Role of Tryptophan in the Spectral and Catalytic Properties of the Copper Enzyme, Galactose Oxidase†

Daniel J. Kosman, Murray J. Ettinger,* Robert D. Bereman, and Ronald S. Giordano

ABSTRACT: Previous results indicate that a tryptophan residue(s) may interact with the sugar substrate and Cu(II) atom of galactose oxidase (Ettinger, M. J., and Kosman, D. J. (1974), *Biochemistry 13,* 1248). We now show that *N*-bromosuccinimide (NBS) reduces enzymatic activity to 2% as two tryptophans are oxidized; only four residues are easily oxidized in the holoenzyme. An enzymatic activity vs. number of residues oxidized profile suggests that this inactivation is probably associated with only one of the first 2 residues oxidized. There is no evidence for chain cleavage or modification of amino acids other than tryptophan. While substrate protection is not afforded by the sugar substrate, the activity-related tryptophan is placed within the active-site locus by spectral evidence. NBS oxidation of two tryptophans results in a marked diminution of the large copper optical-activity transition at 314 nm. Under some reaction conditions, a doubling of ellipticity in the 600-nm region of copper CD is also observed. The effects of the NBS oxidation on the CD spectra of galactose oxidase permit the assignment of the 314-nm CD band to a charge-transfer transition and the 229-nm extremum to a specific tryptophan contribution. The A_{zz} parameter from electron spin resonance spectra is also markedly reduced by the NBS oxidation. Moreover, while cyanide binds to the native enzyme without reducing the Cu(II) atom, cyanide rapidly reduces the Cu(II) atom to Cu(I) in the NBS-oxidized enzyme. These CD and ESR results are taken to suggest that one aspect of the inactivation by NBS oxidation may be a conversion of the pseudosquare planar copper complex in the native enzyme to a more distorted, towards tetrahedral, complex in the inactivated enzyme. Since the inactivation can be accomplished without affecting binding of the sugar substrate, tryptophan oxidation must affect catalysis per se.

Galactose oxidase (D-galactose:O_2 oxidoreductase EC 1.1.3.9) is a single-chain enzyme of molecular weight 68 000 ± 3000 which is secreted extracellularly by cultures of *Dactylium dendroides* (Kosman et al., 1974; Cooper et al., 1959; Nobles and Madhosingh, 1963). This enzyme catalyzes the reaction: D-galactose + O_2 → D-*galacto*-hexodialdose + H_2O_2. ESR[1] and optical spectra establish that the single Cu(II) atom in galactose oxidase belongs to the type II or "nonblue" family of protein cupric coppers[2] (Blumberg et al., 1965; Giordano and Bereman, 1974; Ettinger, 1974). Galactose oxidase is the only known copper protein which contains a single, nonblue Cu(II) atom per molecule with no other prosthetic group.[3]

A principal goal of our work is to identify the endogenous ligands to the Cu(II) atom and any nonligand protein groups within the active site locus which have critical roles in the overall spectral and catalytic properties of the Cu(II) active site. ESR and model studies led to the tentative assignment of the "in-plane" ligands to two nitrogen and two oxygen atoms (Giordano and Bereman, 1974; Kosman et al., 1974; Peisach and Blumberg, 1974). One of the latter nuclei may be contributed by a water molecule (Fabry et al., 1969; Marwedel et al., 1975; Bereman et al., 1977). Recent chemical-modification experiments show that a histidine residue may participate as a general base catalytic group in galactose oxidase and that this group also has a marked influence on the properties of the Cu(II) atom (Kosman et al., 1977; Kwiatkowski et al., 1977). This paper focuses on the critical roles of tryptophan residues in the spectral and catalytic properties of galactose oxidase.

Various spectral measurements suggest that a tryptophan residue or residues may be near the copper atom in galactose oxidase. An optical difference spectrum between the apo- and holoenzymes shows a difference peak at 292 cm which is indicative of an influence of the Cu(II) atom on tryptophan(s) environment(s) (Ettinger, 1974). Fluorescence is also markedly affected by removal of the copper atom (Weiner et al., 1977). Furthermore, dihydroxyacetone, which is an excellent substrate, and the *galacto*-hexodialdose product both have profound effects on the tryptophan optical activity exhibited by galactose oxidase (Ettinger and Kosman, 1974). While it is difficult to imagine how an indole moiety could directly act as a copper ligand, the results reported herein demonstrate that oxidation of probably a single tryptophan residue in galactose oxidase has profound effects on both the catalytic activity and spectral properties of the Cu(II) atom in galactose oxidase.

