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o,o-DITYROSINE IN NATIVE AND HORSERADISH PEROXIDASE-ACTIVATED GALACTOSE OXIDASE[1]

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SUMMARY. Treatment of galactose oxidase with catalytic amounts of horseradish peroxidase results in increases in both enzyme activity and Cu(II)-associated absorbance. This reaction requires O_2 and is reversed upon removal of O_2 or peroxidase. o,o-Dityrosine is detected in amino acid hydrolysates of peroxidase-treated galactose oxidase as a ninhydrin peak. Furthermore, even native enzyme contains this species as detected by fluorescence measurements. Peroxidase treatment increases the amount of dityrosine present. The dityrosine forms an intramolecular crosslink, the first such crosslink found in a non-structural protein. The peroxidase-catalyzed formation of the dityrosine and putative precursor radical(s) is thought to involve a tyrosyl ligand to the Cu(II) in galactose oxidase. Such a radical may be involved in the activation observed.

Introduction. Galactose oxidase (E.C. 1.1.3.9; GOase[2]), an enzyme secreted by the fungus *Dactylium dendroides*, contains a single cupric ion, and catalyzes the oxidation by O_2 of virtually any primary alcohol to the corresponding aldehyde (1-3). Several investigators (4,5) have observed that the activity of this enzyme increases in the presence of horseradish peroxidase (E.C. 1.11.1.7; HRP). In this paper, we report that this activation appears to be the result of a catalytic, oxidative modification of GOase that results in the coupling of two tyrosyl residues to form o,o-dityrosine. The coupling of phenolic compounds is known to be catalyzed by HRP (6-8). We also report preliminary evidence that native GOase itself contains variable quantities of o,o-dityrosine. Although o,o-dityrosine has been observed as a stable *inter*molecular bond in insect and mammalian structural proteins (9,10), to the best of our knowledge,

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[2]Abbreviations used: GOase, galactose oxidase; HRP, horseradish peroxidase; DDCA, diethyldithiocarbamic acid.

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this is the first time that it has been observed as an apparently labile, naturally occuring *intra*molecular crosslink in a non-structural protein.

Materials and Methods. Galactose oxidase was purified as described (2) with some modification (11). The activity of galactose oxidase was determined by its oxygen consumption at 25°, using a Model 53 oxygen electrode system (Yellow Springs Instrument) with 0.1 M galactose (Sigma) and 100% oxygen (Ohio Medical Products) as substrates in 0.1 M pH 7.0 phosphate buffer; or by measuring aldehyde formation at 314 nm (ε = 2,690) with 57.7 mM 3-methoxybenzyl alcohol (Aldrich, redistilled), and atmospheric oxygen as substrates under the same conditions. Difference spectra and spectral assays were done on a Cary 17-D spectrophotometer. o,o-Dityrosine was prepared as described (6) using 30% H_2O_2 (Merck), L-tyrosine (Calbiochem), and horseradish peroxidase (Sigma, Type VII), and it was purified by preparative cellulose thin-layer chromatography (Brinkman). Pulsed-power electrophoresis of HRP-treated GOase was performed as described (2). Amino acid analyses were done on 22h 6N HCl hydrolysates using a Beckman 120-C amino acid analyzer and the elution system of Hare (12). Apo-galactose oxidase for these analyses was made by treating approximately 1 mg/ml of protein with a few crystals of DDCA (Sigma) and rapidly diluting this with several ml of methanol. The denatured protein was then collected by centrifugation and washed several times with methanol to remove residual DDCA. Fluorescence measurements and spectra were obtained on an SLM Model 8000 spectrofluorometer.

Results. Treatment of GOase with as little as 0.01 mole % HRP results in a 2-15 fold increase in enzyme activity ($k_{cat_{app}}$ increases while Km_{app} remains unaltered for the substrates O_2, D-galactose, and 3-methoxybenzyl alcohol). Increases in the intensities of the Cu(II)-associated absorption maxima also result from HRP treatment, as seen in a difference absorption spectrum (Fig 1).

