Fluorescence Properties of the Copper Enzyme Galactose Oxidase and Its Tryptophan-Modified Derivatives†

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ABSTRACT: Galactose oxidase contains a single nonblue Cu(II) atom and 18 tryptophan residues per molecule. Removal of the copper atom reveals that it has an approximately 29% quenching effect on the relative quantum yield of fluorescence. While saturating concentrations of the sugar substrate of galactose oxidase also reduce the quantum yield, the second substrate, oxygen, has no significant effect on fluorescence in the absence of the sugar substrate. *N*-Bromosuccinimide (NBS) inactivates galactose oxidase as two tryptophans are oxidized (Kosman, D. J., Ettinger, M. J., Bereman, R. D., and Giordano, R. S. (1977), *Biochemistry, 16*). Oxidation of two tryptophans also leads to a disproportionately large decrease in fluorescence intensity. A 23% reduction in quantum yield with blue-shift occurs with oxidation of 0.85 tryptophan equiv and a further 25% quenching is obtained as the reaction proceeds to 2.0 residues oxidized. Fluorescence experiments with the modified enzyme show that it contains at least one tryptophan residue which is unreactive towards NBS, but which also interacts with the Cu(II) atom and substrate. These results taken together substantiate the postulate that one or more tryptophan residues, the Cu(II) atom, and the sugar substrate mutually interact within the native enzyme. Energy-transfer calculations suggest that this residue(s) which must be within a relatively hydrophobic environment is at least 12 Å from the Cu(II) atom.

Galactose oxidase (D-galactose:O_2 oxidoreductase, EC 1.1.3.9) is a single-chain enzyme of molecular weight 68 000 ± 3000 which contains one Cu(II) atom, 18 tryptophans, 23 tyrosines, and 22 phenylalanines per molecule (Kosman et al., 1974). Several observations place one or more tryptophan residues within the copper-containing active-site locus of galactose oxidase. An optical difference spectrum between apogalactose oxidase as the sample and the holoenzyme as reference contains a positive peak at 292 nm (Ettinger, 1974). Thus, inclusion of the copper atom appears to force at least one tryptophan residue into a somewhat less hydrophobic environment. The distinctive tryptophan optical activity exhibited by the native enzyme is dramatically altered by binding of either the substrate dihydroxyacetone or the product, *galacto*-hexodialdose (Ettinger and Kosman, 1974). Moreover, galactose oxidase is inactivated without peptide-chain cleavage as two tryptophan residues are oxidized by reaction with NBS[1] (Kosman et al., 1977a). The inactivation profiles imply that probably only one of the first 2 residues oxidized affects activity. Since this inactivation by indole oxidation is accompanied by large changes in copper optical activity and ESR hyperfine splitting parameters (Kosman et al., 1977a), at least one of the oxidized residues is most likely in close proximity to the copper atom.

Given the high sensitivity of fluorescence to tryptophan environment in proteins, we turned to this method to further characterize the interactions between copper and tryptophan(s) and between substrates and tryptophan(s), the environment(s) of the active-site tryptophan(s), and the nature of the inactivation by NBS. Copper–tryptophan interactions per se have general relevance to copper proteins, since this type of interaction has been postulated to play a role in the spectral properties of "blue" copper proteins (Finazzi-Agró et al., 1973; Morpurgo et al., 1972). Fluorescence measurements with enzyme plus substrate are also particularly relevant, since they may detect substrate–tryptophan interactions which cannot be detected by changes in optical activity (Ettinger and Kosman, 1974). Fluorescence data also can be used to estimate the distance between the Cu(II) atom and a tryptophan residue.

Materials and Methods

Galactose oxidase was isolated from fungal cultures as previously described (Kosman et al., 1974). The holo- and apoenzymes were chromatographically purified to homogeneity (Kosman et al., 1974). D-Glucose and NBS were purchased from Sigma, β-methyl D-galactopyranoside was from Mann, and *N*-acetyltryptophan methyl ester was from Cyclo Chemicals, Inc.

Fluorescence Spectra. Fluorescence emission spectra were recorded on a standard Aminco-Bowman spectrophotofluorimeter. Spectra of protein samples and *N*-acetyltryptophan methyl ester as a standard were recorded several times over a 2-h period to yield an average spectrum recorded for fluctuation in lamp intensity. Measured fluorescence was corrected at 5-nm intervals by the method of Chen (1967) for variation in phototube and monochromator response. Thus, the ordinate of the plots, designated fluorescence, is proportional to quanta emitted by the sample; excitation was always at 280 nm. Bandwidth for both excitation and emission was set at 12 nm by a literature method (Melhuish, 1962). Relative quantum yields were estimated from the areas under the corrected spectra by Simpson's rule and the absorbancy at the exciting wavelength (Chen, 1965). To calculate the absolute quantum

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[1] Abbreviations are: NBS, *N*-bromosuccinimide; ESR, electron spin resonance; CD, circular dichroism.

