Galactose Oxidase Pro-Sequence Cleavage and Cofactor Assembly Are Self-Processing Reactions

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Galactose oxidase is a member of a growing class of proteins with novel posttranslationally modified redox-active amino acids (see Figure 1).[1] The unusual nature of these modifications has stimulated interest in the mechanisms by which such cofactors are generated. Recently, the biogenesis of the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor of amine oxidase has been defined.[2] The oxidation of tyrosine to TPQ requires only copper ions and dioxygen, and is not dependent on any accessory proteins.[3] Analogous experiments with galactose oxidase have been hampered by the lack of sufficient quantities of pure precursor (unprocessed, copper-free) protein. Here we report the isolation of an apo, pro-enzyme form of galactose oxidase, and demonstrate that cleavage of the pro-sequence and assembly of the characteristic Tyr•-Cys cofactor are self-processing reactions.

Figure 1 illustrates the critical features of the galactose oxidase active site.[4] In the oxidized (active) state tyrosine 272, which is posttranslationally cross-linked to cysteine 228 via a thioether bond, is oxidized to a radical. Thus the [Cu(II) Tyr•-Cys] unit acts as a two-electron acceptor in the oxidation of a wide variety of alcohols to the corresponding aldehydes. The posttranslational cross-link is believed to modulate the reactivity and redox potential of the tyrosyl radical.[5,6] C228 may aid in stabilization of the radical by virtue of the electron-donating properties of the sulfur atom.[5] The oxidized form of galactose oxidase displays a characteristic set of electronic transitions (vida infra) that are also observed in glyoxal oxidase, a galactose oxidase homologue.[7]

Heterologous expression[8] of the *Fusarium* protein in *Aspergillus nidulans* under copper-limited conditions resulted in the appearance of multiple protein forms (Figure 2). The molecular weights of the SDS-PAGE bands in Figure 2a, established to be galactose oxidase by Western blotting,[8] were estimated as 70.2, 68.5, and ∼65.5 kDa. N-terminal sequencing established that the fastest migrating protein (lower band, ∼65.5 kDa) corresponds to mature, wild-type galactose oxidase. Mature galactose oxidase migrates on SDS-PAGE with an anomalous molecular weight (65.5 kDa as compared to 68.5 kDa predicted by the sequence), owing to the thioether bond, which produces a stable loop thus preventing full unfolding on treatment with SDS.[8] The middle band (Figure 2a) has an estimated M_r that correlates with the mass of the mature galactose oxidase amino acid sequence, suggesting that it is a form of galactose oxidase lacking the thioether bond. This behavior is mirrored by the variant C228G, which is unable to generate a thioether bond.[8] Finally, the upper band (Figure 2a), having an estimated M_r of 70.2 kDa, corresponds to the pro-form with the pro-sequence attached, which was confirmed by the N-terminal sequence data (Table 1). These data suggest that pro-sequence cleavage and thioether bond formation are separable reactions in vivo.

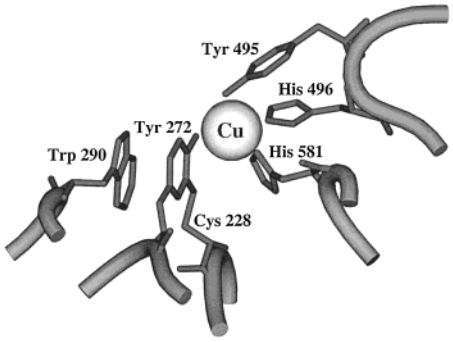

Figure 1. The active site of mature galactose oxidase.

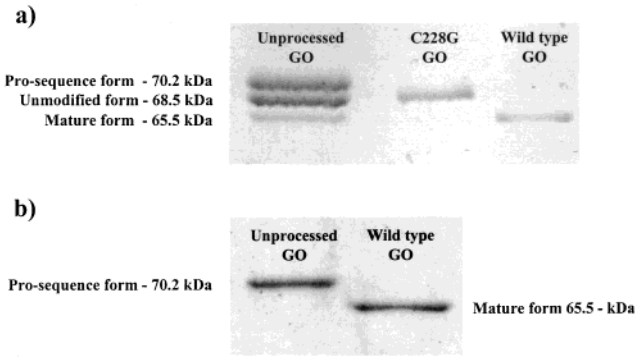

Figure 2. 10% SDS-PAGE of galactose oxidase obtained under both (a) limited and (b) metal-free conditions.

Purification of a homogeneous form of unprocessed galactose oxidase was achieved for the first time by performing growth of the organism and protein purification under strictly metal-free conditions (Figure 2b).[9] Addition of Cu(II) and aerobic incubation result in conversion to the mature form as monitored by SDS-PAGE (Figure 2b). This suggests that both pro-sequence cleavage and thioether bond formation are copper-mediated reactions. Cleavage of the pro-sequence is not due to extraneous or intrinsic protease activity because the reaction occurred when a protease-inhibitor cocktail (Sigma P-8215) was present during the incubation with Cu(II). Importantly, this cocktail contains phenanthroline; adjusting the phenanthroline concentration to exceed the copper concentration in the reaction completely blocked pro-sequence cleavage, establishing that this reaction is copper-

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(9) All glassware was washed with concentrated nitric/sulphuric acid (1:1) and the plasticware was soaked in 0.1 M EDTA. Solutions were prepared either by using Chelex-100 resin (BioRad)-treated double deionized water or by using a batch Chelex-100 resin treatment method. Metal-free galactose oxidase was purified from filtered growth media using a batch protein-loading step prior to cellulose phosphate ion exchange chromatography. The protein was eluted with a linear gradient (10 to 100 mM potassium phosphate, pH 7.3). Fractions were screened using both absorbance measurements and SDS-PAGE. Selected fractions were concentrated and exchanged into 20 mM PIPES, pH 6.8, using ultrafiltration. The purification was conducted in the presence of 1 mM EDTA.

