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Pulse Radiolysis Studies on Galactose Oxidase

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Single-Cu-containing galactose oxidase in the $GOase_{semi}$ state (Cu^{II}, no $Tyr^{\bullet}$ radical) reacts with pulse radiolysis generated formate radicals $CO_2^{\bullet-}$ to give an intermediate UV–vis spectrum assigned as $RSSR^{\bullet-}$, peak at 450 nm ($\epsilon = 8100\ M^{-1}\ cm^{-1}$). From a detailed kinetic analysis at 450 nm, pH 7.0, the following steps have been identified. First the strongly reducing $CO_2^{\bullet-}$ (–1.9V) reduces $GOase_{semi}$ ($k_0 \geq 6.5 \times 10^8\ M^{-1}\ s^{-1}$) to a species $GOase_{semi}^{\bullet-}$. This is followed by biphasic reactions (i) $GOase_{semi}^{\bullet-} + GOase_{semi}$ ($k_1 = 1.6 \times 10^7\ M^{-1}\ s^{-1}$) to give $GOase_{semi} + P^{\bullet-}$ and (ii) $P^{\bullet-} + GOase_{semi}$ ($k_2 = 6.7 \times 10^6\ M^{-1}\ s^{-1}$) to give $GOase_{semi}RSSR^{\bullet-}$. There are no significant absorbance changes for the formation of $GOase_{semi}^{\bullet-}$ and $P^{\bullet-}$, which are Cu^{I} (or related) species. However, $GOase_{semi}RSSR^{\bullet-}$ has an absorption spectrum which differs significantly from that of $GOase_{semi}$. The 450 nm peak is characteristic of an $RSSR^{\bullet-}$ radical with two cysteines in close sequence proximity and is here assigned to Cys515–Cys518, which is at the GOase surface and 10.2 Å from the Cu. On chemical modification of the RSSR group with $HSPO_3^{2-}$ to give $RSSPO_3H^-$ and RS^-, absorbance changes are ~50% of those previously observed. The decay of $RSSR^{\bullet-}$ ($0.17\ s^{-1}$) results in the formation of $GOase_{red}$. No $RSSR^{\bullet-}$ formation is observed in the reaction of $GOase_{semi}$ Tyr495Phe with $CO_2^{\bullet-}$, and a single process giving $GOase_{red}$Tyr495Phe occurs. Similarly in the reaction of $GOase_{ox}$ with $CO_2^{\bullet-}$, a single-stage reaction gives $GOase_{semi}$.

Introduction

Galactose oxidase (GOase; EC 1.1.3.9) is a single-Cu-containing enzyme (MW = 68.5 kDa; 639 amino acids) isolated from fungal sources, in this case *Fusarium* (NRRL 2903).[1,2] It is a 2-equiv oxidase, which in the active state ($GOase_{ox}$) has Cu^{II} and a coordinate tyrosyl radical ($Tyr^{\bullet}$) as redox active components.[3] In the enzymic process $GOase_{ox}$ oxidizes primary alcohols RCH_2OH to RCHO in a 2-equiv change giving $GOase_{red}$. Regeneration of $GOase_{ox}$ occurs with O_2, overall reaction 1. The three redox states of the enzyme can be defined as in (2).

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

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

At pH 7.0 reduction potentials of the $GOase_{ox}/GOase_{semi}$ and $GOase_{semi}/GOase_{red}$ couples are 410 [4] and 159 mV,[5] respectively (vs NHE). From X-ray crystallography the Cu^{II} has a square-pyramidal geometry, and is coordinated by radical-forming Tyr-272, His-496, His-581, and X at the exogenous substrate binding site in a square-planar configuration with Tyr-495 as the axial ligand.[6] Structures with X = $CH_3CO_2^-$, H_2O, or OH^- and N_3^- have been determined.[6–8] To obtain stable $GOase_{red}$, $[Ru(NH_3)_6]^{2+}$ has been employed as a reductant, and protonation of the active site is apparent from the rate law.[5] This helps to bring about a decrease in

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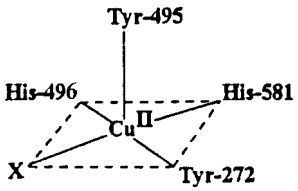
coordination number at Cu^{I}.[9,10] Protons are moreover required in the conversion of O_2 to H_2O_2 as part of the enzyme redox cycle.



To better understand these changes pulse radiolysis (PR) techniques have been used as a means of generating the $GOase_{red}$ state. The use of formate gives uniquely all reducing conditions, with $CO_2^{\bullet-}$ (−1.9 V) the only significant redox active component present. Unexpectedly, in the course of the sequential changes observed, a disulfide $RSSR^{\bullet-}$ radical form of $GOase_{semi}$ is observed.