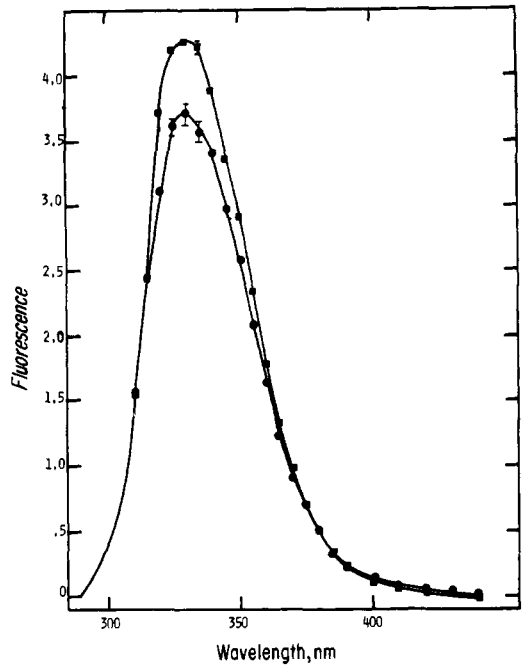

FIGURE 1: Corrected fluorescence emission spectra of galactose oxidase (●) and of the apoenzyme (■) recorded in 0.1 M potassium phosphate buffer, pH 7.0. The protein concentration was ~0.065 mg/mL. Error bars present the standard deviation of time-averaged recordings.

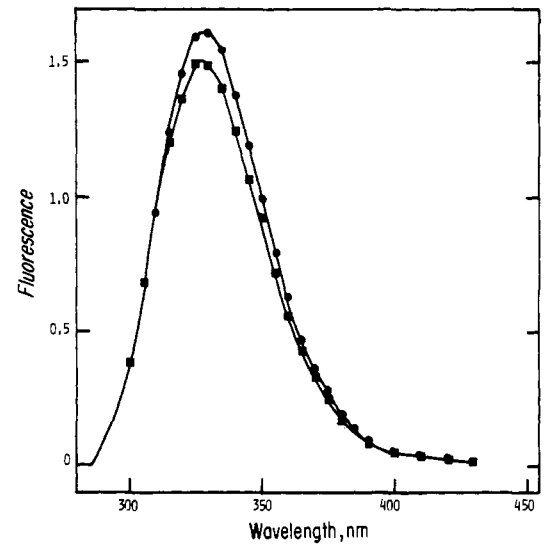

FIGURE 2: Corrected fluorescence emission spectra of a deoxygenated solution of galactose oxidase (●) and a deoxygenated solution of the enzyme with 0.2 M β-methyl D-galactopyranoside (■) in 0.1 M potassium phosphate, pH 7.0. The protein concentration was 0.1 mg/mL.

yield of the sample, *N*-acetyltryptophan methyl ester was used as a model. This tryptophan derivative was also utilized to test for nonspecific fluorescence effects of substrates. A temperature-regulated cuvette holder in conjunction with a circulating water bath kept the sample at 25 ± 0.3 °C.

Deoxygenated solutions of sugar substrate and oxygen-saturated enzyme solutions were prepared in a manner similar to that reported for analogous CD experiments (Ettinger and Kosman, 1974).

Reaction with NBS. Oxidation of galactose oxidase with NBS at pH 4.15 in 5 or 100 mM sodium acetate buffer was performed and analyzed as described elsewhere (Kosman et al., 1977a). In experiments designed to correlate progressive oxidation of tryptophan residues with fluorescence, two different procedures were employed. In the first, two solutions of galactose oxidase of absorbancy at 280 nm of approximately 0.15 and 0.9 were prepared in the acetate buffer. Each was tritrated with an identical molar ratio of NBS. The more dilute solution was monitored by fluorescence, the other by absorbance measurements at 280 nm; both were monitored for enzymatic activity employing a coupled assay (Kosman et al., 1974). The second procedure involved titrating a solution of galactose oxidase ($A \approx 0.9$ at 280 nm) with NBS. The activity and absorbancy were monitored as before and aliquots were withdrawn, diluted to $A \approx 0.15$ in the acetate buffer, and subjected to fluorescence measurements. Selected aliquots were also dialyzed into 0.1 M phosphate buffer (pH 7.0). The correlation of fluorescence with activity and residues oxidized was quite similar for these two methods.