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Table 1. Summary of Galactose Oxidase Forms

	M_r (kDa)[a]	N-terminal sequence[b]	Is the pro-sequence present?	Is the thioether bond formed?
pro-sequence form	70.2	AVxxxIPEG	yes	no
unmodified form	68.5	ASAPIGxAI	no	no
thioether form	~65.5	ASAPIGS?AI	no	yes

[a] Estimated from SDS-PAGE. [b] Sequencing was performed by standard automated Edman procedures. "x" is an unidentified amino acid. These results are consistent with the nucleotide sequence (Genbank M86819).

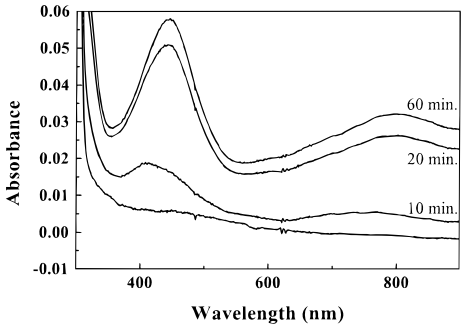
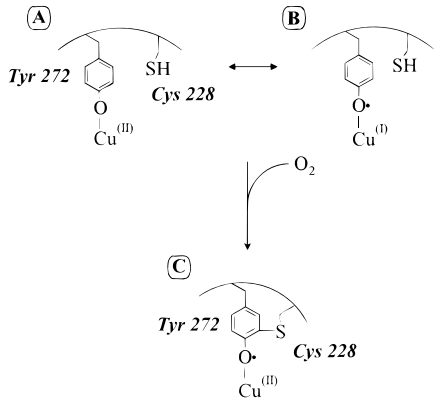


Figure 3. Absorbance spectral changes accompanying the aerobic addition of copper to "metal-free" unprocessed galactose oxidase. Copper sulfate (28 μM) was added aerobically to unprocessed galactose oxidase (28 μM).

dependent. Both processing and activation (on an enzyme activity basis) appear to be copper-specific. Negligible cleavage was observed by SDS-PAGE with Ca(II), Mg(II), or Mn(II). The extent of cleavage detected with Zn(II), Co(II), and Ni(II) was very small and consistent with the estimated trace copper contamination. Finally, no processing was observed under anaerobic conditions, suggesting that oxidative reactions are involved.

As shown in Figure 3, the visible spectrum of the unprocessed protein was featureless prior to the addition of Cu(II).[10] On aerobic exposure to copper sulfate, new transitions are observed at 410 and 750 nm (Figure 3), which are attributable to Cu(II) charge-transfer or ligand-field transitions. The band seen initially at 410 nm shifts to 445 nm, and the broad band initially observed at 750 nm shifts to 800 nm with an increase in its intensity. The characteristic bands at 445 and 800 nm confirm the generation of the tyrosine radical. This electronic spectrum is highly unusual and is assigned as follows: 445 nm, phenolate → Cu(II) charge-transfer and Tyr• $\pi \rightarrow \pi^*$;[11] 800 nm, tyrosinate to tyrosyl ligand-to-ligand charge-transfer mediated by the d_{xz} orbitals on Cu(II).[12] These results unequivocally establish that the formation of the Tyr•-Cys redox cofactor in galactose oxidase is a self-processing reaction requiring only the apo protein, Cu(II), and dioxygen; no other proteins or enzymes are required for the processing and assembly of the catalytically active enzyme.

Scheme 1. Generation of the Thioether Bond in Galactose Oxidase



A possible mechanism for the generation of the thioether bond is outlined in Scheme 1. We suggest that copper coordinates to the two histidine ligands (H496/581) in analogy to suggestions for TPQ biogenesis in *Arthrobacter globiformis* amine oxidase.[13] Activation of the phenol ring is likely to occur via coordination of Y272 to Cu(II) [**A**, Cu(II)−O−Y272], as illustrated by the resonance form **B** [Cu(I)−O−Y•] where the tyrosine ring may be expected to be electron deficient. On addition of molecular oxygen, the reaction may be envisioned to proceed via either a radical or ionic mechanism. Interestingly, the final species (**C**) is the enzymatically active [Cu(II) Tyr•] form. Additional spectroscopic and mechanistic studies are planned to elucidate the mechanism of thioether bond formation. Structural studies of the unprocessed pro-sequence form are underway.

Galactose oxidase joins an increasing number of enzymes that are posttranslationally modified via self-processing reactions to produce the active state. Histidine decarboxylase (and other pyruvoyl enzymes),[14] amine oxidases,[2−3] ribonucleotide reductase,[15] nitrile hydratase,[16] and perhaps cytochrome oxidase[17] are covalently or oxidatively modified to generate a redox cofactor or new active-site functional groups required for catalysis. This capability for self-processing, using available reagents such as metal ions and dioxygen, to generate new types of reactivity might represent a key step in the evolution of enzymes.

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(10) Additionally, inductively coupled plasma atomic emission spectroscopy (ICP-AES) of a similar sample showed the copper content to be below the detection limit of the instrument prior to the addition of Cu(II).

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