pre-enzyme free of any signal sequence is also competent for cofactor biogenesis.

DISCUSSION

The active site of galactose oxidase has evolved two distinct but related functions, cofactor assembly and catalysis. The turnover reaction, which has been the focus of the majority of earlier work (2–4), depends on the presence of a correctly formed redox cofactor in the active site, and a mutational variant lacking the active site Cys\[^{228}\] that is unable to form the Tyr-Cys cross-link is virtually inactive as a catalyst, even though it binds copper (43). Self-assembly of the Tyr-Cys redox cofactor is a second intrinsic, but noncatalytic, reactivity of the active site (29). Little information is presently available on the in vivo maturation of galactose oxidase, which likely occurs as a post-translational event in the trans-Golgi compartment of the secretory pathway. Copper is delivered to this compartment by a Cu(I)-specific transporter (for example, the CCC2 copper transporter in \(S.\) cerevisiae) (44). Previous work has demonstrated that the GAOX biogenesis reaction can occur in vitro when purified prosequence-containing precursor protein is treated with Cu(II) and \(O_2\) (29). However, the observation that chimeric proteins based on a fusion between heterologous leader sequences (Gla or GMF) and the mature galactose oxidase protein sequence are efficiently expressed as functional, cofactor-containing enzyme by \(P.\) pastoris (31) implies that the native signal sequence is not specifically required for cofactor formation. As demonstrated in the present work, the purified recombinant pre-enzyme free of any signal sequence is also competent for cofactor biogenesis.

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and Cu(II) treatment. Copper in both oxidation states appears to support cross-linking and cofactor formation (Fig. 2), but the reactions occur on very different time scales. While the Cu(I)-dependent reaction is completed in seconds ($t_{1/2} = 3.9$ s at pH 7), cofactor formation following addition of Cu(II) occurs over several hours ($t_{1/2} = 5.1$ h), so slowly, in fact, that we cannot rule out the possible involvement of a Cu(I) species arising from reduction of Cu(II) by minor contaminants in the sample. Cu(II) interactions with pre-GAOX are relatively complicated, and in the absence of O$_2$, several distinct complexes are formed on different time scales. An initially formed weakly absorbing complex converts slowly to a distinct form exhibiting strong absorption near 406 nm in the visible spectrum (Fig. 6). The latter absorption feature may be assigned to a Cu(II)-thiolate ligand-to-metal charge transfer excitation based on resonance Raman enhancement of a Cu-S stretch mode (46–48). The measured vibrational frequencies are characteristic of Cu-S stretch modes from tetragonal type II copper sites (49). The spectrum implies coordination by a single thiolate ligand, because tetragonal copper sites with two thiolate ligands display additional combination bands at higher frequency, which are not observed for this sample. The transition energy and intensity for the 406-nm absorption feature is typical of low-coordinated copper thiolate complexes, as have been characterized previously in mutational variants of Cu/Zn superoxide dismutase (46, 47) and azurin (48). The appearance of the 406 nm absorption band reflects a slow isomerization of the active site structure permitting the Cys$^{228}$ sulfur to bind directly to the metal ion. The change in active site structure is also reflected in the perturbed EPR parameters for the protein-bound paramagnetic metal ion (Fig. 7). There is no evidence for stabilization of a protein radical within the anaerobic Cu(II) complex in the EPR spectra for any of these complexes at either cryogenic or ambient temperatures (Fig. 7).

The rapid Cu(I)-dependent biogenesis reaction may more closely resemble the physiological process. This reaction is easily monitored by observing the appearance of the optical absorption signature of fully oxidized mature (cofactor-containing) product following exposure of a preformed pre-GAOXCu(I) anaerobic complex (Fig. 3) to dioxygen. Characterization of biogenesis products formed over a range of copper loading indicates that there are two main subpopulations within the pre-GAOX with relative proportions that vary between preparations (Fig. 3). One subpopulation binds Cu(I) relatively tightly (and is therefore the first to titrate with metal when substoichiometric copper is added) and efficiently forms the cross-linked product on subsequent exposure to O$_2$. Titration of this fraction with O$_2$ (Fig. 4) indicates that two molecules of oxygen are required for the appearance of the oxidized mature enzyme product, which is formed even in the presence of excess unreacted pre-GAOXCu(I) complex. The presence of a second component is indicated by the anomalous behavior of the porphyrin about 0.5–0.8 eq copper loading (Figs. 2 and 3; Table III) and the persistence of a fraction of pre-GAOX in the reaction mixture (Fig. 2, Table II), implying a limit to conversion to cofactor-containing product. EDTA prevents Cu(II) from binding to pre-GAOX (Fig. 9), yet the Cu(I)-dependent biogenesis reaction proceeds in the presence of the chelator, demonstrating that the Cu(I) in solution is efficiently bound by the preenzyme and supports rapid biogenesis of the Tyr-Cys cofactor.

Absorption spectra reported in an earlier study of Cu(I)-dependent cofactor biogenesis imply a substoichiometric conversion to mature galactose oxidase product using propeptide-containing precursor (29).