Oxindole groups were assumed to make no significant fluorescence contribution (Bannister et al., 1972; Imoto et al., 1971). Copper was removed from NBS-oxidized enzyme by the same procedures used with unmodified enzyme (Kosman et al., 1974). A Perkin-Elmer 360 atomic absorption instrument with a 2100 graphite furnace was used to confirm that the copper had been removed from the modified enzyme.

Results

Effect of Copper on Tryptophan Fluorescence. Maximal fluorescence intensity for galactose oxidase occurs at 330 nm (Figure 1). The 20-nm blue shift from the *N*-acetyltryptophan methyl ester model indicates that several of the 18 tryptophans of the enzyme must be within relatively hydrophobic environments (Teale, 1960; Van Duuren, 1961). This is also reflected in the calculated quantum yield of 0.159 relative to 0.07 for the tryptophan model (Holmes and Robbins, 1974). If the excitation wavelength is varied from 270 to 295 nm, no evidence for significant tyrosine to tryptophan energy transfer is detected at a 12-nm bandwidth (Weber, 1961). Thus, as anticipated, fluorescence in galactose oxidase is dominated by its large number of tryptophan residues.

The single Cu(II) atom of the holoenzyme markedly influences the fluorescence intensity of the enzyme. Thus, the quantum yield for the apoenzyme is 29% higher than that of the native enzyme (Figure 1).

Effects of Substrates on Fluorescence. Galactose oxidase catalyzes the oxidation of galactose by molecular oxygen to its aldehyde product. Hydrogen peroxide is the second product. Since all available galactose absorbs at 280 nm, β-methyl D-galactopyranoside, which is also an excellent substrate, was used in fluorescence experiments (Maradufu et al., 1971). Fluorescence spectra obtained in the presence of this substrate in the absence of oxygen show that the sugar substrate reduces the relative quantum yield by 6.7% (Figure 2). The emission spectra in the presence of sugar substrate are slightly blue shifted. Similar quenching by substrate is obtained in pH 7.0 phosphate and pH 4.15 acetate. The effects of β-methyl D-galactopyranoside are specific, since this substrate has no effect on the fluorescence of the tryptophan model. Furthermore, D-glucose (50–400 mM) which is neither a substrate nor an inhibitor of galactose oxidase, has no effect on the enzyme's fluorescence spectrum.

In marked contrast to the effect of galactose, oxygen has no significant specific effect on the fluorescence intensity of the enzyme. Oxygen quenching (up to 5–10%) of tryptophan fluorescence has been reported by others (Parker and Rees, 1960), and the effect of saturating an *N*-acetyltryptophan methyl ester solution with oxygen is a 2% quenching of fluorescence.

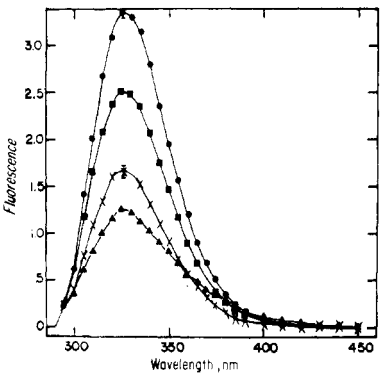

FIGURE 3: Corrected fluorescence emission spectra of deoxygenated solutions of galactose oxidase, unmodified (●) with 0.85 oxidized tryptophans (■), 2.0 oxidized tryptophans (X), and 3.0 tryptophans oxidized (▲) by NBS. Spectra were recorded in 100 mM sodium acetate buffer, pH 4.15, after the modification was performed in 5 mM acetate buffer, pH 4.15. The protein concentration was ~0.14 mg/mL. The error bars represent the standard deviation of time-averaged recordings.

