Autoredox Interconversion of Two Galactose Oxidase Forms $GOase_{ox}$ and $GOase_{semi}$ with and without Dioxygen

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Solutions of galactose oxidase stored in air give in 3−4 h a mix of $GOase_{ox}(Cu^{II}\text{-}Tyr^{\bullet})$ and $GOase_{semi}(Cu^{II}\text{-}Tyr)$, as a result of processes involving the formation and decay of the Cu^{II}-coordinated tyrosyl radical ($Tyr^{\bullet}$). In this work the two reactions have been studied by UV−vis spectrophotometry and separate rate laws defined. The first involves the "spontaneous" autoreduction of $GOase_{ox}$ to $GOase_{semi}$, which in air-free conditions is 100% complete. Rate constants (k_{red}) are dependent on pH, and previously defined acid dissociation constants $pK_{1a} = 5.7$ (exogenous H_2O ligand), and $pK_{2a} = 8.0$ (axial H^+Tyr-495) apply. Values of k_{red}(25 °C) range from 1.55×10^{-4} s^{-1} (pH 5.5) to 2.69×10^{-4} s^{-1} (pH 8.6), $I = 0.100$ M (NaCl). No reaction occurs with N_3^- or NCS^- present in amounts sufficient to give >98% binding at the substrate binding (exogenous) site, while $CH_3CO_2^-$ and phosphate (less extensively bound) also inhibit the reaction. From such inhibition studies K(25 °C) is 161 M^{-1} at pH 6.4 for acetate (previous value 140 M^{-1}) and 46 M^{-1} at pH 7.0 for phosphate. No reaction occurs when the disulfide Cys515-Cys518 (10.2 Å from the Cu) is chemically modified with $HSPO_3^{2-}$, and electron transfer via the disulfide and exogenous position is proposed (source of the electron not established). The conversion of $GOase_{semi}$ to $GOase_{ox}$ only occurs with O_2 present, when a first-order dependence on [O_2] is observed, giving k_{ox}(25 °C) = 0.021 M^{-1} s^{-1} at pH 7.5. This process is unaffected by NCS^- or N_3^- bound at the exogenous site, and a mechanism involving outer-sphere reaction of O_2 to O_2^- followed by a fast step O_2 to H_2O_2 is proposed. As $GOase_{ox}$ is formed, autoreduction back to $GOase_{semi}$ occurs, and at pH 7.5 with O_2 in large excess (1.13 mM) the maximum conversion to $GOase_{ox}$ is 69%. The k_{ox} reaction proceeds to completion with >98% N_3^- bound at the exogenous site.

Introduction

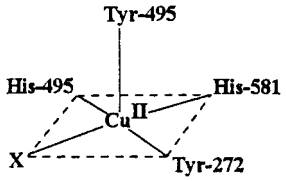
Galactose oxidase (GOase: EC 1.1.3.9) is a water-soluble single Cu containing two-equivalent oxidase (M_w 68.5 kDa; 639 amino acids),[1,2] isolated in this case from a fungal source *Fusariam* (strain NRRL 2903). In the active state it uses Cu^{II} and a coordinated tyrosyl radical (Tyr·) as redox active components.[3] Enzymic activity is observed with primary alcohols RCH_2OH and involves O_2 as a second substrate, to bring about the enzymic reaction (1).

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \qquad (1)$$

Three GOase oxidation states are defined as in (2),

$$\underset{GOase_{ox}}{Cu^{II}\text{-}Tyr^{\bullet}} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \underset{GOase_{semi}}{Cu^{II}\text{-}Tyr} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \underset{GOase_{red}}{Cu^{I}\text{-}Tyr} \qquad (2)$$

where $GOase_{ox}$ and $GOase_{red}$ are involved in the enzymic process (1). In the catalytic cycle the two-equivalent reaction with RCH_2OH gives $GOase_{red}$ which reacts with O_2 to restore $GOase_{ox}$. X-ray crystal structures at resolutions down to 1.7 Å[4,5] have demonstrated that the Cu^{II} has a square-pyramidal geometry, with the radical forming Tyr-272, His-496, His-581, and X^- at the substrate-binding (exogenous) site giving a square-planar geometry and Tyr-495 as the axial ligand. Structures have been determined with $X^- = H_2O$ (or OH^-), $CH_3CO_2^-$, and N_3^-,[4,5] and from EXAFS it has been concluded that the same essential coordination holds for $GOase_{ox}$ and $GOase_{semi}$.[6]



Aqueous solution properties of galactose oxidase have been the subject of recent papers.[7−9] In the course of these studies it was apparent that stored samples of galactose oxidase interconvert to a mixture of $GOase_{ox}$ and $GOase_{semi}$ in autoredox steps.[7,8] Since the net process is the formation and decay of the tyrosyl radical (Tyr·) at Tyr-272, it was of interest to look more closely at these changes. At pH 7.5 the reduction potential for the GOase Tyr·/Tyr couple is 400mV at pH 7.5, decreasing to

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380mV at pH 8.5,[8] which is the smallest value so far observed for the couple. In recent papers we have referred to stored samples as having a 95:5 $GOase_{ox}$:$GOase_{semi}$ composition.[7,8] However, as will emerge in this study, the interconversion is dependent on pH as well as levels of O_2 present. In addition, a nearby disulfide plays an important role, and anions inhibit the $GOase_{ox}$ decay. From UV−vis spectra, formation constants K(25 °C)/M^{-1} have been determined[9] and at pH 7.0 are NCS^- (480), N_3^- (1.98×10^4), and $CH_3CO_2^-$ (104).