We have investigated a number of possible explanations for the substoichiometric conversion of pre-GAOX. One possibility, that a diffusible species generated during the in vitro biogenesis reaction interferes with complete conversion, appears to be ruled out by the observation that the same result is observed at widely different protein concentrations, and is not affected by including scavengers like superoxide dismutase or catalase in the reaction mixture. The sulphydryl content measured for the precursor protein is less than the theoretical amount (Table I), suggesting that a fraction of the free cysteine residues may be blocked. However, ascorbate treatment (to reduce cysteine sulfenyl modifications, reported to be present in the crystal structure of the GAOX precursor (30)) did not alter the yield of cofactor. Other irreversible modifications of protein side chains may still interfere with the biogenesis reaction, or the incompetent fraction may represent an alternative conformation. If so, this alternate conformation must not equilibrate with the competent population, because repeating the Cu(I) + O$_2$ treatment on the biogenesis product did not increase the yield of cross-link or the specific activity. Conformational changes and side chain modifications would have less of an effect during the prompt processing of pre-GAOX that is expected to occur in vivo, accounting for the higher level of conversion observed for the in vitro reaction product. By controlling the amount of copper added to the pregalactose oxidase protein, it has been possible to focus on the competent fraction to investigate the in vitro biogenesis reaction of pre-GAOX.

Gel densitometry has allowed us to correlate formation of a cross-linked biogenesis product with the absorption intensity of the oxidized mature enzyme produced in the reaction. The absorption coefficient of the oxidized product, normalized to copper, is nearly identical to that observed for oxidatively activated as-isolated GAOX ($\epsilon_{445} = 5100 \text{ M}^{-1} \text{cm}^{-1}$). This is substantially less than the theoretical extinction coefficient for the oxidized product predicted by correcting for the apoprotein and unactivatable fractions in the as-isolated enzyme ($\epsilon_{445} = 1 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$). The specific activity of the biogenesis product at lower copper loading (Table III) is also comparable with that found for the as-isolated enzyme. It is clear that in vitro biogenesis leads to a product distribution very similar to that found for the in vivo reaction, with a substantial fraction of unactivatable (catalytically inactive) but cross-linked sites, implying that the complexity observed for the as-isolated protein is an intrinsic feature of the biogenesis reaction itself. Whether this component reflects an alternative cross-linking pattern (for example, Cys$^{228}$-Trp$^{250}$) or irreversible modification of the Tyr-Cys site (for example, oxygenation to form a sulfonyl or sulfonyl derivative (38–40)) will require a more detailed structural characterization of these products.

Kinetic studies are providing important clues to the mechanism of cofactor biogenesis in galactose oxidase. The Cu(I)-dependent reaction is very fast, and rapid reaction techniques (i.e. stopped-flow spectrophotometry) are required to analyze the kinetics. Lacking signals from either the initial pre-GAOXCu(I) complex or any reaction intermediate (Fig. 10), cofactor formation has been monitored through the appearance of oxidized GAOX product in these experiments. This product is likely formed by oxidation of an initial, reduced mature enzyme complex (see below) in a reaction corresponding to the O$_2$ half-reaction of the normal turnover cycle (2). The reaction of fully reduced, mature GAOX (containing Cu(I) and no radical) with dioxygen is known to be extremely rapid ($k_{\text{obs}} = 8 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) (41, 50), independent of pH (50), shows no significant SKIE (41), and is therefore well suited to serve as a non-interfering monitoring reaction. Although oxidized mature GAOX has been reported to be unstable in the presence of the fully reduced, Cu(I)-containing mature GAOX, undergoing rapid bimolecular redox comproportionation ($k_{\text{comp}} = 4.4 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$) to form...
our working hypothesis for the biogenesis mechanism (Scheme 2A) is based on involvement of mononuclear Cu(I) and dioxygen, an odd-electron system, implying that free radical intermediates occur in the formation of the Tyr-Cys cross-link. In the model shown here, Cu(I) is bound in a trigonal complex most likely formed by Tyr272, His496, and His681. Dioxygen then reacts with pre-GAOXCu(I) to generate an oxygenated complex, formulated as Cu(II)-O2•⁻ to Cu(I), forming a fully reduced, cofactor-containing active site (Table 2, step 1). This oxygenated species may abstract a hydrogen atom from Cys228, forming a thiyl free radical, likely as the result of a competition (at lower pH) between commitment to cofactor biogenesis on the one hand, and loss of hydroperoxyl (protonated superoxide) resulting in commitment to formation of the 406 nm pre-GAOXCu(II) complex on the other (Reactions 1 and 2). The spectral data recorded for the low pH reaction (Fig. 11, top) resolve onto a mixture of oxidized GAOX (minority product, rapid formation), 406 nm species (majority product, slow formation), and a weakly absorbing pre-GAOXCu(II) complex (prompt formation) which is an intermediate in conversion to the 406 nm species (Fig. 6). The pH dependence of the biogenesis reaction between pH 6.5 and 8.5 appears to reflect titration of a group in the protein ionizing with a pKₐ = 7.3, higher reaction rates being associated with the deprotonated group (Fig. 12). Because the Cu(I) complex is formed prior to mixing with buffer in these reactions, it seems unlikely that this ionization is associated with the two active site histidine residues that serve as metal ligands in both oxidation states (53). Although other possibilities cannot be ruled out by these results, it is attractive to assign this ionization to Cys228. Cysteine residues in proteins typically exhibit a pKₐ near 8.3, but stabilization of the thiolate (e.g. by hydrogen bonding with Trp290 indole or exposure to solvent) would be expected to perturb the side chain acidity. Hydrogen-bonded cysteines have been shown to titrate with pKₐ values as low as 6.3 in thioredoxin and glutaredoxin (54, 55). At lower pH, protonation may destabilize the initial oxygenated complex, formulated as Cu(II)-O₂•⁻ leading to loss of HO₂•, blocking further reaction and producing pre-GAOXCu(II) rather than mature GAOX as the product.