Experimental Section

Source of GOase. Galactose oxidase (GOase) from the fungal source *Fusarium* (strain NRRL 2903),[11,12] was obtained using an *Aspergillus nidulans* strain (G191/pGOF101).[11,13] Yields of GOase were 25−30 mg/L of culture medium. A similar expression system was used for the Tyr495Phe variant,[14] yield 20−25 mg/L. Final purification was using phosphocellulose column chromatography, and the purity was confirmed by FPLC (Pharmacia) which gave a single peak using a Mono-S cation-exchange column. GOase samples were concentrated and stored at −80 °C. In such samples the enzyme is present as a mix of $GOase_{ox}$ and $GOase_{semi}$.[15,16] UV−vis spectra of wild-type GOase and the Tyr495Phe variant in all three oxidation states and at varying pH's have been reported.[4,5,15] Concentrations of enzyme were determined from the absorbance at 280 nm ($\epsilon = 1.05 \times 10^5$ M^{-1} cm^{-1}).[12] At pH 7.0 $GOase_{semi}$ has an ϵ value of 1635 M^{-1} cm^{-1} at 645 nm.

Other Reagents. Potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, potassium hexacyanoferrate(II), $K_4[Fe(CN)_6]\cdot 3H_2O$, and sodium formate, $NaCO_2H$, were all from BDH (Analar). Cylinder nitrous oxide N_2O, oxygen free, was from BOC. Phosphate buffer, made up from the sodium hydrogen phosphates Na_2HPO_4 and NaH_2PO_4 (Sigma Chemicals), was used in PR experiments. Buffers used for other purposes were the following: 2-morpholinoethanesulfonic acid (Mes, pK_a 6.1, pH 5.5−6.7); 2-(cyclohexylamino)ethanesulfonic acid (Ches, pK_a 9.3, pH 8.6−10.0); 2,6-dimethylpyridine, Fluka (lutidine, pK_a 6.75, pH 5.7−8.0).

Reactant Solutions. For all PR experiments solutions were made up to the required pH using $H_2PO_4^-$ (pK_a 6.68) and HPO_4^{2-}, and the ionic strength adjusted to $I = 0.100$ M. After dialysis into the required buffer, preparation of stored enzyme as $GOase_{semi}$ was achieved by addition of small aliquots of freshly prepared buffered $[Fe(CN)_6]^{4-}$ (∼100 times GOase). A sample of $GOase_{ox}$ was likewise obtained by treatment with $[Fe(CN)_6]^{3-}$. The reactions with $[Fe(CN)_6]^{4-/3-}$ result in the formation of some apo-GOase.[15] After 2−3 min at room temperature excess reagent was removed by either ultrafiltration (4 °C), using a PM-30 membrane (Amicon) in a 10 mL stirred cell or by passing the protein solution down a small desalting column (0.8 × 12 cm) (P-6DG Gel, Bio-Rad). When excess $[Fe(CN)_6]^{4-}$ or $[Fe(CN)_6]^{3-}$ had been removed, the GOase sample (∼1 mL) was transferred to a small vial and sealed with a rubber septum, and O_2 was removed by passing a stream of N_2 over the surface for ∼20 min. Samples (∼0.2 mM) were then frozen in dry ice. Samples of $GOase_{semi}$ Tyr495Phe were prepared by similar procedures. All solutions were made up with glass-distilled water run through a MilliQ filtration system and/or purified by passing through a Sanyo Puri-Fi deionizer, final conductivity ≤ 10 μS cm^{-1}. The pH of solutions was determined using a Radiometer PHM62 pH meter fitted with a Russell CWR/332 combined Ag/AgCl/glass electrode calibrated using standard solutions.

Chemical Modification of GOase. Trisodium thiophosphate (alternative name trisodium phosphorothioate), Na_3SPO_3, was prepared from thiophosphoryl chloride (Acros Organics) according to a method previously described.[17] Thiophosphoric acid has acid dissociation pK_a values of H_3SPO_3 (2.05), $H_2SPO_3^-$ (5.6), and $HSPO_3^{2-}$ (10.3).[18] Reactions with protein disulfides (i.e. cystines) proceed optimally when the thiophosphate is present as $HSPO_3^{2-}$, and conditions of pH 9.5 (10 mM Ches) with $HSPO_3^{2-}$ in 40-fold excess at 25 °C were used (eq 3).

$$RSSR + HSPO_3^{2-} \rightarrow RS{-}SPO_3H^- + RS^- \qquad (3)$$

After 16 h, excess thiophosphate was removed by dialysis (3 × 2 L buffer at pH 7.0) and the protein purified by FPLC using a Mono-S column (Pharmacia), when a single major peak was observed. After FPLC the modified GOase was made up in Mes buffer at the pH required and frozen at −80 °C. The reagent 5,5′-dithiobis(nitrobenzoic acid), DTNB (Sigma), was used to characterize the product.[16]

Electrospray Mass Spectrometry. As a check that $HSPO_3^{2-}$ modification of $GOase_{semi}$ at one or both RSSR disulfides had occurred, electrospray mass spectrometry was carried out. The protein sample was desalted (zero level buffer used), and acetic acid (1%) was added before introduction into a Micromass Autospec M spectrometer. The mobile phase used was acetonitrile (60%)−water (40%). A high nozzle skimmer cone voltage of 96.2 mV was required for detection. Mass spectra were obtained in the positive ion mode and analyzed using Micromass Opus software to give a mean MW of 68 654 Da (unmodified) and 68 755 Da (modified).[16] The difference between the two samples of 101 Da (MW of SPO_3^{2-} 111 Da) indicates that only one disulfide is modified.