Experimental Section

Isolation of Enzyme. GOase was obtained from the fungal source *Fusarium* (strain NRRL 2903)[10,11] and from the *Aspergillus nidulans* (strain G191/pGOF 101) expression system by procedures previously described.[12] The Tyr495Phe variant was obtained from a similar expression system.[7,13] Final purification was achieved using phosphocellulose column chromatography, and purity was confirmed by FPLC analysis.

Buffers. The following buffers were obtained from Sigma Chemicals or as stated: 2-(*N*-morpholino)ethanesulfonic acid (Mes, pK_a = 6.1, pH 5.5−6.7); 2,6-dimethylpyridine (lutidine, Fluka, pK_a 6.75, pH 5.7−8.0); 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches, pK_a 9.3, pH 8.6−1.00). These buffers were selected because they do not coordinate to GOase.[9] Acetate (and in this work phosphate) coordinate to GOase,[4,9] while Hepes and Tris are unsatisfactory for other reasons. In the present work, acetate (acetic acid pK_a 4.74) and phosphate ($H_2PO_4^-$ pK_a 6.68 average[9]) inhibit $GOase_{ox}$ autoreduction.

Preparation of Enzyme Solutions. Procedures were as used previously.[9] Combined $GOase_{ox}$ and $GOase_{semi}$ concentrations were determined from the UV−vis absorbance at 280 nm ($\epsilon = 1.05 \times 10^5$ M^{-1} cm^{-1}).[11] UV−vis spectra of $GOase_{ox}$ and $GOase_{semi}$ vary with pH.[9] To convert to $GOase_{ox}$ or $GOase_{semi}$, an excess of $K_3[Fe(CN)_6]$ (Sigma) or $K_4[Fe(CN)_6]\cdot 3H_2O$ (BDH, Analar), respectively, was added. After 1−2 min, the excess reagent was removed by Amicon filtration or by desalting on a P6DG column (Bio-Rad). However, it has to be borne in mind that loss of Cu is observed on treatment with $[Fe(CN)_6]^{3-/4-}$ (10−20% for each redox cycle) with formation of apo-GOase.[9] The amount of catalytically competent Cu-containing protein was determined from the absorbance at 450 nm ($\epsilon = 8600$ M^{-1} cm^{-1}) for $GOase_{ox}$ at pH 7.0.[7,9] Newly prepared protein gave a Cu content of ∼90% as determined by the biquinoline colorimetric method.[14]

Other Reagents. The sodium salts of thiocyanate (NaNCS; Fluka), azide (NaN_3), acetate ($CH_3CO_2Na\cdot 3H_2O$), hydrogen peroxide, and hydrogenphosphates ($NaH_2PO_4\cdot H_2O$ and/or Na_2HPO_4), were from Sigma or as stated.

Procedures. Reactions were studied at 25.0 ± 0.1 °C in the pH range 5.5−9.0, with ionic strength I made up to 0.100 ± 0.001 M by addition of NaCl. Decay or formation of $GOase_{ox}$ was monitored at the 450 and 810 nm peaks and gave good agreement (±3%). The decay was studied under N_2 (99.99%, liquid N_2 source). Cylinder O_2 gas (99.5% BOC) was used to saturate buffer solutions, and hence by quantitative transfer adjust $[O_2]$ to levels greater than in air, and up to 1.36 mM. Concentrations of O_2 were determined using a Beckman Model 0160 oxygen analyzer, fitted with a thermocompensated 39556 electrode (Instruction Manual 015-082 155-B). The analyzer was calibrated at 25 °C against air-saturated de-ionized water when $[O_2]$ is 0.263 mM. With the ionic strength adjusted to 0.100M (NaCl) the $[O_2]$ was 0.252 mM.

Chemical Modification of Disulfide. The procedure has been described previously.[15] Trisodium thiophosphate, Na_3SPO_3, was prepared from thiophosphoryl chloride.[16] Reaction with protein disulfides is optimum at pH 9.5 (10mM Ches), and in this case $HSPO_3^{2-}$ was in 40-fold excess of $GOase_{semi}$ (40−75 μM) at 25 °C (3). After 16 h,

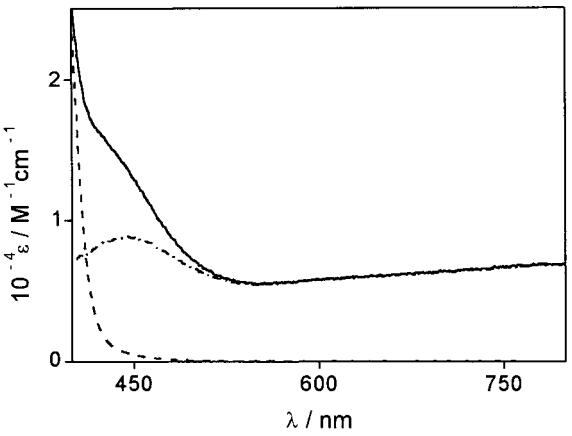

Figure 1. UV/vis spectra of $HSPO_3^{2-}$-modified $GOase_{ox}$ (-·-·-); the reagent dithiobis(2-nitrobenzoic acid, DTNB (- - -), and the product obtained on reaction of $HSPO_3^{2-}$modified $GOase_{ox}$ (23μM) with DTNB (100μM) (—) at 25 °C, pH 7.2 (100 mM phosphate), I = 0.100 M (NaCl).

