tion. The reaction mixture stayed purple as the TlCp disappeared and TlCl precipitated. After 1.5 h the reaction was filtered to remove TlCl (1.25 g) and the solvent was removed in vacuo to give a crude purple solid. The crude product was recrystallized from toluene/pentane (1:4) at −30 °C; yield 1.17 g (78%). Its ^{1}H NMR spectrum was identical with that of a previously prepared sample.[3]

(32) Nb(η^5-C_5Me_5)(CHCMe$_3$)Cl$_2$. Nb(CHCMe$_3$)(THF)$_2$Cl$_3$ (1.24 g, 3.00 mmol) was dissolved in 15 mL of benzene. TlC_5Me_5 (1.02 g, 3.00 mmol, see preparation 33) in 5 mL of benzene was added dropwise with stirring. The reaction was stirred for 1 h, and the mixture was filtered to remove TlCl (0.71 g). The solvent was removed from the filtrate in vacuo to give 1.06 g of a purple oil. The oil was sublimed at 65 °C (0.01 μm) onto a 0 °C probe to give the product as purple microcrystals; yield 0.97 g (88%). On this scale the product can be obtained only in low yield (since it is extremely soluble in pentane) by dissolving the purple oil in minimal pentane and cooling to −30 °C for several days.

^{1}H NMR (C_6D_6): τ 4.26 (br s, 1, C*H*CMe$_3$), 8.05 (s, 15, C_5Me_5), 8.88 (s, 9, CHC*Me*$_3$). ^{13}C NMR ($C_6D_5CD_3$, −30 °C, 15 MHz, ^{1}H-gated decoupled): δ 250.2 (br d, J_{CH} = 91 Hz, *C*HCMe$_3$), 121.1 (s, C_5Me_5), 48.8 (s, CHC*Me*$_3$), 31.6 (q, J_{CH} = 126 Hz, CHC*Me*$_3$), 13.0 (q, J_{CH} = 127 Hz, C_5Me_5). IR (Nujol): 2455 (m, ν_{CH_a}) cm^{-1}.

(33) TlC_5Me_5. A mixture of 1.42 g of LiC_5Me_5[16] and 3.80 g of Tl_2SO_4 in 25 mL of THF was stirred for 3 days at 25 °C. All solids were filtered off and the solvent was removed in vacuo. The yellowish residue was sublimed at 70 °C (0.01 μm) to give a bright yellow solid (1.8 g) whose ^{1}H NMR spectrum showed it to be a ca. 1:3 mixture of TlC_5Me_5 (τ 7.80 (d, J_{HTl} = 18 Hz)) and $C_{10}Me_{10}$ (τ 8.2 (6), 8.3 (6), 8.8 (3)). Recrystallization from pentane at −30 °C yielded 0.4 g of dark yellow-orange, pure TlC_5Me_5.

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Stereoelectronic Properties of Metalloenzymes. 6. Effects of Anions and Ferricyanide on the Copper(II) Site of the Histidine and the Tryptophan Modified Forms of Galactose Oxidase

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Abstract: A study of the coordination chemistry of three chemically modified forms of the copper(II)-containing enzyme, galactose oxidase, has been carried out. Alkylation of one unique histidine residue by iodoacetamide yields an inactive enzyme which does not bind CN^- or F^-, a property characteristic of the native enzyme. Oxidation by *N*-bromosuccinimide in 5 mM acetate buffer produces an inactive enzyme which binds F^- and CN^- in a manner very similar to that of the unmodified enzyme. However, after oxidation in 100 mM acetate buffer, CN^- causes a chemical reduction and F^- interacts with the copper(II) ion only weakly. Apparently, the different ionic strengths of 5 and 100 mM acetate buffers allow two and three tryptophans, respectively, to be oxidized. Taken together, these results suggest that different factors affect anion and substrate binding and further suggest that different sites at the one copper(II) center take part in these two processes. The $Fe(CN)_6^{3-}$ ion interacts with all modified forms and the unmodified enzyme to produce similar changes in the optical spectra. However, the ESR spectrum does not disappear with either of the modified forms as has been observed for the unmodified galactose oxidase. This represents definitive evidence that the unique optical changes are not due to the presence of Cu(III) in galactose oxidase. The additional observation of organic radical signals in the ESR spectra of the modified forms in the presence of $Fe(CN)_6^{3-}$ suggest that the loss of the copper ESR signal in the native enzyme under similar conditions may be due to a magnetically coupled copper(II)–radical pair or perhaps a copper(II)–ferricyanide dimer pair.