Materials and Methods

Galactose oxidase was isolated from fungal cultures and the apo- and holoenzymes were purified as described previously (Kosman et al., 1974).

D-galactose, NBS, and D-raffinose were purchased from Sigma.

Reaction with NBS. The oxidation of galactose oxidase was

† From the Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214. *Received April 7, 1975.* This is a contribution from the Bioinorganic Graduate Research Group of the Departments of Biochemistry and Chemistry. Supported in part by National Science Foundation Grant BO40662.

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NBS, *N*-bromosuccinimide; CD, circular dichroism; ESR, electron spin resonance.

[2] The classification of Cu(II) sites in proteins into two classes was originally based on comparisons of $A_{\parallel}$ values for the different Cu(II) types in the "blue", multicopper proteins (see Malkin and Malmström, 1970). Although the Cu(II) site in galactose oxidase is not necessarily similar to the "nonblue" sites in these other proteins, such general classifications still remain useful for purposes of comparison.

[3] Although uricase and ribulose diphosphate carboxylase were purported to contain copper (Mahler, 1963; Wishnick et al., 1969), neutron activation and flameless atomic absorption analyses show that neither protein contains copper (Schallinger, L., and Ettinger, M. J., unpublished results).

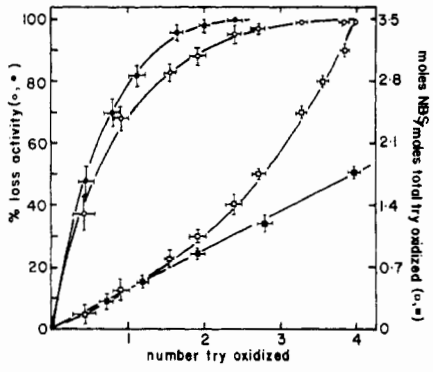

FIGURE 1: Correlation of the number of tryptophans oxidized with loss of activity and tryptophan reactivity. Experimental details are given under Materials and Methods. The individual types of experiments are: oxidation of hologalactose oxidase in 5 mM sodium acetate buffer (●); oxidation of hologalactose oxidase in 100 mM sodium acetate buffer (○ and □); oxidation of apogalactose oxidase in 100 mM sodium acetate buffer (■). The ranges indicated are for from 3 to 5 separate experiments.

accomplished and monitored by the method of Spande and Witkop (1967). The reaction was carried out in 5 mM sodium acetate buffer at pH 4.15 at a protein concentration of 0.5 mg/mL. Where indicated, products were also isolated after reaction in 100 mM acetate buffer at the same pH. Enzyme activity was monitored by a spectrophotometric, coupled assay (Kosman et al., 1974). The number of tryptophans oxidized was determined by the decrease in absorbance at 280 nm (Spande and Witkop, 1967) using the value 104 000 for the molar extinction coefficient (Kosman et al., 1974). Spectral studies were performed after exhaustive dialysis against 100 mM sodium phosphate buffer at pH 7.0. Enzyme subjected to the identical procedures except for the actual addition of NBS served as a control.

Amino Acid Analysis. Following hydrolysis of the apoenzyme by aqueous *p*-toluenesulfonic acid in the presence of tryptamine to preserve tryptophan (Liu and Chang, 1971), analyses were performed on a Beckman 120C autoanalyzer (Spackman et al., 1958).

Gel Electrophoresis. Disc gel electrophoresis was carried out in 7.5% acrylamide prepared in the pH 8.6 Tris–glycine buffer of Rustum et al. (1971) containing 0.1% each of sodium dodecyl sulfate and β-mercaptoethanol. Sample preparation followed the previous procedures (Kosman et al., 1974).