$$RSSR + HSPO_3^{2-} \rightarrow RSSPO_3H^- + RS^- \quad (3)$$

excess thiophosphate was removed and the protein purified by FPLC Pharmacia chromatography (Mono-S column), giving a single major peak.[17] Electrospray mass spectrometry on $GOase_{semi}$ gave a mean M_w of 68654 Da (unmodified) and 68755 Da (modified), the difference of 101 Da (M_w of SPO_3^{3-} 111 Da) indicates modification of one disulfide per GOase molecule. In addition, using Ellman's reagent 5,5′-dithio-(2-nitrobenzoic acid) (DTNB; Sigma) for the colorimetric determination of the free (polypeptide) cysteine (RS^-) in (3) was carried out (4).[18]

$$(NO_2)R'SSR'(NO_2) + RS^- \rightarrow NO_2R'SSR + NO_2R'S^- \quad (4)$$

Thus at pH 7.2 (100mM phosphate), DTNB (5mM) in the presence of EDTA (0.10mM; disodium dihydrogen ethylediaminetetraacetate) reacts with RS^- to give an absorbance at 412 nm ($\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}). Unmodified GOase gave no positive test for RS^-, but with $HSPO_3^{2-}$-modified protein one RS^- per GOase was confirmed, Figure 1. One (of two) GOase disulfides is therefore modified by $HSPO_3^{2-}$. From the X-ray structure, the disulfide Cys515-Cys518 nearest the Cu is most exposed, and most likely modified. The second disulfide Cys18-Cys27 is 40.5 Å from the Cu. From UV−vis measurements, N_3^- binds at the exogenous position of $HSPO_3^{2-}$-modified $GOase_{ox}$: formation constant K(25 °C) = 1.95×10^4 M^{-1} (cf. 1.92×10^4 M^{-1} for unmodified $GOase_{ox}$[9]) at pH 7.5, I = 0.100 M.

Results

Air-Free Autoreduction of $GOase_{ox}$. UV−vis scan spectra, Figure 2, indicate 100% reduction in 3−4 h at pH 7.5 (5). A

$$GOase_{ox}(Cu^{II}\text{-}Tyr^{\bullet}) + e^- \rightarrow GOase_{semi}(Cu^{II}\text{-}Tyr) \quad (5)$$

similar behavior was observed at other pH's. First-order plots (e.g., inset of Figure 2) gave k_{red} values listed in Table S1 (Supporting Information). Variations of k_{red} with pH are observed (Figure 3). Assuming that the trend in k_{red} at high pH's decreases to zero at pH ∼4.0, a pK_a value of 6.7(2) is obtained. This is in satisfactory agreement with recent (average) pK_{2a}

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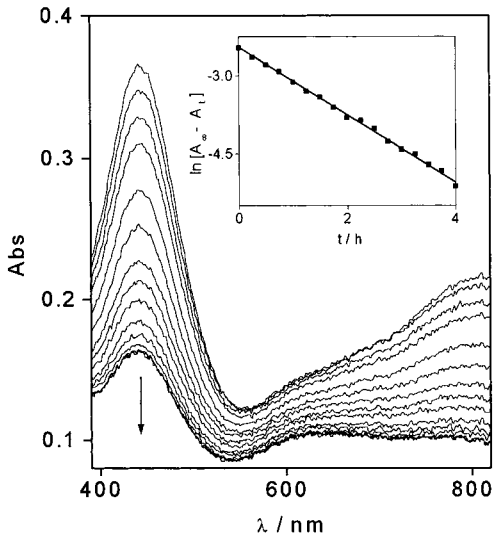

Figure 2. Scan spectra recorded every 15 min for the autoreduction (25 °C) of $GOase_{ox}$ (42 μM) at pH 7.5 (10 mM lutidine) in the absence of O_2, $I = 0.100$ M (NaCl). The inset shows a first-order plot of ln $(A_t - A_\infty)$ against time.

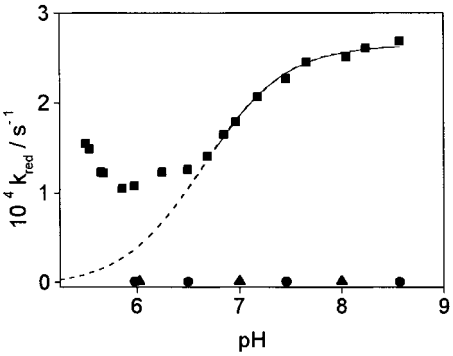

Figure 3. The variation of first-order rate constants k_{red}(25 °C) with pH for the autoreduction of $GOase_{ox}$ (■) monitored at 450 and 810 nm in O_2-free conditions, $I = 0.100$ M (NaCl). No reaction is observed for the autoreduction of $HSPO_3^{2-}$-modified $GOase_{ox}$ (▲) or O_2-free $WTGOase_{semi}$ (●).

values of 6.9,[7−9] assigned as acid dissociation of H^+Tyr-495. The trend observed at the lower pH's (Figure 3) most likely corresponds to acid dissociation of the H_2O at the exogenous site, $pK_{1a} = 5.7$.[9]

In air, the $GOase_{ox}$ decay is less extensive and well short of the 100% levels observed for air-free conditions (see below). The autoreduction of $GOase_{ox}$ isolated from *Fusarium* NRRL 2903 (native source) was monitored in the absence of O_2 at pH 7.5 and showed the same reactivity as wild-type GOase isolated from the *A. nidulans* overexpression system.