Colorimetric Method Using Ellman's Reagent. Further confirmation of the modification was carried out using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; 5 mM), at pH 7.2 (100 mM phosphate) in the presence of 0.10 mM EDTA (disodium dihydrogen ethylenediaminetetraacetate). This reacts with the polypeptide cysteine displaced by the $HSPO_3^{2-}$ (eq 3) to give an intense yellow colored thionitrobenzoic acid derivative, absorbance at 412 nm ($\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}) (eq 4).[19] With the modified protein, reaction at one cysteine per GOase was indicated.

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No absorbance changes were observed with unmodified $GOase_{semi}$.

$$\underset{\text{DTNB}}{NO_2R'SSR'NO_2} + \underset{\text{cysteine}}{RS^-} \rightarrow NO_2R'SSR + \underset{\text{colored product}}{NO_2R'S^-} \quad (4)$$

Pulse Radiolysis Procedure. Initial PR experiments were carried out on a Van de Graaff accelerator at the University of Leeds Cookridge Radiation Research Centre, using a multipass cell (6.9 cm light path length) and a 2.5 MeV ($\sim 4 \times 10^{-16}$ J) beam of electrons. Pulse lengths were 0.6 μs, and the yields of reducing radicals generated by the pulse were determined by standard procedures.[20] All runs were performed under anaerobic conditions in N_2O-saturated phosphate buffer (40.5 mM) at pH 7.0, containing 0.010 M sodium formate, $I = 0.100$ M. Under these conditions the radical products $OH^\bullet$ and $H^\bullet$ are converted to the strongly reducing $CO_2^{\bullet-}$ (−1.9 V) as the only radical species present (eqs 5 and 6).

$$OH^\bullet + HCO_2^- \rightarrow H_2O + CO_2^{\bullet-} \quad (5)$$

$$H^\bullet + HCO_2^- \rightarrow H_2 + CO_2^{\bullet-} \quad (6)$$

Small amounts of ~0.2 mM $GOase_{semi}$ were added (under a stream of N_2O) to the N_2O-saturated buffer solution to give final concentrations 7.4−18.0 μM. Reactions were monitored in the range 380−510 nm at 22 ± 1 °C with $[CO_2^{\bullet-}] < 0.6$ μM. Absorbance vs time traces were recorded on a Gould 4072 100 MHz Transient Digitizer, which was used in conjunction with a Sun SPARC Station IPC computer for storage and processing. The PR traces were subject to first-order kinetic analysis using the TREAT program.[21]

Phosphate is used as buffer in pulse radiolysis studies because organic buffers give free-radical products. However while this work was in progress it was found that phosphate interacts with $GOase_{ox}$.[16] A formation constant $K = 46$ M^{-1} was determined at pH 7.0, and since Cu^{II} is present also in $GOase_{semi}$, a similar value is expected. Moreover, from titrations carried out at pH 7.0 (40.5 mM phosphate), $I = 0.100$ M (NaCl), formate interacts with $GOase_{semi}$, formation constant K (25 °C) = 186(1) M^{-1}, which as in the case of acetate is assigned to binding at the exogenous site.[15] With phosphate (40.5 mM) and formate (0.010 M), ~50% complexing takes place.

In view of the above, it was necessary to carry out checks on the various PR procedures used. This was most readily done by decreasing the concentration of phosphate, since procedures are crucially dependent on the presence of 0.01 M formate. The checks carried out were using a Linacc accelerator at the Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex, U.K., using N_2O-saturated phosphate buffer (2 mM) at pH 7.0. The ionic strength was adjusted to $I = 0.100 \pm 0.001$ M with $NaClO_4$. Concentrations of $GOase_{semi}$ were in the range 4.9−19.8 μM. A dose of ~3.7 Gy and pulse length of 0.2 μs were used. The kinetics were monitored at 450 nm with $[CO_2^{\bullet-}] \sim 2.2$ μM. The pattern of behavior was found to be the same, with some changes in numerical values. Values of k_{1obs} and k_{2obs} from the latter studies are listed in this work. Other effects were minimal and the original data retained.