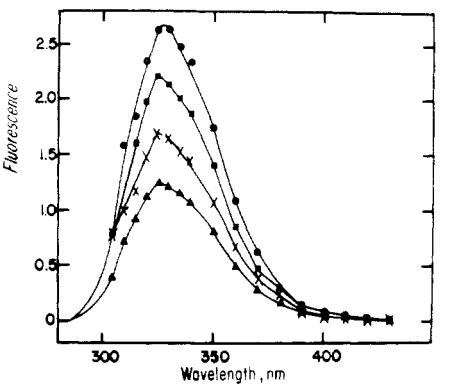

FIGURE 4: Corrected fluorescence emission spectra of deoxygenated solutions of galactose oxidase, unmodified (●), with 0.85 oxidized tryptophans (■), 2.1 oxidized tryptophans (X), and 2.7 tryptophans (▲) oxidized by NBS. Spectra were recorded in 100 mM sodium acetate buffer, pH 3.97, after the modification was performed in 100 mM sodium acetate buffer, pH 3.97. The protein concentration was ~0.086 mg/mL.

An oxygen-saturated solution of galactose oxidase shows a 3.2% decrease in quantum yield relative to a solution at air concentration of oxygen.

Effects of NBS Oxidation on Fluorescence. Galactose oxidase activity plateaus at 2% of native enzyme activity as two tryptophans are oxidized by NBS in 5 mM acetate buffer and activity is ultimately abolished by further modification (Kosman et al., 1977a). These first two residues oxidized apparently also make a disproportionately large contribution to the total fluorescence of galactose oxidase. A 23% reduction in quantum yield occurs with oxidation of 0.85 tryptophan equiv and an additional 25% quenching is observed as the reaction proceeds to 2.0 residues oxidized (Figure 3). In contrast to these results, oxidation of the third equivalent of tryptophan results in a diminution of fluorescence which more closely approximates $\frac{1}{18}$ of the total fluorescence of the native enzyme, i.e., 5.3% (Figure 3). The fluorescence spectrum of galactose oxidase with 0.85 tryptophan equiv oxidized is blue shifted with respect to the native enzyme's spectrum, as evidenced by the greater quenching to the red of the fluorescence maximum (Figure 3). A further blue shift in the fluorescence spectrum is associated with the oxidation to two tryptophans, while no additional spectral shift is observed as a third tryptophan equiv is oxidized (Figure 3).

When the NBS oxidation is carried out in 100 mM acetate buffer, relatively large changes in fluorescence are again associated with modification of the first 2 equiv of tryptophan (Figure 4). At 0.85 residue oxidized there is a 17% reduction in quantum yield and an additional 18% reduction occurs as oxidation proceeds to 2.10 residues oxidized. A greater change (11.9%) in fluorescence occurs as a third equivalent is oxidized in 100 mM acetate than occurs when the NBS reaction is in 5 mM acetate (Figure 4). This is the expected result if only the two readily oxidized tryptophans exhibit unusually high fluorescence and if in 100 mM acetate a third tryptophan reacts with NBS at a comparable rate. Thus, greater heterogeneity of the oxidation mixture in 100 mM acetate is indicated not only by absorbance and activity measurements (Kosman et al., 1977a), but also by fluorescence. These differences can be attributed to ionic strength efforts. While barely significant differences in enzyme fluorescence are detected between 100 mM acetate buffer (pH 4.15) and 100 mM phosphate buffer (pH 7.0), a significant decrease and red shift is observed between the fluorescence of the enzyme in 100 mM and 5 mM acetate. Similar dependence on ionic strength is seen in phosphate at pH 7.0.

Effects of Substrate on the Fluorescence of NBS-Oxidized Enzyme. No significant interaction of an unmodified tryptophan residue with β-methyl D-galactopyranoside is detectable in 0.1 M phosphate, pH 7.0, after oxidation of two tryptophans in 5 mM acetate (Figure 5A). This result corresponds to the fact that substrate binding is not detected at the copper site by CD under these conditions (Kosman et al., 1977a). However, sugar substrate interaction with an unmodified tryptophan residue is detected in 0.1 M acetate, pH 4.15, after the NBS reaction in 5 mM acetate (Figure 5B). A 10% increase in the quantum yield of the derivatized enzyme occurs in the presence of 0.2 M β-methyl D-galactopyranoside. Since the effect is comparable in magnitude to the effect of substrate on the fluorescence of the native enzyme, it is unlikely that the effect obtained with the modified enzyme reflects substrate binding to any unmodified enzyme contaminant. After NBS oxidation in 100 mM acetate, the sugar substrate affects the fluorescence of an unmodified tryptophan residue(s) even in 0.1 M phosphate, pH 7.0 (Figure 5C). At pH 7.0 or at pH 4.15, substrate quenches the fluorescence of the enzyme derivatized in 100 mM acetate by a similar magnitude as substrate quenches the fluorescence of the native enzyme.