Effect of Anions on $GOase_{ox}$ Decay. Knowing 1:1 formation constants K for the reactions of X^- with $GOase_{ox}$,[9] it is possible to calculate levels of $X^- = N_3^-$ (5mM), NCS^- (0.4M) required to give >98% complexing at the exogenous site with pH in the range 5.76−8.05. Under these conditions, no $GOase_{ox}$ decay was observed in >8 h. Acetate (0−30 mM) and phosphate (0−50 mM) are less effective inhibitors and provide information regarding the extent of coordination of these anions.

Previously, formation constants K(25 °C) for the 1:1 reaction of $CH_3CO_2^-$ with $GOase_{ox}$ have been determined from UV−vis changes,[9] and at pH 6.41 K is 140(6) M^{-1}. In the present studies, k_{obs} was determined for the acetate (X^-) inhibition of $GOase_{ox}$ autoreduction, at pH 6.4, Table S2 (Supporting Information). The reaction sequence can be written as (6)−(7),

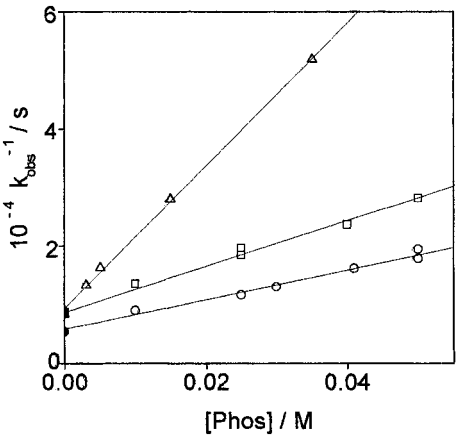

Figure 4. The effect of phosphate on rate constants k_{red}(25 °C) for the autoreduction of $GOase_{ox}$ (10 μM) at pH 5.7 (△), 6.1 (□), and 7.0 (○), $I = 0.100$ M (NaCl). The solid symbols indicate rate constants determined at zero phosphate.

$$GOase_{ox} + X^- \overset{K}{\rightleftharpoons} GOase_{ox}\,X^- \tag{6}$$

$$GOase_{ox} \xrightarrow{k_1} GOase_{semi} \tag{7}$$

and the rate law expressed as in (8),

$$\text{rate} = k_{obs}[GOase_{ox}]_T = k_1\frac{[GOase_{ox}]_T}{1 + K[X^-]} \tag{8}$$

where $[GOase_{ox}]_T$ is the total $GOase_{ox}$. On rearranging, (9) is obtained,

$$\frac{1}{k_{obs}} = \frac{K[X^-]}{k_1} + \frac{1}{k_1} \tag{9}$$

and a graph of k_{obs}^{-1} vs $[X^-]$ is linear. At pH 6.4, with $X^- = CH_3CO_2^-$, K is 161(15) M^{-1}. Rate laws in which the autoreduction of $GOase_{ox}\,X^-$ is also considered give less satisfactory fits. No UV−vis changes are observed on addition of phosphate to $GOase_{ox}$, but inhibition of the $GOase_{ox}$ autoreduction is again observed. A listing of k_{obs} values is included in Table S2 (Supporting Information). Using the same procedure as for $CH_3CO_2^-$, K(25 °C)/M^{-1} values of 142(3) (pH 5.7), 46(4) (pH 6.1), and 46(7) (pH 7.0) were determined, Figure 4. The pK_{1a} (5.7) for the $GOase_{ox}$ endogenous H_2O, pK_a (6.9) for H^+Tyr-495, and pK_a (6.68) for $H_2PO_4^-$ are possible contributors, and a less detailed analysis was made.

Decay of $HSPO_3^{2-}$-Modified $GOase_{ox}$. At pH's in the range 6.0−8.0, UV−vis readings at 450 nm indicate that no autoreduction of $HSPO_3^{2-}$-modified $GOase_{ox}$ is observed. Figure 5 compares the behavior observed with that for unmodified $GOase_{ox}$ at pH 7.5.

Decay of $GOase_{ox}$ Tyr495Phe Variant. At pH 6.0 and 8.0 (10mM buffer) a maximum of 9% decay was observed over 16 h, indicating a much slower reaction as compared to that of WT $GOase_{ox}$, which is complete in 3−4 h under identical conditions.