Enzyme after Pulsing. Samples of $GOase_{semi}$ which had been used in PR experiments gave the same rate constants for oxidation with $[Fe(CN)_6]^{3-}$ as those previously reported.[4]

Results

Pulse Radiolysis Reaction Scheme. Experiments on $WTGOase_{semi}$ demonstrate the formation and decay of an intensely absorbing species with $\lambda_{max} = 450$ nm, Figures 1 and 2. The transient absorption spectrum over the range 380−510 nm shown in Figure 2A was recorded 50 ms after the pulse. The absorbance at 450 nm was independent of $[GOase_{semi}]$ from 8 to 18 μM and gave $\epsilon = 8.1 \times 10^3$ M^{-1} cm^{-1}, where both features are characteristic of a protein disulfide radical $RSSR^{\bullet-}$. When viewed on faster sweep speeds (<100 ms) the growth of absorption at 450 nm exhibits sigmoidal character, which suggests that consecutive

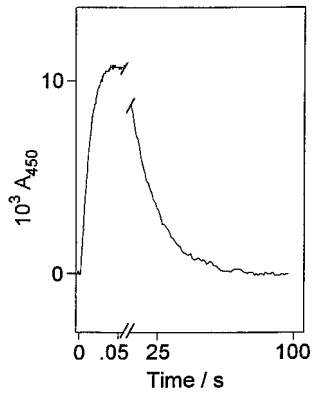

Figure 1. Absorbance changes monitored at 450 nm following $CO_2^{\bullet-}$ pulse radiolysis of $GOase_{semi}$ (8.0 μM) at pH 7.0. The rate constants k_1 and k_2 contribute to the first phase (absorbance increase) in which $GOase_{semi}RSSR^{\bullet-}$ is formed. The absorbance decay corresponds to the intramolecular process k_3 giving $GOase_{red}$. Note that the initial and final absorbance readings are identical.

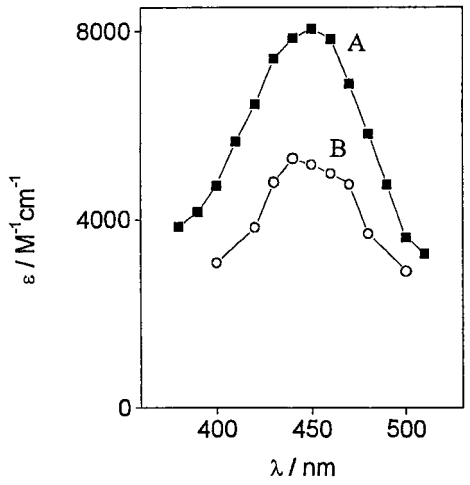

Figure 2. UV−vis spectra of the $RSSR^{\bullet-}$ component recorded post-$CO_2^{\bullet-}$ (<0.6 μM) reduction of $GOase_{semi}$ (8.0 μM) and following completion of k_1 and k_2, pH 7.0, $I = 0.100$M. Spectrum A was obtained with $WTGOase_{semi}$, and spectrum B is the corresponding spectrum using thiophosphate-modified $GOase_{semi}$.

Table 1. First-Order Rate Constants (22 °C), k_{1obs} and k_{2obs}, for a Two Consecutive Reaction Fit to Absorbance (A) Increases at 450 nm vs Time (Eq 7) with Runs at pH 7.0 (2 mM Phosphate) and $I = 0.100$ M ($NaClO_4$)

$[GOase_{semi}]/\mu M$	k_{1obs}/s^{-1}	k_{2obs}/s^{-1}
19.8	322	134
	241	149
11.8	243	111
	195	78.5
	196	79.0
	180	71.3
4.9	84	42.8
	117	26.0

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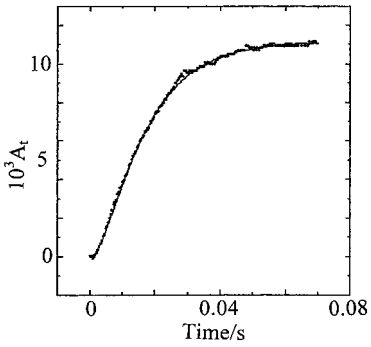

Figure 3. Biphasic fit to the absorbance increase (22 °C) monitored at 450 nm (post-reaction of $CO_2^{\bullet-}$), giving first-order rate constants k_{1obs} and k_{2obs} corresponding to eqs 9 and 10 with [$GOase_{semi}$] in large excess, pH 7.0 (2 mM phosphate), $I = 0.100$ M ($NaClO_4$).

Table 2. First-Order Rate Constants k_3 (22 °C) for the Decay of the $GOase_{semi}$ $RSSR^{\bullet-}$ Intermediate at 450 nm, pH 7.0, Except As Indicated, with $I = 0.100$ M

[$GOase_{semi}$]/μM	k_3/s^{-1}	[$GOase_{semi}$]/μM	k_3/s^{-1}
8.0	0.172	18.0	0.170
10.3	0.164		0.230[a]
14.0	0.174		0.049[b]

[a] pH 6.0. [b] pH 8.0.

steps are involved with a nonabsorbing intermediate. Equation 7 describes the growth of absorption resulting from two consecutive first-order reactions, where k_{2obs} is the rate constant for the slower step, $R = k_{1obs}/k_{2obs}$, and k_{1obs} is the rate constant of the faster step.