Introduction

The enzyme galactose oxidase (D-galactose:O_2 oxidoreductase, EC 1.1.3.9, herein referred to as GOase) has recently been the subject of intense study to determine the role of the single type II copper atom in the catalytic mechanism. This enzyme is a single-chain protein of molecular weight 68 000 ± 3000 daltons[1] and catalyzes the reaction: D-galactose + O_2 → D-galactohexodialdose + H_2O_2.[2] From electron spin resonance (ESR),[3] circular dichroism (CD),[4] optical and ligand binding studies,[5] it has been suggested that the inner-sphere coordination of the copper consists of two nitrogen atoms and two oxygen atoms in a pseudo-square-planar array. The two nitrogen atoms have been assigned as histidine imidazoles[3] whereas one oxygen has been suggested to be an exchangeable water molecule or hydroxide ion.[6,7] The origin of the other oxygen atom has not been determined. Even though these studies have shed considerable light on the nature of the copper center, it is equally important to understand the role of other amino acid residues which contribute to the binding and catalysis occurring at the active site. They must be complementary to the coordination chemistry of the copper atom.

Recent chemical modifications of GOase have led to the identification of at least one tryptophan[8] and one histidine[9] residue

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(1) Kosman, D. J.; Ettinger, M. J.; Weiner, R. E.; Massaro, E. J. *Arch. Biochem. Biophys.* **1976**, *165*, 456.
(2) Amaral, D.; Kelly-Falcoz, F.; Horecker, B. *Methods Enzymol.* **1966**, *9*, 87.
(3) Bereman, R. D.; Kosman, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7322.
(4) Ettinger, M. J. *Biochemistry*, **1974**, *13*, 1242.
(5) Giordano, R. S.; Bereman, R. D.; Kosman, D. J.; Ettinger, M. J. *J. Am. Chem. Soc.* **1974**, *96*, 1023.
(6) Melnyk, D. L.; Ettinger, M. J.; Bereman, R. D. *Proc. Int. Cong. Biochem., 6th* **1979**, *Abstr. No. 03-1-5153.*
(7) Bereman, R. D.; Shields, G., submitted for publication.
(8) Kosman, D. J.; Ettinger, M. J.; Bereman, R. D.; Giordano, R. S. *Biochemistry* **1977**, *16*, 1597.

0002-7863/80/1502-6244$01.00/0

at the active site. The histidine moiety is not a copper ligand.[10] Alkylation of the histidine renders the protein inactive although it is indicated from fluorescence and CD studies that substrate still binds. It has been suggested that the histidine influences activity through a critical role both in maintaining the copper chelate conformation and by participating in the oxidation step of the *catalytic* mechanism as a base catalytic group.[9] A maximum of four tryptophans can be oxidized by *N*-bromosuccinimide. The oxidation of two tryptophans, at least one of which is associated with the active site, causes the enzyme to have a reduced affinity for substrate and also leaves the enzyme almost entirely inactive.[8] A fluorescence Förster energy-transfer calculation suggests that one of these tryptophans is a minimum of 12 Å from the copper center.[11] A critical link between the reactive tryptophan and histidine residues has been established from the observation that tryptophan oxidation prevents histidine alkylation and modification of the histidine residue affects the specific fluorescence of the reactive tryptophan residues.[9] In addition, the histidine and the copper centers have been related because the apoenzyme cannot be alkylated specifically, whereas the copper's influence on the tryptophan has been observed by fluorescence studies.[11]

We have undertaken a study of the chemical behavior of the copper(II) centers in these modified forms of GOase for several reasons. Even though the modifications produce an inactive enzyme, the copper(II) center represents the best "model" of that of the protein. In addition, changes in reactivity of the copper(II) may be related to the role of the copper and/or modified residues in the enzymatic reaction.