Reaction of $GOase_{semi}$ to $GOase_{ox}$. No absorbance changes are observed in air-free conditions. In air and at higher O_2 levels, UV−vis scan spectra indicate conversion to $GOase_{ox}$, Figure 6 (10). The formation of $GOase_{ox}$ is incomplete, and the extent of reaction depends on the pH and $[O_2]$, Table S3 (Supporting

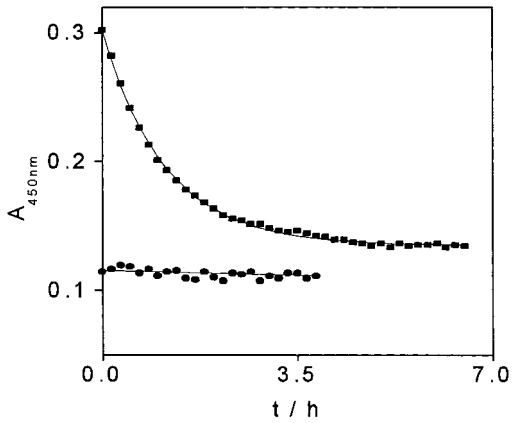

Figure 5. A comparison of UV−vis absorbance changes (25 °C) at 450 nm for the autoreduction of 34.8 μM $GOase_{ox}$ (■), with the corresponding reaction of 13.1 μM $HSPO_3^{2-}$-modified $GOase_{ox}$ (●) at pH 7.5 (10 mM lutidine), $I = 0.100$ M (NaCl).

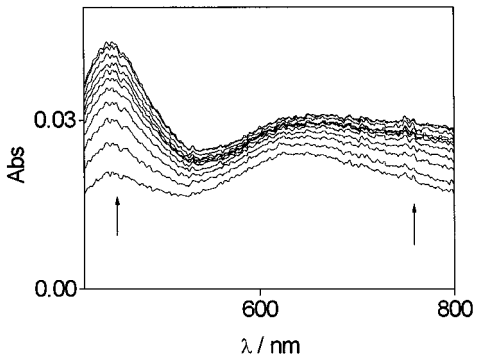

Figure 6. UV−vis scan spectra at 60 min intervals for the reaction of $GOase_{semi}$ (15 μM) to $GOase_{ox}$ in air ($[O_2] = 0.75$ mM) at 25 °C, pH 7.5 (10 mM lutidine), $I = 0.100$M (NaCl).

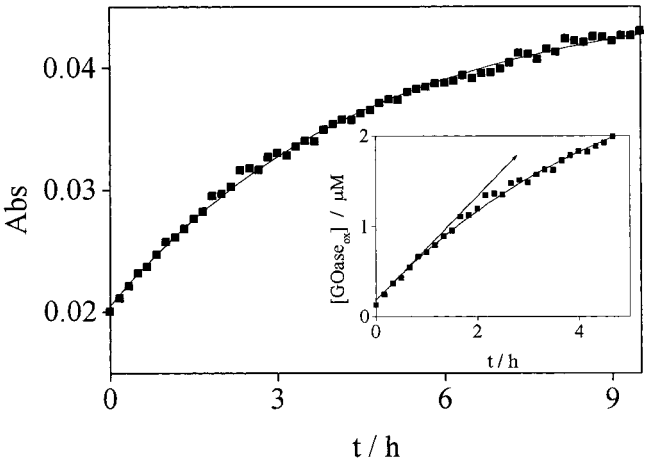

Figure 7. Absorbance (450 nm) with time changes for the oxidation of $GOase_{semi}$ (11.0 μM) to $GOase_{ox}$ with O_2 (0.75 mM) at pH 7.5 (10 mM lutidine), $I = 0.100$ M (NaCl). The inset illustrates the initial slope method used to determine rates with no $GOase_{ox}$ present.

Information). Figure 7 shows absorbance vs time changes for a run at pH 7.5, and the procedure for determining the initial rate

$$GOase_{semi} + O_2 \rightarrow GOase_{ox} + O_2^- \qquad (10)$$

($t = 0$) is illustrated in the inset. On dividing the rate by $[GOase_{semi}]_o$, values of k_{obs}/s^{-1} are obtained, Table S4 (Supporting Information). These exhibit first-order dependencies on $[O_2]$, Figure 8. No reaction is observed at zero $[O_2]$. The slopes

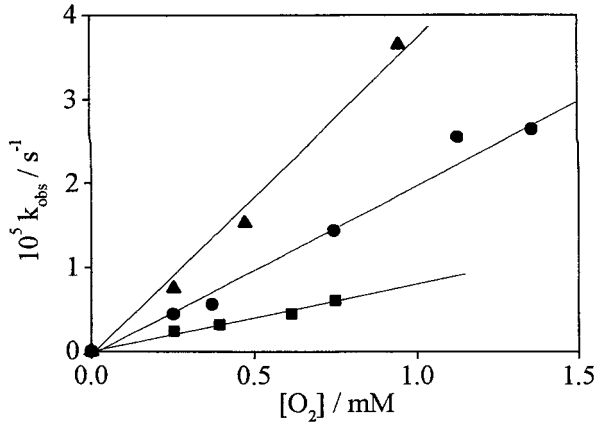

Figure 8. The dependence of first-order rate constants k_{obs}(25 °C) for the O_2 oxidation of $GOase_{semi}$ on $[O_2]$, at 6.0 (□), 7.5 (●), and 8.6 (▲) (10 mM lutidine), $I = 0.100$ M (NaCl).