Analysis of the sigmoidal traces according to eq 7 using a nonlinear least-squares fitting program yields values of k_{1obs} and k_{2obs} as listed in Table 1. The quality of fit is illustrated in Figure 3. Both k_{1obs} and k_{2obs} increase with [$GOase_{semi}$] as shown in Figure 4a,b.

$$A_t = A_\infty\{1 + \exp(-Rk_{2obs}t) - R\exp(-k_{2obs}t)\}/(R-1) \quad (7)$$

At the [$GOase_{semi}$] employed, the values of k_{1obs} are too small for this reaction to compete effectively with the self-recombination of $CO_2^{\bullet-}$ ($2k = 1.3 \times 10^9$ M^{-1} s^{-1}).[22] Therefore, we must conclude that, in addition to the two-stage process, there is a faster stage, which involves a nonabsorbing species. This is believed to be a Cu^I or Cu^I-related species which we designate $GOase_{semi}^{\bullet-}$ (eq 8).

Since this species is formed in competition with the self-decay of $CO_2^{\bullet-}$, it can be concluded that $k_0 \geq 6.5 \times 10^8$ M^{-1} s^{-1}. Thus, the two consecutive steps giving rise to the sigmoidal character follow reaction 8.

$$CO_2^{\bullet-} + GOase_{semi} \xrightarrow{k_0} GOase_{semi}^{\bullet-} \quad (8)$$

The slope of Figure 4a gives $k_1 = 1.8(\pm0.1) \times 10^7$ M^{-1} s^{-1}, and from Figure 4b $k_2 = 6.7(\pm0.4) \times 10^6$ M^{-1} s^{-1}. Therefore, after the initial reduction of the protein (eq 8), the formation of $RSSR^{\bullet-}$ involves two consecutive bimolecular steps as in (9) and (10). Alternatively, a least-squares linear regression treatment for Figure 4a suggests a slope of

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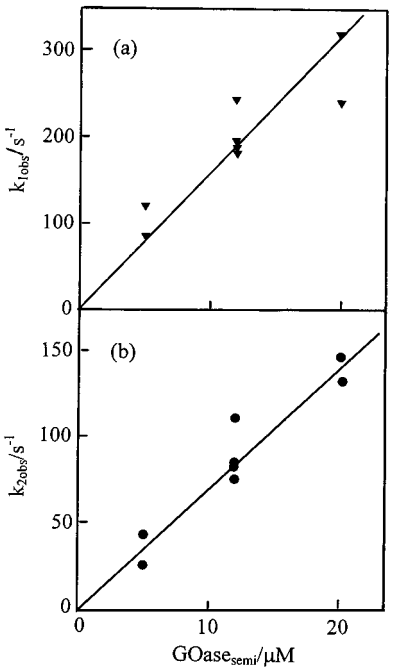


Figure 4. Dependencies of (a) k_{1obs} and (b) k_{2obs} on [$GOase_{semi}$] at 22 °C, pH 7.0 (2 mM phosphate), $I = 0.100$ M ($NaClO_4$).

Table 3. Summary of Kinetic Data (22 °C) from Pulse Radiolysis Studies on $GOase_{semi}$, pH 7.0, with $I = 0.100$ M

reaction	rate constant
$CO_2^{\bullet-} + GOase_{semi} \rightarrow GOase_{semi}^{\bullet-} + CO_2$	$k_0 \geq 6.5 \times 10^8$ M^{-1} s^{-1}
$CO_2^{\bullet-} + CO_2^{\bullet-} \rightarrow$ products	$2k = 1.3 \times 10^9$ M^{-1} s^{-1} [a]
$GOase_{semi}^{\bullet-} + GOase_{semi} \rightarrow P^{\bullet-}$	$k_1 = 1.8 \times 10^7$ M^{-1} s^{-1}
$P^{\bullet-} + GOase_{semi} \rightarrow GOaseRSSR^{\bullet-} + P$	$k_2 = 6.7 \times 10^6$ M^{-1} s^{-1}
$GOaseRSSR^{\bullet-} \rightarrow$ products	$k_3 = 0.17$ s^{-1}

[a] Reference 22.