The relationships of the copper center with the histidine-alkylated and tryptophan-oxidized forms of GOase have been investigated here by using the exogenous ligands, cyanide and fluoride, as probes of the copper atoms since they have been shown to have an extensive and informative coordination chemistry with GOase.[3,5,7] It has been suggested that these anions bind at an equatorial site at the copper center in GOase because of the large changes they cause in the spin Hamiltonian parameters. The properties of anion binding to the modified proteins are compared to those of the native enzyme. In addition, the interaction of ferricyanide with the modified forms of GOase has been studied. This anion has been shown to cause unique spectral changes in native GOase, and these spectral changes have been assigned to the production of Cu(III). Additionally, an increase in the activity of the enzyme is observed.[12,13] Those observations also led to a postulate that Cu(III) is the active copper form in the native enzyme.[13] It was thought that knowledge of how this anion interacts with the modified forms of GOase might further our understanding of the interaction of ferricyanide with the native GOase.

Materials and Methods

Galactose oxidase was purified from cultures of *Dactylium dendroides* following literature procedures.[1] *N*-Bromosuccinimide (NBS) was purchased from Eastman Chemical, and potassium fluoride, sodium cyanide, and potassium ferricyanide were purchased from Baker and used without further purification. Iodoacetamide was purchased from Sigma and recrystallized from benzene prior to use.

Reaction with *N*-Bromosuccinimide. Oxidation of GOase with NBS at pH 4.15 in 5 or 100 mM sodium acetate buffer was performed and analyzed as described elsewhere.[8] The number of tryptophans oxidized was determined by the decrease in absorbance at 280 nm, as described by Spande and Withop,[14] by using a value of 104 000 for the molar extinction coefficient. In all cases, the enzyme was titrated with NBS until a maximum of 2% of the original activity remained as determined by a coupled assay.[1] All protein solutions were dialyzed exhaustively into 100 mM sodium phosphate buffer, pH 7.0, prior to spectroscopic analysis.

Reaction with Iodoacetamide. GOase was incubated with 5 mM iodoacetamide in 100 mM sodium phosphate buffer, pH 7.0, at room temperature as described previously.[9] The extent of reaction was determined by coupled assay activity measurements, and the reaction was allowed to proceed until virtually no activity remained. Excess iodoacetamide was removed by exhaustive dialysis. Previous work has shown that exactly one histidine residue is alkylated by this procedure.

Electron Spin Resonance Spectra. ESR spectra were obtained with a Varian E-9 spectrometer operating near 9-GHz microwave frequency. Spectra were calibrated with diphenylpicrylhydrazyl radical (DPPH) as an internal standard in conjunction with a Hewlett-Packard microwave frequency meter. Vanadyl acetylacetonate was employed to calibrate the hyperfine splittings. Various microwave power settings and modulation levels were checked to determine where the best resolution could be obtained. Microwave powers of 30–40 mW and modulation levels of 3.2 G represented the best instrument settings.

Anion Titrations. Enzyme samplers were concentrated to 0.2–0.5 mM. Ligand solutions were prepared in 100 mM sodium phosphate buffer at pH 7.0, and the titrations were carried out as previously described.[5]

Optical Spectra. Optical spectra were obtained on a Cary 17 recording spectrophotometer, using a 0–0.1 absorbance scale slidewire to increase the sensitivity. Enzyme samples of approximately 1 mg/mL were prepared in 100 mM phosphate buffer, pH 7.0, and passed through a 0.1-μm Millipore filter prior to spectral measurements. Ferricyanide was added to both the sample and reference cells to record a difference spectrum in an attempt to eliminate its absorbance from the resulting spectra.

Table I. ESR Parameters of GOase and Its Derivatives

	$A_\parallel$	$g_\parallel$	$A_\perp$	$g_\perp$
native[a]	175.0	2.277	small	2.055
NBS (5 mM)	174.4	2.284	small	2.054
NBS (100 mM)	166.3	2.275	small	2.059
alkylated	175.0	2.265	small	2.052
native + CN^-	155.8	2.226	small	2.050
NBS (5 mM) + CN^-	160.6	2.245	small	2.045
NBS (100 mM) + CN^-	reduction			
alkylated + CN^-	no reaction			
native + F^- [a]	159.7	2.305	small	2.050
NBS (5 mM) + F^-	169.1	2.279	small	2.045
NBS (100 mM) + F^-	mixture of species			
alkylated + F^-	no reaction			

[a] From ref 3.