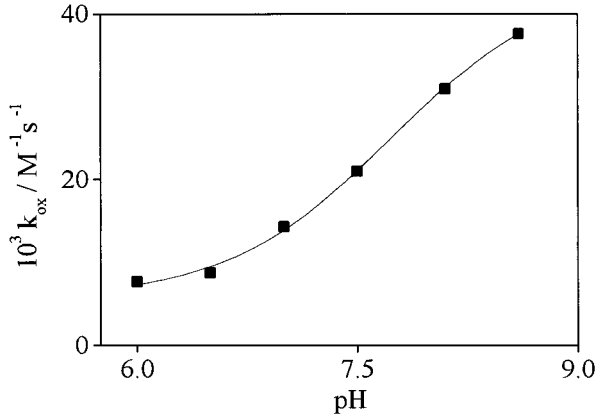

Figure 9. The variation of k_{ox}/M^{-1} s^{-1} (25 °C) for the O_2 (0.252 mM) oxidation of $GOase_{semi}$ with pH, monitored at 450 and 810 nm (average shown), $I = 0.100$ M (NaCl).

give k_{ox}/M^{-1} s^{-1} values of 0.0077(1) (pH 6.0, 10mM Mes), 0.021(1) (pH 7.5, 10mM lutidine), and 0.038(1) (pH 8.6, 10mM Ches). A plot of all k_{ox} values vs pH (Figure 9) gives a $pK_a = 7.7(2)$, in agreement with previous $GOase_{semi}$ pK_{2a} values of 8.0 (1) ascribed to H^+Tyr-495.[17] At low pH, $k_H = 6(1) \times 10^{-3}$ M^{-1} s^{-1} makes little or no contribution, and at high pH $k_o = 44(3) \times 10^{-3}$ M^{-1} s^{-1} from a fit to (11).

$$k_{ox} = \frac{k_o K_a + k_H[H^+]}{K_a + [H^+]} \qquad (11)$$

It is assumed that formation of O_2^- in (10) is followed by O_2^- oxidation of a second $GOase_{semi}$ in a fast step, giving H_2O_2. To allow for such contributions, experimental k_{ox} values as listed need to be halved.

There is no evidence for the reaction $GOase_{semi} + O_2$ (reactant in large excess) conforming to simple equilibration kinetics. Thus apparent k_{eq} values can be determined from pseudo-first-order plots and the applicability of $k_{eq} = k_{ox}[O_2] + k_{red}$ tested. Values of k_{red} (Table S1) are 4-fold those for k_{eq}, and there is no evidence for such an equation applying. The results provide additional support for the two separate processes k_{ox} and k_{red}.

Effect of Anions on O_2 Oxidation of $GOase_{semi}$. On addition of $[N_3^-] = 9.8$mM, sufficient to give >98% complexing at the exogenous binding site at pH 7.5, no effect on the $GOase_{semi}$ reaction with O_2 (1.36 mM) was observed. The rate constant

k_{ox} is unchanged at 0.021 M^{-1} s^{-1} and with ~10% allowance for Cu-free protein 100% formation of $GOase_{ox}$ is observed in 17 h.

O_2 Oxidation of $HSPO_3^{2-}$-Modified $GOase_{semi}$. A sample of $HSPO_3^{2-}$-modified $GOase_{semi}$ was prepared at pH 7.5 and oxidation in the presence of O_2 (0.252 mM) monitored. The rate constant k_{ox} was unchanged (0.020 M^{-1} s^{-1}), and 72% formation of $GOase_{ox}$ was observed.

Reaction of $GOase_{semi}$ with H_2O_2. Addition of H_2O_2 to $GOase_{semi}$ in small amounts, i.e., 0.5−2.0 protein equivalents, gave no UV−vis changes. With a 20-fold (1mM) excess of H_2O_2, up to 24% changes occurred, but some precipitation was also noted. The $GOase_{semi}$ could be recovered by reaction with $[Fe(CN)_6]^{4-}$ It is concluded that H_2O_2 is not at sufficiently high levels to bring about $GOase_{semi}$ oxidation. During the catalytic cycle, H_2O_2 is formed, but little or no reaction with $GOase_{red}$ is apparent.

Discussion

From the autoredox studies described in this paper, there are two contributing reactions: (a) the intramolecular k_{red} process, $GOase_{ox} + e^- \rightarrow GOase_{semi}$ as in (5); and (b) the second-order reaction of $GOase_{semi}$ with O_2 (k_{ox}) as in (10). For O_2-free conditions, (a) proceeds to completion. However, the reaction of $GOase_{semi}$ with O_2 gives $GOase_{ox}$, and (a) then contributes alongside (b). As a result, maximum formation of $GOase_{ox}$ falls well short of 100%. Thus, at pH 7.5 the reaction of $GOase_{semi}$ (25 μM) with O_2 (1.13 mM) gives a maximum $GOase_{ox}$ formation of 69%.

First-order rate constants k_{red} are dependent on pH, Figure 3, and from a fit of data a pK_a of 6.7(2) is obtained. Similar acid dissociation pK_a values have been obtained in previous studies on $GOase_{ox}$ (average 6.9)[7−9] and have been assigned to acid dissociation of H^+Tyr495 (pK_{2a}), (12).

$$H^+\text{Tyr-495} \overset{K_{2a}}{\rightleftharpoons} \text{Tyr-495} + H^+ \qquad (12)$$