End-Group Analysis. The dansylation procedure described by Gray (1967) was followed. Following hydrolysis in 6 N HCl, the dried sample was extracted with acetone and spotted on polyamide plates (Gallard-Schlesinger). The plates were developed first with a solvent mixture of 90% formic acid and H_2O (3:200). Based upon R_f values in the first dimension, selected standards were chosen and spotted for development in the second dimension, using a solvent consisting of *n*-heptane, 1-butanol, and acetic acid (3:3:1). Glycine was often visualized in the chromatograms, but could be removed by sodium dodecyl sulfate gel filtration of the protein on Sephadex G-50 prior to dansylation. The spot corresponding to this amino acid was the only one affected by this treatment.

Copper Determinations. Copper contents were determined with a Perkin-Elmer 360 atomic absorption spectrophotometer equipped with a 2100 graphite furnace. Optimal conditions for drying, charring, and atomization were established with the unmodified protein. Copper standards were prepared from a commercial standard (Alfa).

Protein Concentration. Protein concentrations were determined from the $E_{280\ nm}^{1\%,\ 1\ cm} = 15.4$ (Kosman et al., 1974).

TABLE I: Amino Acid Composition of Native and NBS-Oxidized Galactose Oxidase.[a]

Amino Acid	Moles of Residue/68 000 g of Protein	
	Native	NBS Oxidized[b]
Lys	25.9	25.8
His	7.8	7.9
Arg	28.2	28.0
Cys	4.5	4.6
Asp	76.1	75.9
Thr	60.8	61.1
Ser	68.9	68.8
Glu	32.9	33.0
Pro	42.1	42.1
Gly	70.0	69.9
Ala	43.0	43.0
Val	39.1	39.0
Met	12.9	12.8
Ile	32.0	31.9
Leu	27.2	27.2
Tyr	22.7	22.6
Phe	21.8	21.9
Trp	17.8	15.3[b]

[a] Hydrolyses were for 22 h in 3 N *p*-toluenesulfonic acid, 0.5% tryptamine (Liu and Chang, 1971). Moles of residue/68 000 g are calculated using Ala = 43.0. [b] Spectrophotometric titration (Spande and Witkop, 1967) indicated 2.3 residues of Trp were oxidized by NBS.

Corrections were made for absorbance changes after tryptophan oxidation with the NBS-modified enzyme.

CD Spectra. Spectra were recorded as described previously (Ettinger, 1974). Although the CD spectrum of galactose oxidase contains an extremum at 610 nm (Ettinger, 1974), spectra were not recorded at higher wavelengths to avoid damage to the electrooptic plate supplied by the manufacturer. Slit widths were programmed to maintain a constant 15-Å bandwidth. solutions for binding studies were prepared as previously described (Ettinger and Kosman, 1974). Spectra given in figures are averages from two to five separate experiments. When the NBS reaction was carried out in 100 mM acetate, products contained 2.1 to 2.5 oxidized tryptophans. After reaction in 5 mM acetate, spectra were obtained on a product with 2.0 residues modified.

ESR Spectra. ESR spectra were determined and analyzed as previously reported (Giordano and Bereman, 1974). Enzyme concentrations ranged between 15 and 45 mg/mL. The effect of the CN^- on the NBS-modified enzyme was investigated in such a way that 10 μL of a standard CN^- solution, when added to 100 μL of sample, yielded the CN^- to NBS–galactose oxidase molar ratios indicated. After the samples were mixed for 5 min, they were frozen to 120 K and spectra were recorded.

Results

Inactivation by NBS Oxidation. Oxidation of 2 of the 18 tryptophan residues in galactose oxidase results in a product which has approximately 2% of the native enzyme's activity (Figure 1). This inactivation is accomplished with a 3.5 molar ratio of NBS to tryptophan residues modified. A total of four tryptophans are readily susceptible to NBS oxidation, at which point residual enzyme activity is below 1%. Addition of further large excesses of NBS causes enzyme activity to drop below the limits of detection. With such treatment, protein absorbance increases, indicating the onset of tyrosine oxidation. Thus,