Results

Cyanide and Fluoride Binding to NBS-Oxidized Galactose Oxidase. Anion binding to GOase is altered after selective tryptophan oxidation under some but not all oxidative reaction conditions. Kosman et al. have shown that when GOase is oxidized in 5 mM acetate buffer, a product results which has approximately 2% of the native enzyme's activity.[8] On the basis of spectroscopic analysis, 2 of the 18 tryptophan residues in the enzyme have been oxidized under these conditions.[8] However, at least one additional residue is more reactive in 100 mM acetate buffer than in 5 mM acetate buffer, and a composite total of three residues must be oxidized before the enzyme reaches 2% of its original activity.[8]

When GOase is treated with NBS in 5 mM acetate buffer and then dialyzed back into 100 mM phosphate buffer, pH 7.0, it reacts with cyanide and fluoride in a manner *similar* to that of the native protein. That is, when CN^- is added to the enzyme solution in a ratio of 1:1, cyanide:GOase, there is an immediate change in the ESR spin Hamiltonian parameters (see Table I). As higher concentrations of cyanide are added, there are no further alterations in the spectrum. Fluoride requires higher concentrations for saturation to be reached in the NBS-modified form than in GOase but also results in changes in the spin Hamiltonian parameters which are similar to those observed for the native GOase. This is not an unexpected result because the spin Hamiltonian parameters of the 5 mM acetate–NBS-oxidized GOase are not very different from the native enzyme (see Table I). Furthermore, it has been observed that no net change occurs in the copper optical activity in the 350–600-nm region as compared to the native protein when GOase is modified in 5 mM acetate buffer.[8] These

(9) Kwiatkowski, L. D.; Siconolfi, L.; Weiner, R. E.; Giordano, R. S.; Bereman, R. D.; Ettinger, M. J.; Kosman, D. J. *Arch. Biochem. Biophys.* **1977**, *182*, 712.
(10) Bereman, R. D.; Peisach, J.; Mims, W., unpublished results.
(11) Weiner, R. E.; Ettinger, M. J.; Kosman, D. J. *Biochemistry* **1977**, *16*, 1602.
(12) Dyrkacz, G. R.; Libby, R. D.; Hamilton, G. A. *J. Am. Chem. Soc.* **1976**, *98*, 626.
(13) Hamilton, G. A.; Adolf, P. K.; deJersey, J.; DuBois, G. C.; Dyrakacz, G. R.; Libby, R. D. *J. Am. Chem. Soc.* **1978**, *100*, 1899.
(14) Spande, T. F.; Witkop, B. *Methods Enzymol.* **1967**, *11*, 498.

all seem to indicate that there is little change in the inner coordination sphere environment of the copper atom when GOase is treated with NBS under these conditions.

When GOase is treated with NBS in 100 mM acetate buffer, however, the spin Hamiltonian parameters (Table I) suggest that the copper center has been more perturbed. Consistent with this suggestion are the CD results which show that the copper optical activity around 600 nm is approximately doubled after NBS oxidation of two tryptophan residues in 100 mM acetate buffer.[8] When GOase is oxidized with NBS in 100 mM acetate buffer, the interaction of the metal center with cyanide and fluoride is also altered. When cyanide is added to the enzyme solution at increasing ratios of cyanide to GOase, the intensity of the ESR signal constantly decreases, leaving only a small fraction of the signal by a 10:1 ratio. The loss of the ESR signal under the conditions of excess cyanide indicate that the copper(II) has been reduced to copper(I). The ESR signal which remains at a 10:1, cyanide:GOase ratio, is sufficiently weak that it is difficult to identify the g and A values. Yet, it appears to be similar, if not identical, with that of the ESR signal resulting from cyanide addition to the GOase oxidation product in 5 mM acetate. Since spectroscopic analyses of the oxidation product in 100 mM acetate show that typical preparations were modified to the extent of 3.1–3.4 trytophan residues, it appears that a total of four residues are accessible to oxidation in 100 mM acetate buffer. The typical preparation then has a mixture of enzymes consisting of a large fraction which has three tryptophans oxidized, a small fraction with only two residues oxidized, and therefore also a small fraction with four residues oxidized. The reaction between the oxidation product in 100 mM acetate buffer and fluoride shows that only a very small fraction binds (two tryptophans oxidized) whereas the remainder does not appear to interact with the copper at all, even at very high fluoride concentrations.