It is concluded from Figure 3 (by extrapolation of the high pH data as shown) that little or no autoreduction occurs with Tyr-495 protonated and OH^- present as the exogenous ligand. The UV−vis spectrum of $GOase_{ox}$ is pH dependent, and the peak at 800 nm has been assigned to an interligand Tyr272-Cu-Tyr495 charge-transfer process (CT), via the Cu d_{xy} orbital.[19] The 4-coordinate Cu^{II} present in $GOase_{ox}$ Tyr495Phe has a much less strong absorption at 800 nm and also gives negligible autoreduction. In recent studies, Parrinello and co-workers have used dynamic density functions and mixed quantum-classical calculations on GOase (and model complexes) to consider the electron distribution over the Cu^{II}-linked Tyr-495 and Tyr-272.[20] At high pH (Tyr-495 unprotonated) the unpaired electron of the $Tyr^•$ is delocalized over Tyr-495, but at low pH (Tyr-495 protonated) the delocalization is solely over Tyr-272. Other comments on this effect have been made.[21−24] At the lower pH's, the trend in k_{red} is reversed (Figure 3), with uptake of an electron by the exogenous Cu^{II}-H_2O (pK_a 5.7) more favorable than that of Cu^{II}-OH^-, (13).

$$Cu^{II}\text{-}H_2O \overset{K_{1a}}{\rightleftharpoons} Cu^{II}\text{-}OH^- + H^+ \qquad (13)$$

Similarly no autoreduction of $GOase_{ox}$ is observed with >98% complexing of N_3^- and NCS^- at the exogenous site,[5] and inhibition is apparent with the less extensively complexed $CH_3CO_2^-$ and phosphate. Therefore, the anions OH^-, N_3^-, NCS^-, $CH_3CO_2^-$, and phosphate all have inhibiting effects on autoreduction, (5). Interestingly, the 800 nm $GOase_{ox}$ peak is diminished on addition of N_3^-, NCS^-, and $CH_3CO_2^-$, while phosphate has little or no effect on the UV−vis spectrum. The latter is in contrast to changes observed for phosphate with the Trp290His variant (the active site of which is easier to access)[25] and suggests that in the present case the phosphate interaction is outer-sphere. It is concluded that anions at or near the $GOase_{ox}$ active site inhibit autoreduction.

The phosphate inhibition of autoreduction has enabled K(25 °C) for the interaction of phosphate with $GOase_{ox}$ to be determined. The procedure was first checked by studying the inhibition of $CH_3CO_2^-$ at pH 6.4. At pH 7.0, K(25 °C) for the interaction with phosphate is 46 M^{-1}. Hence, the percentage of $GOase_{ox}$ associated with phosphate at different phosphate levels, 10 mM (32%) and 50 mM (70%), can be calculated and may relate to a previous report.[26] At pH 7.0 K/M^{-1} for phosphate is less than values for NCS^- (480), N_3^- (1.98×10^4), and $CH_3CO_2^-$ (104), and the tetrahedral phosphate appears to have greater difficulty in accessing the Cu^{II} site. The effect of phosphate buffer (10 and 50 mM levels) on K for the complexing of NCS^- and N_3^- to $GOase_{ox}$ is small, suggesting that the phosphate is readily displaced.[9] The interaction with phosphate is rapid and occurs within the mixing time, with no further changes apparent over 7 days.

An important outcome of this study is that (5) is not the reverse of (10) and that equimolar amounts of H_2O_2 are not effective in the redox changes observed. Nothing is known about the source of electrons for (5), but information has been obtained regarding the pathway for electrons to the active site of $GOase_{ox}$. An $RSSR^{•-}$ radical has been generated in pulse radiolysis studies on $GOase_{semi}$ when a relatively slow decay (0.17 s^{-1}) to the Cu^{II} of $GOase_{semi}$ is observed. There are two disulfide groups in GOase, of which Cys515-Cys518 is the more exposed, and nearest to the Cu.[4,5] The nearest S-atom is 10.2 Å from the Cu, Figure 10. To explore the possible involvement of the disulfide in autoreduction, $HSPO_3^{2-}$ modification was carried out, which cleaves the S−S bond to give a new disulfide, $RSSPO_3^{2-}$. With this modification in place, autoreduction of $GOase_{ox}$ no longer occurs. While it is possible that radicals still form at the modified disulfide, the pathway through to the $Cu^{II}/Tyr^•$ active site is disrupted. Reduction potentials for the RSSR/$RSSR^{•-}$ couple have been reported to be −420 mV at pH 7.[28] Given that the reduction potential for $GOase_{ox}/GOase_{semi}$ is 400 mV, the intramolecular redox change in (14) is favorable by 820 mV.

$$RSSR^{•-} + GOase_{ox} \rightarrow RSSR + GOase_{semi} \qquad (14)$$

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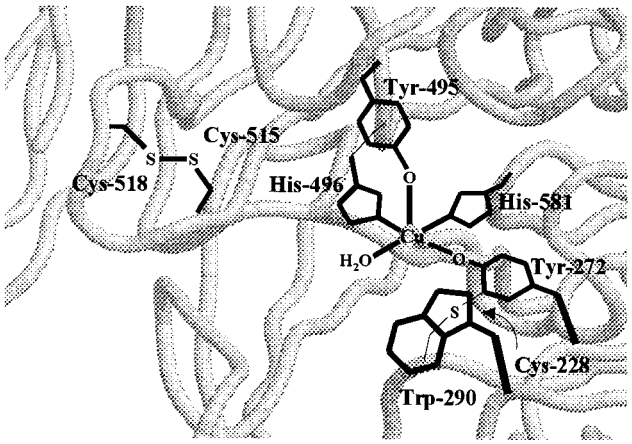

Figure 10. Structure of galactose oxidase showing the relative positions of the disulfide at Cys515-Cys518 and the Cu^{II} active site.[4]

It appears therefore that $RSSR^{\bullet-}$ is involved as a transient source of the electron for autoreduction of the $Tyr^{\bullet}$ and that this and the anion inhibition effects define a pathway for electron transfer to the active site.