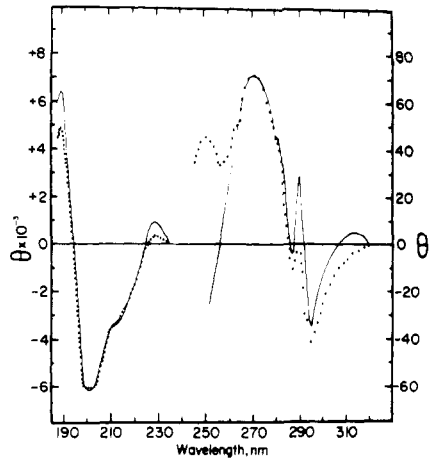

FIGURE 2: Ultraviolet CD spectra of galactose oxidase (—) and NBS-modified galactose oxidase after 2.0 residues were oxidized in 5 mM acetate buffer, pH 4.15 (• • •). Spectra were recorded at ~1.5 mg/mL of protein in 0.1 M sodium phosphate buffer, pH 7.0. Overlapping spectra were recorded in cells of 0.1-, 0.01-, and 1.0-cm path length. Note different ellipticity scales for far- and near-UV regions.

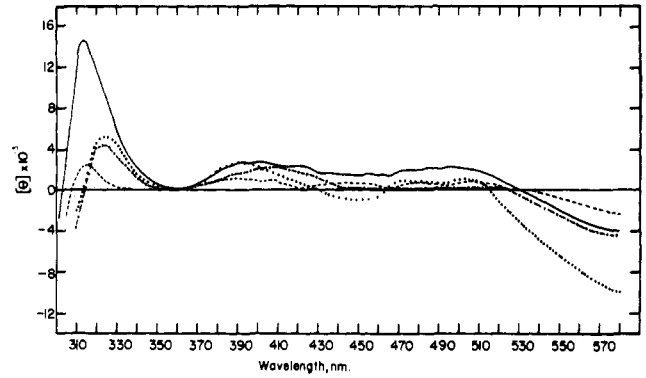

FIGURE 3: Copper CD spectra of unmodified galactose oxidase exposed to the reaction conditions (—), NBS-modified galactose oxidase after reaction in 100 mM acetate (• • •), NBS-modified galactose oxidase after reaction in 5 mM acetate (- • -); each spectrum obtained after dialysis into 0.1 M sodium phosphate buffer, pH 7.0; and of unmodified galactose oxidase in 5 mM acetate buffer, pH 4.15 (- - -). All spectra were recorded at ~1.5 mg/mL of protein in a 5-cm path-length cell.

the plateau in enzymatic activity at two residues modified most likely reflects a discrete modification of two tryptophan residues, rather than a composite total arising from a modification of several different residues in different enzyme molecules. At least one additional residue seems to be more reactive in 100 mM than 5 mM acetate buffer, since activity does not reach 2% until a composite total of two to three residues are oxidized under these conditions (Figure 1). In contrast to the native enzyme, the apoenzyme reacts linearly with increasing NBS concentration (Figure 1). Thus, the high accessibility and/or reactivity of the reactive residues in the native enzyme is markedly dependent on the precise conformation of the copper-containing enzyme, even though only small differences can be detected between the protein conformations of the apo- and holoenzymes (Ettinger, 1974).

Sugar substrates do not prevent the inactivation by NBS modification. The enzyme is still inactivated by a 3.3 molar excess of NBS in the presence of 0.2 M D-galactose or in 5 mM raffinose. In the presence of either substrate, an increase, rather than the normal decrease, in absorbance at 280 nm occurs with inactivation, which suggests that some modification of tyrosine residues may contribute to inactivation under these conditions.

Analysis of Product. In sodium dodecyl sulfate gel electrophoresis under reducing conditions, the galactose oxidase product with two tryptophans oxidized still migrates as a single component. Thus, there is no evidence for any peptide bond cleavage associated with the NBS reaction. N-terminal analyses of the native and modified protein indicate that a single N-terminal alanine is present in each sample. Amino acid analysis demonstrates that the NBS reaction is confined to tryptophan residues (Table I). Furthermore, both the native enzyme and the product with two tryptophans modified contain 1 g-atom of copper per mole.