When cyanide is added to iodoacetamide-treated GOase at 1:1 and 10:1 ratios of cyanide:GOase, no changes can be observed in the ESR spectrum. Thus, at these concentrations, cyanide does not interact with the copper center of the alkylated GOase. At higher ratios of cyanide:GOase (50:1 and 100:1), a loss of intensity of the ESR signal is observed as well as a loss of intensity of the visible optical spectrum, indicating a reduction of Cu(II) to Cu(I). Fluoride does not cause any noticeable changes in the ESR spectrum of the histidine-alkylated GOase even at very large concentrations of fluoride, again showing a lack of anion–copper interaction.

The ESR spectrum of the histidine-alkyated GOase is quite similar to that of the native protein, indicating that the inner-sphere ligands have not changed. It has been recently confirmed by pulsed electron spin resonance experiments that the histidine which is alkylated is not one of the two copper–ligand groups. Small changes in the copper geometry, however, have been suggested previously from the large reduction of the copper optical activity and visible absorption bands, perhaps indicating the formation of a more centrosymmetric environment about the copper. Such a geometrical change could cause protein groups to move in such a way that the copper center is no longer accessible to anions.

Ferricyanide Interaction with the NBS-Oxidized Galactose Oxidase. It has been noted previously that the addition of ferricyanide to a solution of native GOase causes a disappearance of the ESR signal due to Cu(II).[12] At higher concentrations of ferricyanide, the formation of a sharp radical signal at $g = 2.00$ is also observed in the ESR spectrum.[15] It has been suggested that the loss of the ESR signal and change in optical features were due to the presence of Cu(III).[12,13]

When ferricyanide is added to a solution of GOase modified by NBS in 5 mM or 100 mM acetate buffer in a ratio 20:1, $Fe(CN)_6^{3-}$:GOase, the ESR spectrum remains *unchanged* in both intensity and spin Hamiltonian values, although the formation of a radical signal is observed at $g = 2.00$. (See Figure 1). The addition of 0.5 M ammonium sulfate to the enzyme solutions caused no changes in the ESR results even though it has been

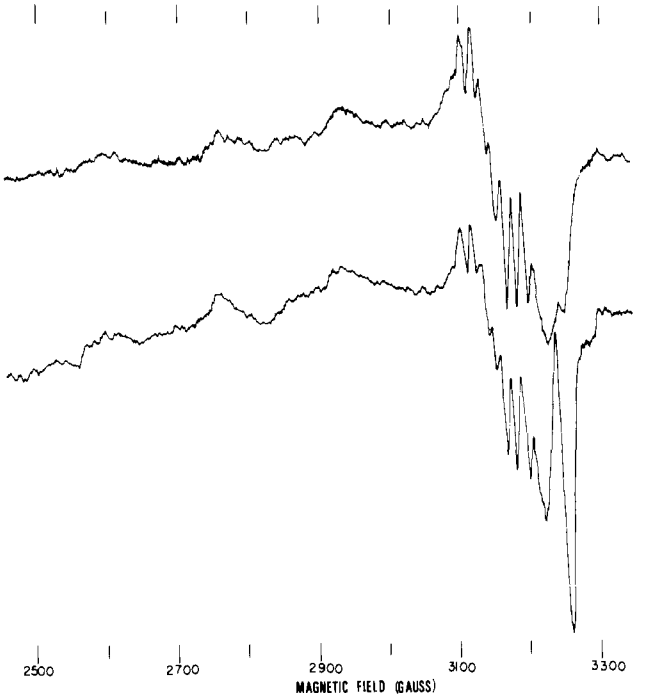

Figure 1. ESR spectrum at 120 K of NBS-oxidized galactose oxidase (upper spectrum) and the same sample in the presence of $Fe(CN)_6^{3-}$ (5 mM acetate oxidation product).

suggested that sulfate alters the interaction of native GOase with ferricyanide.[16] Since the ESR results indicated that ferricyanide was not interacting with the copper center when the enzyme was modified, an optical spectrum of the Cu(II) absorbances in the modified protein in the presence of ferricyanide was obtained to compare with that of GOase.[13] Although it is technically difficult to obtain these spectra due to the high absorbance of ferricyanide around 400 nm, the spectrum at higher wavelengths was observed to change in approximately the same manner as has been observed for the interaction of ferricyanide with native GOase. That is, increases in absorbance are noted at 445, 630, and 800 nm. The only difference noted is that the increases at each of these wavelengths is only about 75–80% of that observed with the native enzyme. Because these optical changes can occur *without* a reduction in the ESR signal intensity, the change *cannot* be due to an oxidation of Cu(II) to Cu(III) as had been previously assigned.[13] These results further support the concept that the effect of ferricyanide on the optical absorbance and paramagnetic characteristics of GOase are distinct.[17]