At lower pH, the reaction becomes slower (Fig. 12), and below pH 7 the product absorption spectrum changes, with shift in the absorption maximum to 435 nm (Fig. 11). The reaction at lower pH is also more complex, requiring multiple kinetic phases to adequately fit the data. This behavior may be understood as the result of a competition (at lower pH) between commitment to cofactor biogenesis on the one hand, and loss of hydroperoxyl (protonated superoxide) resulting in commitment to formation of the 406 nm pre-GAOXCu(II) complex on the other (Reactions 1 and 2). The spectral data recorded for the low pH reaction (Fig. 11, top) resolve onto a mixture of oxidized GAOX (minority product, rapid formation), 406 nm species (majority product, slow formation), and a weakly absorbing pre-GAOXCu(II) complex (prompt formation) which is an intermediate in conversion to the 406 nm species (Fig. 6). The pH dependence of the biogenesis reaction between pH 6.5 and 8.5 appears to reflect titration of a group in the protein ionizing with a pKₐ = 7.3, higher reaction rates being associated with the deprotonated group (Fig. 12). Because the Cu(I) complex is formed prior to mixing with buffer in these reactions, it seems unlikely that this ionization is associated with the two active site histidine residues that serve as metal ligands in both oxidation states (53). Although other possibilities cannot be ruled out by these results, it is attractive to assign this ionization to Cys228. Cysteine residues in proteins typically exhibit a pKₐ near 8.3, but stabilization of the thiolate (e.g. by hydrogen bonding with Trp290 indole or exposure to solvent) would be expected to perturb the side chain acidity. Hydrogen-bonded cysteines have been shown to titrate with pKₐ values as low as 6.3 in thioredoxin and glutaredoxin (54, 55). At lower pH, protonation may destabilize the initial oxygenated complex, formulated as Cu(II)-O₂•⁻ leading to loss of HO₂•, blocking further reaction and producing pre-GAOXCu(II) rather than mature GAOX as the product.

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dized mature GAOX, as observed (step 5). This is a two-electron biogenesis reaction, requiring two reductants (identified here as Cu(I) and cysteine thiol). Replacing Cu(I) by Cu(II) in this mechanism (for example, in the Cu(II)-dependent biogenesis experiments described above) would require an additional (exogenous) 1e\(^-\) equivalent to reduce dioxygen to the stable H\(_2\)O\(_2\) product following one-electron oxidation of a protein side chain. This model accounts for the pH sensitivity of the biogenesis reaction in terms of ionization of Cys\(^{228}\) at high pH (Scheme 3). The deprotonated thiolate group has a lower reduction potential (\(E^0(\text{RS/RS}^-) = 0.8\) V versus normal hydrogen electrode, compared with \(E^0(\text{RSh/RS} = 1.37\) V) (57) and would react more readily (via electron transfer, ET) to generate a thiyl free radical and a coordinated peroxide. If thiyl radical formation is more readily (via electron transfer, ET) to generate a thiyl free radical species (with a sensitivity of \(k = 29, 30\), predicts the appearance of spectroscopic features as-

of a tyrosine free radical within a pre-enzyme Cu(II) complex

considered to be less likely. A previous proposal for Cu(II)-de-

active site complex having Cys228 directly coordinated to copper

which is not an intermediate in the biogenesis reaction.

require any preproptide leader sequence to be present. Cofactor biogenesis utilizes some features of the active site (like the copper coordination environment) that are retained in the mature protein and contribute to the catalytic reaction. However, other features (e.g. the special reactivity of the unmodified Cys\(^{228}\) thiol and the Tyr\(^{272}\) side chains) are unique to the pre-enzyme complex. The self-processing in vitro formation of the Tyr\(^{272}\) Cys cofactor in galactose oxidase shows that the

path to a functional enzyme, involving formation of reactive intermediates and structures with special geometric constraints, must be templated within the structure of the active site.

We have proposed a mechanism for cofactor biogenesis that accounts for the significant experimental characteristics (copper oxidation state dependence, oxygen stoichiometry, pH sensitivity, and solvent isotope sensitivity) that we find for this reaction. Further work will be required to investigate the nature of reactive intermediates formed during galactose oxidase cofactor biogenesis in more detail.

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