Ultraviolet CD Spectra of NBS-Oxidized Galactose Oxidase. The most striking difference between the far-ultraviolet CD spectra of NBS-modified galactose oxidase and native enzyme is at the 229-nm extremum (Figure 2). Prior to these modification studies, no unambiguous assignment could be given to this band (Ettinger, 1974). However, the observed 50% diminution of the 229-nm extremum in the spectrum of the product with two tryptophans modified suggests that this band is associated with tryptophan optical activity. The far-ultraviolet CD spectra indicate that there is no marked change in protein conformation associated with the NBS modification.

The sharp extrema at 290 and 295 nm in the near-ultraviolet CD spectrum of galactose oxidase can be assigned to tryptophan optical activity (Ettinger, 1974). Modification of two tryptophans results in a specific reduction in the 290-nm extremum (Figure 2), and smaller changes in the 295-nm region. The only other change induced by NBS oxidation is the appearance of a new peak near 250 nm in the spectrum of the modified protein (Figure 2). This optical activity probably arises from oxindole groups; the absorption spectrum of oxindole has a maximum at 250 nm (Green and Witkop, 1964). The fact that changes in the near-ultraviolet CD upon NBS oxidation are small demonstrates again that oxidation of two tryptophan residues does not lead to gross changes in protein conformation.

Copper Optical Activity in NBS-Oxidized Galactose Oxidase. The Cu(II) atom in galactose oxidase exhibits unusually large optical activity which is markedly sensitive to substrate or product binding and to changes within the active-site environment (Ettinger and Kosman, 1974; Kosman et al., 1977). Oxidation of two tryptophan residues leads to a large reduction in the 314-nm CD band (Figure 3). Copper optical activity between 350 and 550 nm is also markedly affected by tryptophan oxidation (Figure 3). The effect of NBS modification on copper optical activity in the 600-nm region is somewhat dependent on the reaction conditions. After modification in 5 mM acetate, no net change in this region is observed, while the ellipticity is approximately doubled after NBS oxidation of the same number of residues in 100 mM acetate (Figure 3).

Acetate itself has large effects on the copper optical activity of galactose oxidase, by virtue of acetate anion binding to the Cu(II) atom (Figure 3). However, since the CD effects of acetate on the native enzyme can be reversed by dialysis, the effects of acetate on the inactivation profile and on CD following the NBS modification cannot simply be due to an acetate–copper interaction. Moreover, fluorescence spectra of galactose oxidase in various buffers demonstrates that the effects dependent on acetate concentration are largely ionic-strength effects (Weiner et al., 1977).

ESR Parameters of NBS-Oxidized Galactose Oxidase and the Effects of Cyanide. That oxidation of two tryptophan

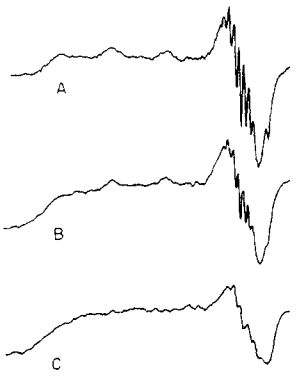

FIGURE 4: Effect of cyanide on the ESR spectrum of NBS-modified galactose oxidase with 2.0 residues oxidized. (A) In the absence of cyanide; (B) at a 1:1 molar ratio of cyanide to modified enzyme; (C) at a 10:1 molar ratio of cyanide to modified enzyme.

TABLE II: Spin Hamiltonian Parameters for Native and NBS-Oxidized Galactose Oxidase.[a]

Native Galactose Oxidase	NBS-Oxidized Galactose Oxidase
$g_{xx} = 2.058$	$g_{xx} = 2.065$
$g_{yy} = 2.048$	$g_{yy} = 2.055$
$g_{zz} = 2.273$	$g_{zz} = 2.267$
$A_{xx} = 28.8$	$A_{xx} = 30.6$
$A_{yy} = 30.1$	$A_{yy} = 31.7$
$A_{zz} = 176.5$	$A_{zz} = 166.0$

[a] Hyperfine splittings in Gauss.

residues is sufficient to markedly affect the Cu(II) atom in galactose oxidase is also demonstrated by the large effect the modification has on the nuclear hyperfine splitting parameters for galactose oxidase (Table II). A substantial reduction in the A_{zz} parameter occurs with the oxidation of two tryptophan residues.