Ferricyanide Interaction with the Histidine-Alkylated Galactose Oxidase. The interaction of ferricyanide with the histidine-alkylated form of GOase again produces no change in the intensity or spin Hamiltonian parameters of the ESR spectrum. However, the formation of a radical signal centered about $g = 2.00$ is again observed. In this case the radical appears to be either based on a nitrogen center ($I = 1$) or coupled to two protons since three lines are observed. (See Figure 2). Such proton coupling could arise from a radical centered on a tyrosine,[18] whereas a nitrogen-centered radical may suggest that the position of the radical is at or near the modified histidine. It is not certain whether the ferricyanide is interacting with the protein in the same vicinity as in the native or NBS-modified protein or whether the modification causes the ferricyanide to interact at another site in the enzyme. The optical absorption spectrum was again obtained. There were no observed differences between the absorbance of the modified enzyme and that of the interaction of the modified

(15) Ettinger, M. J.; Reinhammer, B. R. M., personal communication.

(16) Ettinger, M. J.; Kosman, D. J., personal communication.

(17) Bereman, R. D.; Ettinger, M. J.; Kosman, D. J.; Kurland, R. J. *Adv. Chem. Ser.*, **1977**, *No. 162*, 263.

(18) Sjoberg, B.-M.; Reichard, P.; Graslund, A.; Ehrenberg, A. *J. Biol. Chem.* **1978**, *253*, 6863.

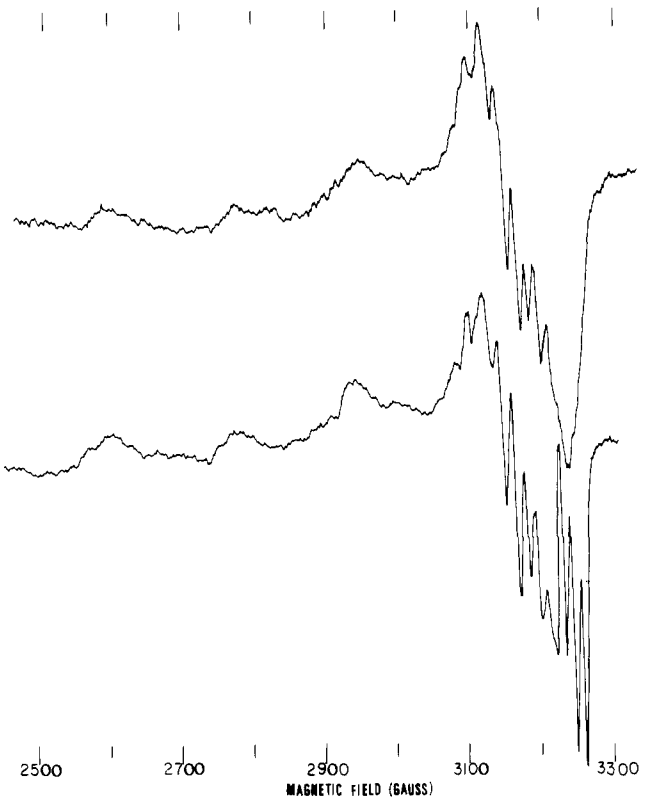

Figure 2. ESR spectrum at 120 K of alkylated galactose oxidase (upper spectrum) and the same in the presence of $Fe(CN)_6^{3-}$.

enzyme with ferricyanide in the visible region. Because the copper absorbances in the alkylated enzyme are weak, it may not be possible and/or necessary to observe differences in the spectra to preclude a ferricyanide–copper(II) interaction similar to that observed for the NBS-modified enzyme. We therefore can not draw any conclusions about the interaction of the histidine-alkylated form of GOase with ferricyanide based on optical results.

Discussion

The oxidation in 5 mM acetate buffer of two tryptophans of GOase has left an enzyme whose properties about its metal center remain basically unchanged either in spectral properties or chemical properties in terms of anion binding. Yet a major difference between the modified and native enzymes is that the specific oxidation of two tryptophans has left an enzyme which has a reduced affinity for sugar substrates[9] and is inactive. It is clear then that there are different factors involved in the binding of substrates to GOase and in the binding of anions to the enzyme.