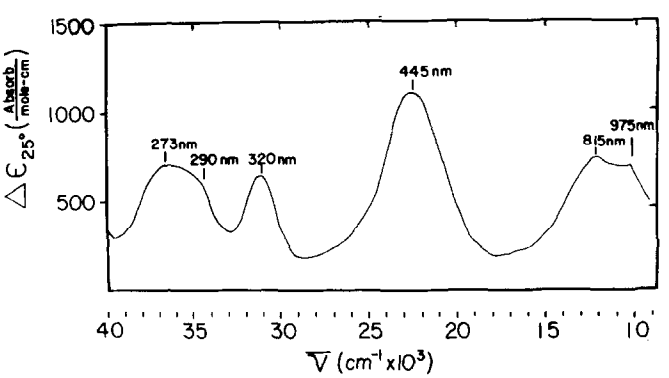


Figure 1. Difference absorbance spectrum resulting from the modification of GOase by HRP, expressed as units of molar extinction coefficient, and corrected for contributions from the simultaneous change in the HRP spectrum (5). Conditions: 10 µM GOase in 0.1 M phosphate pH 7.0 with 10 nM HRP in 1 cm cells (230-400 nm); and 33 nM HRP in 4.5 cm cells (300-1100 nm).

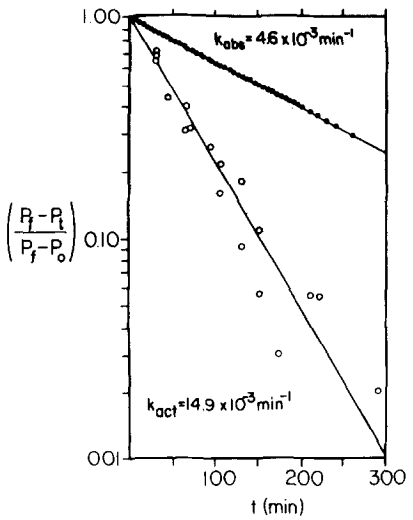
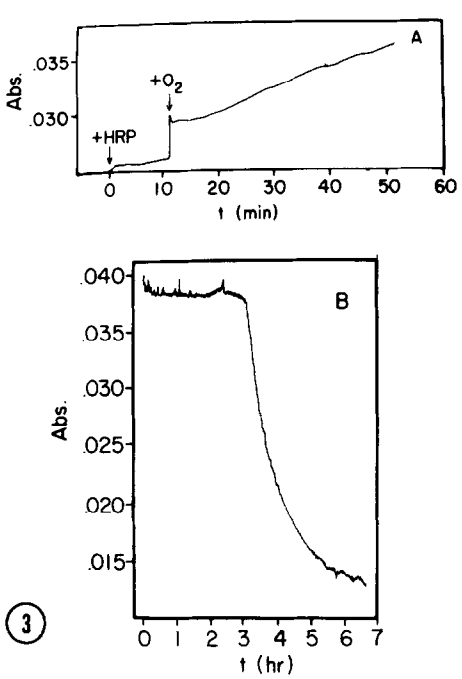


Figure 2. First order rate curves for modification of GOase by 3 nM HRP. The axis labeled $\frac{(P_f-P_t)}{(P_f-P_o)}$ represents the fraction of GOase that has not yet been modified. Modification followed by the increase in absorbance at 445 nm (P_t= absorbance at any time, t), ●——●; modification followed by the increase in GOase activity (P_t = rate of oxygen consumption, using galactose as substrate, at any time, t), o——o.

The positive peaks at 320, 445, and 800, as well as at 280 nm, all increase at the same rate in an apparent first-order process. The activity increase is also a psuedo first-order process, but the calculated rate constant is 2-3 times that determined for the spectral change (Fig 2). These psuedo first-order rate constants vary linearly with HRP concentration over the range 3 nM to 170 nM. Furthermore, these changes are inhibited by cyanide, a known inhibitor of HRP (13). Oxygen is absolutely required, as shown by the data in Fig 3. In addition, the increases in activity and Cu(II)-associated absorbance are stable only in the presence of HRP. This is shown by loss of these modification characteristics when HRP is removed by phosphocellulose chromatography, or when the reaction is terminated by the removal of oxygen (Fig 3B).

o,o-Dityrosine is identified as a product of the HRP treatment by its appearance as a new ninhydrin peak in the amino acid chromatogram for GOase. This peak appears between Phe and His in the same position as an o,o-dityrosine standard (Fig 4). Dityrosine is also present in GOase that is untreated with HRP, but in such small amounts that it is not visualized as a ninhydrin peak.