Cyanide binds stoichiometrically to galactose oxidase without changing its redox state when present at an equimolar concentration to the enzyme (Giordano et al., 1974). The effects of cyanide on the ESR spectra of NBS-oxidized galactose oxidase reveal profound changes within the copper chelate of the enzyme. When CN^- ion is added at molar ratios of 0.5:1, 1:1, 6:1, 10:1, or 20:1 to the modified enzyme, the ESR signal markedly decreases in intensity (Figure 4). At a 10:1 molar ratio, most of the signal due to the modified enzyme disappears. However, the shape of the ESR signal that remains, for example, at a 1:1 ratio of CN^- to modified enzyme, is identical to that of the modified enzyme alone (Figure 4). This fact, coupled to the fact that no evidence for copper removal by cyanide is obtained by atomic absorption analyses, indicates that the reduction of the ESR signal with cyanide is due entirely to a rapid reduction of the Cu(II) atom to Cu(I). Cyanide is known to form stable Cu(I) complexes (Malkin and Malmström, 1970).

Galactose Binding to NBS-Oxidized Galactose Oxidase. Selective tryptophan oxidation does appear to affect galactose binding under some, but not all, reaction conditions. Binding of 0.2 M D-galactose to galactose oxidase in a deoxygenated solution can easily be detected by following changes in copper CD spectra (Ettinger and Kosman, 1974). The reduction in copper optical activity by galactose after modification in 100 mM acetate (Figure 5) is comparable to its effect on the

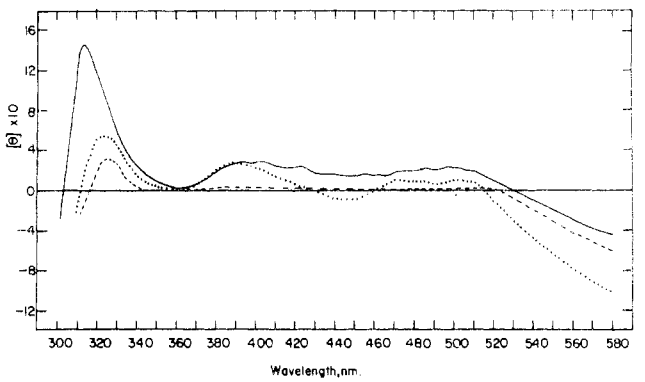

FIGURE 5: Copper CD spectra of unmodified galactose oxidase exposed to the reaction conditions (—), NBS-modified galactose oxidase after reaction in 100 mM acetate buffer, pH 4.15 (· · ·), and a deoxygenated solution of the latter which also contains 0.2 M D-(+)-galactose (- - -). Spectra were recorded at ~1.5 mg/mL of protein in 0.1 M sodium phosphate buffer, pH 7.0, in a 5-cm path-length cell.

spectrum of the native enzyme. However, binding is not detected by CD after modification in 5 mM acetate.

Fluorescence measurements can be used to detect the binding of β-methyl D-galactopyranoside (Weiner et al., 1977). Binding measurements by fluorescence confirm that NBS oxidation in 5 mM acetate yields an enzyme derivative with reduced affinity for the sugar substrate, while modification in 100 mM acetate does not affect subsequent binding in 100 mM, pH 7.0, phosphate (Weiner et al., 1977).

Discussion

The simplest interpretation of the activity vs. number of tryptophans oxidized profile in 5 mM acetate buffer is that two residues in galactose oxidase are about equally reactive towards NBS, but only one of these residues is actually associated with enzymatic activity. Thus, the 60% reduction of activity at a composite total of 1 equiv oxidized would correspond to 60% of enzyme molecules with only the activity-associated tryptophan oxidized and 40% with only the other tryptophan oxidized. At 2 equiv oxidized and 2% activity, these residues are presumed to be modified on 98% of the enzyme molecules. However, similar reaction profiles would be obtained if two residues were involved which influenced activity identically.