As a third tryptophan is oxidized by NBS, the GOase undergoes several structural changes at the copper center as is evidenced by CD data and ESR spectral changes.[8] The anions tested do not bind to the metal center except in small quantities, and this is likely due to small inhomogeneities in the enzyme oxidation preparation. Yet substrate binding studies show that even after tryptophan oxidation in 100 mM acetate buffer, the substrate has been observed by fluorescence measurements to bind normally to the enzyme.[9] This observation can be understood when it is noted that the early substrate binding measurements were carried out on enzyme which had been modified in 100 mM acetate buffer but only to the extent of *two* tryptophans. The oxidation in 100 mM acetate buffer, however, is specific for a composite total of *three* tryptophan moieties. It is therefore quite possible that the modified enzyme preparation and 100 mM acetate buffer used in these earlier studies may have consisted of a mixture. As much as one third of the enzyme may not have had the one specific moiety oxidized which is critical to substrate binding.

Because we have been able to selectively oxidize first two and then three tryptophan residues to GOase, it is possible to observe the different roles for these residues. It appears that one (but perhaps both) of the first two residues oxidized is crucial for substrate binding to GOase whereas the third, different tryptophan is necessary to maintain a copper(II) coordination environment necessary for anion binding. This provides further evidence that the site for substrate binding is not *exactly* the same as for anion binding, which is thought to occur at an equatorial site of the copper(II). Conversely, the anion-binding study of the histidine-alkylated GOase, coupled with previous substrate-binding experiments, shows that factors which affect equatorial anion binding to GOase do not necessarily affect substrate binding. Interestingly, this work shows that a loss of anion binding can be brought about by geometrical changes at the copper center which can be observed by ESR, CD, and optical measurements. However, it appears that the loss of the ability to bind substrates is associated with changes at the copper center which cannot be observed by these methods or by changes somewhere else in the protein. Recently, ^{19}F NMR relaxation studies of GOase in the presence of substrates and/or cyanide show that both substrates and cyanide bind at the copper(II) but at different coordination sites. This suggests an axial site for substrates and an equatorial site for cyanide.[19,20]

The binding of ferricyanide to the NBS-modified protein clearly shows that changes in the optical spectrum brought about by the binding ferricyanide *cannot* be associated with a Cu(III) species because these changes are not accompanied by a decrease in intensity of the Cu(II) ESR signal.

Since the anion-binding ability of the NBS-oxidized enzyme which was prepared in 5 mM acetate buffer is quite similar to that of the unmodified enzyme, and since the optical and ESR spectral parameters are similar to those of the unmodified enzyme, it is difficult to imagine that $Fe(CN)_6^{3-}$ interacts in a much different fashion with this modified enzyme than with GOase. Clearly, however, the results of the interaction as judged by the ESR spectra are different. It may be that the reduction in ESR signal intensity due to ferricyanide interacting with native GOase is caused by a coupling of unpaired spin density on the metal center with that on the observed radical rather than by an oxidation of Cu(II). A small conformational change in the modified protein would cause an increase in distance between these unpaired spins and consequently prevent the spin–spin couple. This suggestion however can only be possible if the ferricyanide undergoes reduction at a rate equivalent to the disappearance of the copper ESR signal. This reduction of ferricyanide by native GOase has been followed optically, and its rate does not appear to be fast enough to support this hypothesis.[17] Another explanation may be that ferricyanide forms a complex with the copper center causing a coupling of unpaired spin density and consequently the loss of ESR signal in the native enzyme. Spin pairing in such a complex might be quite sensitive to the orientation of the ferricyanide with respect to the copper center. Again, conformational changes in the protein brought about by tryptophan oxidation may be sufficient to uncouple the two metal centers. If such a complex forms, it would be expected that ferricyanide must not bind equatorially to the copper because the enzyme modified by NBS in 100 mM acetate buffer does not appear to bind cyanide and fluoride but does not interact with ferricyanide.

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(19) (a) Kurland, R. J.; Marwedel, B. J. *J. Phys. Chem.* **1979**, *83*, 1422. (b) Marwedel, B. J.; Kosman, D. J.; Bereman, R. D.; Kurland, R. J., submitted for publication.

(20) Winkler, M. E.; Bereman, R. D.; Kurland, R. J., submitted for publication.