$k_{1a} = 1.1(\pm0.3) \times 10^7$ M^{-1} s^{-1} and intercept $k_{1b} = 65.6(\pm33.7)$ s^{-1}. However, the error in the intercept is large and may well stem from the reaction of $GOase_{semi}$ with small (and variable) trace amounts of O_2.

$$GOase_{semi}^{\bullet-} + GOase_{semi} \xrightarrow{k_1} GOase_{semi} + P^{\bullet-} \quad (9)$$

$$P^{\bullet-} + GOase_{semi} \xrightarrow{k_2} P + GOase_{semi}RSSR^{\bullet-} \quad (10)$$

Kinetics of $RSSR^{\bullet-}$ Decay. UV−vis absorbance vs time changes give a good fit to a single first-order process (k_3), Figure 5. Values of k_3 at pH 7.0 are independent of [$GOase_{semi}$] consistent with an intramolecular process, Table 2, and give $0.17(\pm0.02)$ s^{-1}. On increase of the pH, k_3 decreases, Table 2.[4] The rate constant is assigned to electron transfer over a distance 10.2 Å to the Cu. In previous studies the spontaneous decay of $GOase_{ox}$, $k_{red} = 1.9 \times 10^{-4}$ s^{-1} at pH 7.0,[16] has been observed and suggests an intramolecular reaction with $RSSR^{\bullet-}$. It is possible that the step involving generation of $RSSR^{\bullet-}$ may contribute in this case. A summary of kinetic data is given in Table 3.

Pulse Radiolysis of Chemically Modified $GOase_{semi}$. Studies on $GOase_{semi}$ (7.6 μM), chemically modified with thiophosphate $HSPO_3^{2-}$, were also carried out. As in the case of unmodified $GOase_{semi}$, it was not possible to measure k_0

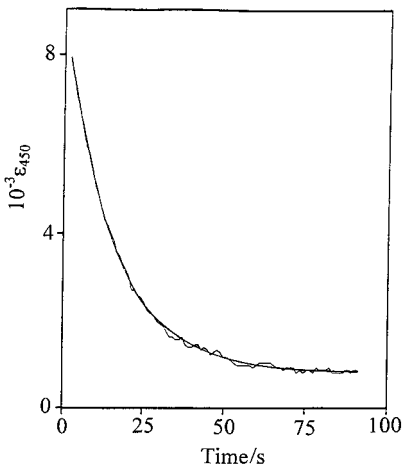


Figure 5. Uniphasic fit giving k_3 (22 °C) for the absorbance decay of the $GOase_{semi}RSSR^{\bullet-}$ radical at 450 nm, $I = 0.100$ M.

directly, and other stages were as already defined. The spectrum of the intermediate formed as in (11) is included in Figure 2. While still characteristic of a disulfide radical, a broader peak with maximum at 440 nm and with absorption coefficient ~50% less ($\epsilon \sim 5 \times 10^3$ M^{-1} cm^{-1}) is obtained. The disulfide radical can be formulated as $RSSPO_3H^{\bullet-}$, where R is either one of Cys-515/Cys-518. The formation of two different products may account for the broader peak.

Pulse Radiolysis of Other GOase Forms. These experiments provide an important contribution in terms of the effect of Cu coordination on the reaction. The same procedures were used as for Figures 1 and 2, pH 7.0 (40.5 mM phosphate), $I = 0.100$ M. In the case of the 4-coordinate $GOase_{semi}$ Tyr495Phe variant (8.0 μM), the pulse was set to give $[CO_2^{\bullet-}] < 0.6$ μM. Initial absorbance changes were fast ($k > 10^9$ M^{-1} s^{-1}) and corresponded to reduction of the Cu^{II}. No intermediate UV−vis spectrum corresponding to the formation of $RSSR^{\bullet-}$ was observed. The product $GOase_{red}$ Tyr495Phe is therefore formed in a single stage, summarized by (11).

$$CO_2^{\bullet-} + GOase_{semi} \rightarrow CO_2 + GOase_{red} \qquad (11)$$

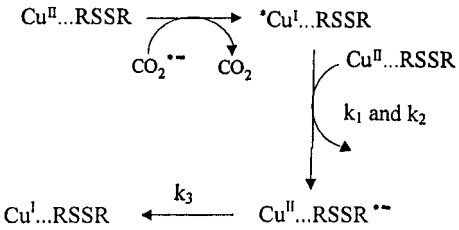
A similar procedure was used for the reaction of $GOase_{ox}$ (6.3 μM) with $CO_2^{\bullet-}$. Absorbance changes at 450 nm showed a rapid decrease corresponding to the $GOase_{ox}$ reduction (eq 12). No other reaction steps were observed, and retention of 5-coordinate Cu^{II} is apparent.

$$CO_2^{\bullet-} + GOase_{ox} \rightarrow CO_2 + GOase_{semi} \qquad (12)$$

Discussion

The most significant observation in the studies on WT $GOase_{semi}$ is the formation of an $RSSR^{\bullet-}$ intermediate with intense absorbance at 450 nm. The reaction steps identified are summarized in Scheme 1. Disulfide radicals $RSSR^{\bullet-}$ are fairly well understood having been detected in studies on the apo-riboflavin binding protein apo-neocarzinostatin and bovine immunoglobin.[23] Mixed disulfide radicals, originating