The effects of tryptophan oxidation on the copper optical activity of galactose oxidase may aid in the interpretation of the native enzyme's copper CD spectrum (Ettinger, 1974). That the 314-nm band corresponds to a charge-transfer transition is consistent with the effects of NBS oxidation on copper optical activity. Tryptophan oxidation specifically reduces the 314-nm band, while ellipticity in the 600-nm region either increases or does not change. These effects are in marked contrast to effects of exogenous ligands which invariably lead to a uniform reduction in copper optical activity (Ettinger and Kosman, 1974). Thus, one can infer that the 314-nm transition(s) is dominated by a charge-transfer transition that is markedly influenced by a tryptophan residue(s), while the 610-nm band is either a pure d–d transition or involves a different charge-transfer contribution. Since it is difficult to imagine how a tryptophan indole can be an endogenous ligand to the Cu(II), this effect on the charge-transfer transition is most likely mediated by a critical change in copper-chelate conformation which precludes the charge-transfer transition interaction. The fact that no net changes occur in the 600-nm region following modification in 5 mM acetate suggests that

no changes in ligands to the copper atom occur under these conditions.

The marked reduction in the ESR A_{zz} parameter is indicative of reduced electron density along the perpendicular axis of the pseudosquare planar copper chelate within the enzyme. The change is in the same direction and of the same magnitude as those changes induced by exogenous, π-bonding ligands (Giordano et al., 1974). However, since, as expected (Peisach and Blumberg, 1974), such ligand exchange also decreases g_{zz}, the decrease in A_{zz} only upon NBS treatment suggests that the reduction in axial unpaired electron density is due not to ligand exchange but to a change in chelate conformation.

Consistent with this suggestion is the increase observed in the 600-nm region of the CD spectrum following oxidation of two tryptophans in 100 mM acetate. To date, tryptophan oxidation is the only perturbation of the native enzyme which results in an increase in this band. A distortion of the putative pseudosquare planar chelate in the native protein would be expected to increase the rotatory strength of a d–d transition. In as much as Cu(I) is more stable than Cu(II) in a tetrahedral ligand field (Malkin and Malmström, 1970), the ability of cyanide to reduce the copper atom in NBS-oxidized galactose oxidase is also consistent with a less centrosymmetric copper chelate within the modified enzyme.

Any inactivation of an enzyme may be associated with effects on binding, chemical catalysis, or both. While substrate binding is affected by NBS oxidation, the modification appears to also inhibit catalysis per se. The binding properties of modified enzyme are markedly dependent on the concentration of acetate present during the reaction with NBS. Initial modifications were performed in 100 mM acetate until it was realized that reaction in 5 mM acetate results in a sharper plateau in enzymatic activity at two residues modified. The fact that NBS-oxidized galactose oxidase binds D-galactose at its copper atom following reaction in 100 mM acetate and dialysis into phosphate while this product still has only 2% enzyme activity demonstrates that tryptophan oxidation does affect chemical catalysis per se. Moreover, fluorescence experiments show that even the product following reaction in 5 mM acetate can bind the sugar substrate in 100 mM acetate (Weiner et al., 1977). Fluorescence results also indicate that the differences in the reaction and reaction product which are related to acetate concentration most likely reflect general ionic strength effects. That this influence of acetate involves some indirect effect is also indicated by the fact that the copper site itself is saturated in either 5 or 100 mM acetate, as evidenced by copper CD.

In summary, oxidation of the activity-related tryptophan(s) presumably interferes with its interactions with the Cu(II) atom and protein groups within the active-site locus, which in turn markedly alters the characteristic geometry and electronic structure of the copper chelate which presumably are essential for its catalytic properties. Under conditions where affinity for substrate is decreased by tryptophan oxidation, concommitant changes in protein conformation apparently occur which prevent access to the active-site copper. Such changes must be quite subtle, since they are markedly dependent upon the concentration of acetate in the NBS reaction mixture. The effects that removal of the copper atom have on the spectral properties of tryptophan residue(s) (Ettinger, 1974; Weiner et al., 1977) and the effect of sugar substrate or product binding on tryptophan fluorescence and optical activity (Ettinger and Kosman, 1974; Weiner et al., 1977) are indicative of the close reciprocal nature of whatever the molecular interactions between copper and tryptophan are. Moreover, the tryptophan(s) appears to influence the entire active site, since selective tryptophan oxidation also dramatically alters the reactivity of an active-site histidine (Kosman et al., 1977).

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