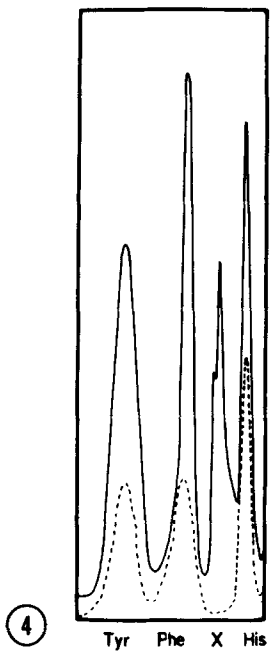


Figure 3. The oxygen requirement for GOase modification. (A) HRP is added to GOase in a Thunberg cuvette that has been purged with argon, and the spectral change at 445 nm is monitored. The cuvette is then opened to air at the time indicated. The 0.003 absorbance discontinuity at this point is due to a slight change in cuvette position. (B) After aerobically modifying GOase with HRP in a Thunberg cuvette, the cuvette was flushed with argon at t = 0 and absorbance monitored at 445 nm. As seen, this resulted in complete bleaching within 7 h. The absorption was restored by addition of oxygen.

Figure 4. Chromatogram of 6N HCl hydrolysates of HRP modified and unmodified GOase, in the vicinity of the dityrosine peak (labeled "X") (8); ——— modified GOase; -----, unmodified GOase.

However, it can be detected by its characteristic fluorescence (excitation at 320 nm, emission at 400 nm). In either case, dityrosine is seen in the chromatogram only when GOase copper is rapidly removed by DDCA in the presence of HRP prior to acid hydrolysis.

HRP treatment does not change the electrophoretic mobility of GOase or its elution from a column of Sephadex G-100. Thus, o,o-dityrosine forms an *intra*molecular rather than an *inter*molecular crosslink in this protein.

Discussion. o,o-Biphenols have been characterized as the product of HRP oxidation of a variety of phenolic substrates (6,7) including tyrosine residues in proteins (8). Our data indicate that at least two GOase tyrosines are sus-

ceptible to such coupling to form an intramolecular crosslink. Furthermore, untreated GOase appears to contain small quantities of o,o-dityrosine. We assume that this dityrosine is derived from the same pair of residues that are involved in the HRP modification.

The GOase Cu(II) may be interacting with this pair of tyrosines. Amundsen et al. (14) suggest that the 320 and 445 nm absorption bands of N_2O_2-Cu(II) complexes are due to phenolate ← Cu(II) charge transfer transitions. Available evidence suggests that the Cu(II) site in GOase is an N_2O_2 system (3,15), in which one oxygenous ligand remains unassigned. Our working hypothesis, based in part on the 320 and 445 nm spectral changes, is that this ligand site is occupied by one of the two tyrosines involved in the dityrosine crosslink found in GOase and further induced by HRP.

We assume that the simultaneous increases in GOase activity, Cu(II)-associated absorbance, and o,o-dityrosine content indicate a common origin. However, due to difficulties in quantitating the dityrosine in GOase, the basis of this relationship is not clear. The more rapid increase in activity suggests that the immediate effect of HRP treatment is to generate a higher concentration of some active enzyme form, which then undergoes a slow reaction, perhaps a protein conformational change, causing the observed increases in Cu(II)-associated absorbance. Significantly, only an increase in transition probability occurs, with no change in transition energies. This could be due to either an increase in the concentration of the enzyme form which exhibits these transitions or to subtle conformational changes which increase the allowedness of these Cu(II) transitions without altering their energy.

We presume that the effect of the Cu(II) on either the o,o-dityrosine or on some precursor is related to the mechanism of both HRP-induced dityrosine formation and GOase activation. Dityrosine formation is thought to result from the dimerization of two phenol radicals, or by the reaction of a single phenol radical with another phenol to yield an adduct radical which then undergoes a subsequent one-electron oxidation (6, 9, 16). Since a phenol radical - Cu(II)

system is a two-electron acceptor, it could hypothetically represent an active, oxidized form of GOase. Removal of the Cu(II) would allow the phenol coupling reaction to go to completion, forming the dityrosine observed by amino acid analysis. Of relevance to this model of GOase, is the presence of an apparently catalytic phenolic tyrosine free radical in the B2 protein of E. coli ribonucleotide reductase (17). Efforts to detect or trap such an organic free radical in GOase are in progress.

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