Scheme 1



from a single cysteine modified with a sulfur-containing reagent HSX to give RSSX, have also been observed in studies on hemerythrin[24] and the α-subunit of hemoglobin.[25] All such radicals are identified by a UV−vis peak in the range 380−450 nm ($\epsilon \sim 10^4$ M^{-1} cm^{-1}). In the present studies the peak at 450 nm ($\epsilon = 8.1 \times 10^3$ M^{-1} cm^{-1}) provides evidence for the formation of $RSSR^{\bullet-}$. The absorbance at 450 nm cannot be assigned to a thiyl radical ($RS^{\bullet}$), which has a weak absorbance with maximum at around 300 nm.[26] The reduction potential of an RSSR /$RSSR^{\bullet-}$ couple has been reported to be −410 mV.[27] $GOase_{ox}$ also has an absorbance peak at 450 nm, and we have considered therefore the possibility that this product is formed by oxidation of $GOase_{red}$. However in the PR, the yield of $CO_2^{\bullet-}$ in N_2O-saturated 0.01 M formate solutions is 6.6×10^{-7} mol J^{-1}, while the yield of H_2O_2 (a possible oxidant) is only 0.71×10^{-7} mol J^{-1}. Hence there is only enough H_2O_2 to oxidize ~10% of the $GOase_{red}$ to $GOase_{ox}$. Similarly if one considers $GOase_{ox}$ formation from $GOase_{semi} + H_2O_2$, the absorption is 10× bigger than could be attributed to this reaction. We conclude that neither of these reactions accounts for the changes observed.

The 450 nm absorbance is assigned to the 3 electron S−S in $RSSR^{\bullet-}$ bond which gives rise to a $\sigma-\sigma^*$ transition.[28,29] The energy associated with the transition is dependent on geometric and electronic constraints imposed on the disulfide. In PR studies on metallothioneins (61 amino acids, 20 of which are cysteines), with cysteines in close proximity, the $RSSR^{\bullet-}$ generated also has an absorbance maximum at 450 nm ($\epsilon = 9 \times 10^3$ M^{-1} cm^{-1}).[28,30] GOase has two disulfides at Cys18−Cys27 and Cys515−Cys518. The smaller separation of the cysteines and greater (surface) accessibility in the latter case suggest that it is the more likely site for reaction.[7] The nearest S atom of Cys515−Cys518 is 10.2 Å from the Cu,[7] which compares with a distance of 40.5 Å in the case of Cys18−Cys27, Figure 6.

One major question to address is why $WTGOase_{semi}$ should react with $CO_2^{\bullet-}$ by a multistage process, while the $GOase_{semi}$ Tyr495Phe variant and $GOase_{ox}$ should involve single-stage processes. A lower coordination number (4, 3, or 2)[31,32] is

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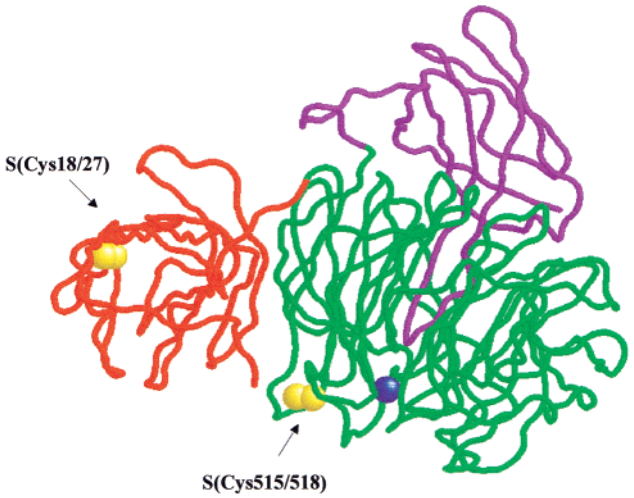

Figure 6. Space-filling model from crystallographic information as reported in ref 7 and from the Brookhaven Data Base, illustrating the proximity of the disulfide group Cys515−Cys518 (yellow) to the Cu^{II} active site (blue) of $GOase_{semi}$.

likely for the d^{10} Cu^{I} in $GOase_{red}$, where from XANES and EXAFS studies 3-coordination is favored.[33] Recent studies on the formation of $GOase_{red}$ by reduction of $GOase_{semi}$ with $[Ru(NH_3)_6]^{2+}$ indicate that protonation and dissociation of Tyr-495 occur.[5] The 4-coordinate Cu^{II} in the $GOase_{semi}$ variant is better prepared for the change to 3-coordinate $GOase_{red}$. Also in the case of the $GOase_{ox}$ reduction to $GOase_{semi}$ (both 5-coordinate Cu^{II}), a single stage process is observed. The more complex outcome in the present studies is therefore attributed to the more demanding 5 to 3 change in coordination. While $CO_2^{\bullet-}$ reacts rapidly with $GOase_{semi}$, it has no H atoms or H^+ component to help adjust the Cu coordination sphere. Hence an alternative route for reaction is observed.