Studies on the autoxidation of $GOase_{semi}$ provide evidence for a quite different process, with a rate law first order in $[GOase_{semi}]$ and $[O_2]$ which defines k_{ox}. Modification of the disulfide as described above has no effect on the reaction. Likewise, when N_3^- is complexed >98% at the exogenous site the rate law (and k_{ox}) is unaffected. This establishes an entirely different kind of redox process. The O_2 clearly does not bind at the exogenous site of $GOase_{semi}$ and outer-sphere electron transfer from Tyr-272 to the O_2 needs to be considered. It is possible that the O_2 can approach sufficiently close to residues Trp-290/Cys-228 (making use of the Cu^{II} access channel) or Tyr-495/Tyr-272 (Figure 10) for such an electron-transfer process. The situation contrasts with that of the catalytic cycle when O_2 binds to the Cu^{I} of $GOase_{red}$, and in a two-equivalent oxidation gives H_2O_2 and $GOase_{ox}$ as products.[7] The contrasting affinities of Cu^{I} and Cu^{II} for O_2 are of interest. In other experiments it has been established (and here confirmed) that rate constants for the oxidation of $GOase_{semi}$ with, for example, $[Fe(CN)_6]^{3-}$ are unaffected by pK_{1a}, i.e., whether H_2O or OH^- is present at the exogenous site.[8] Therefore, in these reactions also an alternative pathway for electron transfer from Tyr-272 is required with no involvement of the exogenous position.

At pH 7.5 the second-order rate constant (k_{ox}) for the reaction of O_2 with $GOase_{semi}$ is 0.021 M^{-1} s^{-1}. For an outer-sphere reaction, the rate-determining step is $O_2 + e^- \rightarrow O_2^-$, where O_2^- presumably reacts rapidly with a second $GOase_{semi}$ to give H_2O_2. The pK_a of 4.8 for HO_2[29,30] is effective outside the range studied. From $E^{\circ\prime}$ values for the O_2/O_2^- couple (−160 mV; pH independent),[31−33] and for $GOase_{ox}/GOase_{semi}$ (400 mV at pH 7.5), the first stage of oxidation by O_2 is thermodynamically uphill. However, with the inclusion of the second stage $O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$ (E° = 695mV at pH 7.0),[30,34,35] the overall change is favorable. Similar features are apparent in the O_2 oxidation of eight ruthenium(II) am(m)ine complexes, Ru^{III}/Ru^{II} reduction potentials 51−533 mV ($E^{\circ\prime}$). Rate constants (k) have been determined (pH 1−2, I = 0.100 M)[36] and range from 0.0077 M^{-1} s^{-1} for the reaction of $[Ru(NH_3)_4(phen)]^{2+}$ (533 mV) to 63 M^{-1} s^{-1} for $[Ru(NH_3)_6]^{2+}$ (51 mV). The results give a linear free-energy plot of ln k vs $E^{\circ\prime}$ for the Ru^{III}/Ru^{II} couple, and the reactions have been assigned as outer-sphere $O_2 \rightarrow O_2^-$ processes.[36] The rate constant of 0.11 M^{-1} s^{-1} for $[Ru(NH_3)_5(isn)]^{2+}$ (387 mV; isn = isonicotinamide) is of interest in the context of the present studies, since it compares with 0.021 M^{-1} s^{-1} (similar driving force) for the reaction of O_2 with $GOase_{semi}$.

To summarize, two reactions contribute to the autoredox interconversion of $GOase_{ox}$ and $GOase_{semi}$. The reactions are of interest because they define relatively simple processes leading to the decay and formation of the Cu^{II}-coordinated radical Tyr· at Tyr-272. One of these, which in air-free conditions proceeds to 100% completion, involves a spontaneous intramolecular reduction of $GOase_{ox}$. Electron transfer to the Tyr-272 radical can be blocked by chemical modification of the nearby (10.2 Å) Cys515-Cys518 disulfide, confirming this group as a transient source of an electron. Anion coordination of OH^-, N_3^-, NCS^-, $CH_3CO_2^-$, and phosphate at or (in the phosphate case) near the exogenous site blocks autoreduction and furthers define an electron-transfer pathway to $Tyr^{\bullet}$. The second reaction involves $O_2 \rightarrow O_2^-$ oxidation of $GOase_{semi}$, with the regeneration of $Tyr^{\bullet}$ and formation of $GOase_{ox}$. This reaction is believed to be outer-sphere and in the vicinity of Tyr-272 with no bonding of O_2 to the Cu^{II}. Anions such as N_3^- at the exogenous site have no effect, and a different electron-transfer pathway from Tyr-272 possibly via other amino acid residues through to the O_2 is occurring. In its turn $GOase_{ox}$ undergoes autoreduction, and in air a mixture of the two states is obtained.

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Supporting Information Available: Listing of rate constants and extent of oxidation of $GOase_{semi}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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