At 450 nm there is very little difference in the absorbance of $GOase_{semi}$ and $GOase_{red}$,[5] and the only significant UV−vis changes are brought about by the formation of $GOase_{semi}RSSR^{\bullet-}$. In the $CO_2^{\bullet-}$ stage of reaction a metastable Cu^{I} site is very likely formed, but we use the $GOase_{semi}^{\bullet-}$ description since there remains a possibility that the electron may reside on a nearby ligand and not on the Cu. There is too little information to fully address the question as to why two intermediates $GOase_{semi}$ and $P^{\bullet-}$ should be involved. One possibility centers around the 50% complexing of formate HCO_2^- (0.01 M) at the exogenous site. Effects of phosphate buffer have been excluded by the experiments using decreased levels (2 mM). The $CO_2^{\bullet-}$ reduction is most likely outer-sphere and unlikely, therefore, to provide an explanation of the two stages. One stage reactions are moreover observed with $GOase_{red}$ Tyr495Phe and $GOase_{ox}$. The bimolecular nature of k_1 and k_2 rules out intramolecular electron transfer from incipient Cu^{I} to the Cys515−Cys518.

There is no evidence for $RSSR^{\bullet-}$ involvement in the reaction of the enzyme with primary alcohols RCH_2OH. The 2-equiv RCH_2OH reduction of $GOase_{ox}$ is achieved by acid dissociation to RCH_2O^-, followed by H atom and e^- transfer to give RCHO. The rate law for the $[Ru(NH_3)_6]^{2+}$ reduction of $WTGOase_{semi}$ to $GOase_{red}$ gives an $[H^+]$ dependence consistent with a single proton uptake and dissociation of Tyr-495.[5] The Cu^{I} coordination in $GOase_{red}$ is therefore Tyr-272, His-496, and His-581, with no exogenous H_2O/OH^- present. Although two protons are required for the O_2 ($\rightarrow H_2O_2$) oxidation of $GOase_{red}$ back to $GOase_{ox}$, the rate law is independent of $[H^+]$, rate constant 1.0×10^7 M^{-1} s^{-1}.[10] This suggests that the first proton is provided by the uncoordinated H^+Tyr-495, and that HO_2^- is then displaced by solvent H_2O with release of H_2O_2.

Conclusions

The different Cu^{II} and Cu^{I} coordination geometries provide what is potentially a significant barrier to redox change between $GOase_{semi}$ and $GOase_{red}$. At the outset the aim was to take advantage of $CO_2^{\bullet-}$ (−1.9 V) as a strong reductant and explore effects on the reduction of 5-coordinate $WTGOase_{semi}$ to 3-coordinate $GOase_{red}$. However, a metastable product is formed, written here as $*Cu^{I}\cdots RSSR$ or (without commitment to the Cu^{I} state) $GOase_{semi}^{\bullet-}$. The bimolecular steps k_1 and k_2 which follow have similar rate constants ($\sim 10^7$ M^{-1} s^{-1}), involve a second molecule of $GOase_{semi}$, and give $GOase_{semi}RSSR^{\bullet-}$. The intramolecular decay of this intermediate ($k_3 = 0.17$ s^{-1}) occurs by a process in which $RSSR^{\bullet-}$ (−410 mV) reduces $GOase_{semi}$ to $GOase_{red}$ (159 mV). The S to Cu separation is 10.2 Å, and the driving force, 569 mV (i.e. 410 + 159 mV) at pH 7.0. The driving force is greater than that for the $[Ru(NH_3)_6]^{2+}$ (51 mV) reduction of $GOase_{semi}$ of 108 mV (i.e. −51 + 159 mV).[5] The second-order rate constant in the latter case of 1130 M^{-1} s^{-1} (pH 7.0) reflects the favorable electron self-exchange for the reaction of $[Ru(NH_3)_6]^{2+}$ + $[Ru(NH_3)_6]^{3+}$ (820 M^{-1} s^{-1}),[34] and possibly closer approach of the $[Ru(NH_3)_6]^{2+}$ to the Cu^{II} site. The formation of $RSSR^{\bullet-}$ can be regarded as an escape route for the electron from the metastable PR product. With no provision of H atoms by the $CO_2^{\bullet-}$ reductant it is difficult for the 5 → 3 change in coordination number to take place as part of the initial rapid step. However in the reduction of 4-coordinate $GOase_{semi}$ Tyr495Phe loss of the exogenous H_2O/OH^- is sufficient to bring about the 4 → 3 geometry change and no $RSSR^{\bullet-}$ is formed. Likewise the $CO_2^{\bullet-}$ reduction of $GOase_{ox}$ to $GOase_{semi}$ is more direct (no geometry change at the Cu^{II}). In the $WTGOase_{semi}$ studies no intramolecular electron transfer in the reverse direction from $GOase_{red}$ to RSSR is observed.

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