Effects of Copper on the Fluorescence of NBS-Oxidized Enzyme. To determine whether an unmodified tryptophan residue still also interacts with the copper atom after NBS inactivation of the enzyme, the fluorescence spectrum was obtained with a sample of oxidized galactose oxidase after removal of its copper atom. The derivatized apoenzyme gives a 27% higher quantum yield than the holo-modified enzyme (Figure 6). Thus, the apparent effect of copper on the fluorescence of an unmodified tryptophan residue(s) is comparable to the influence of the Cu(II) atom on the fluorescence of native galactose oxidase.

Distance between the Copper Atom and a Tryptophan in the Native Enzyme. A distance between the copper atom and an active-site tryptophan can be estimated if a Förster-type energy transfer (Förster, 1959) is assumed to contribute to the quenching effect of the copper atom. Energy transfer is indicated by the overlap between the fluorescence emission spectrum and the copper absorbance transition at 314 nm (Ettinger, 1974). The efficiency of transfer, E, is given by:

$$E = \frac{(R_0/R)^6}{1 + (R_0/R)^6} \quad (1)$$

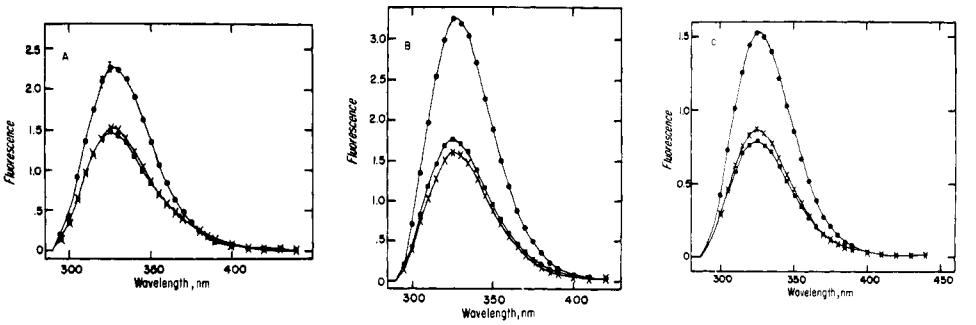

FIGURE 5: Corrected fluorescence emission spectra. (A) Deoxygenated solution of galactose oxidase, unmodified (●), with two tryptophans oxidized by NBS in 5 mM sodium acetate, pH 4.15 (X), and the modified enzyme with 0.2 M β-methyl D-galactopyranoside (■), recorded in 0.1 M potassium phosphate buffer, pH 7.0. (B) Deoxygenated solutions of the enzyme, unmodified (●), with two tryptophans oxidized by NBS in 5 mM sodium acetate, pH 4.15 (X), and the modified enzyme with 0.2 M β-methyl D-galactopyranoside (■) recorded in 0.1 M sodium acetate, pH 4.15. The protein concentration was ~0.1 mg/mL and error bars represent the standard deviation of time-averaged recordings. (C) Deoxygenated solution of the enzyme, unmodified (●), with 2.1 tryptophans oxidized by NBS in 0.1 M sodium acetate, pH 3.97 (X), and the modified enzyme with 0.2 M β-methyl D-galactopyranoside (■) recorded in 0.1 M sodium acetate, pH 3.97.

where R is the distance between the donor–acceptor pair and R_0 is the distance at which the energy transfer is 50% efficient. R_0 is evaluated by the expression:

$$(9.79 \times 10^3)(\kappa^2 Q_D J n^{-4})^{1/6} \text{Å} \qquad (2)$$

where n is the refractive index of the medium taken as 1.5 and κ^2 is the dipole orientation factor. When the donor is free to rotate and the acceptor is fixed, which is probably the most appropriate assumption for the tryptophan → Cu(II) transfer, κ^2 ranges from ⅓ to ⅔ (Wu and Stryer, 1972). J, the spectral overlap integral, was approximated by:

$$J = \sum_\lambda F_D(\lambda) \sum_A (\lambda)\, \lambda^4\, \Delta\lambda / \sum_\lambda F_D(\lambda) \Delta\lambda \qquad (3)$$

where terms were summed over 2-nm intervals, $F_D(\lambda)$ is the corrected fluorescence of the donor at λ and $\sum(\lambda)$ is the molar extinction coefficient of the acceptor at λ. F_D was taken as the difference in fluorescence between the apo- and holoenzymes. Q_D is the quantum yield of the donor in the absence of transfer which can be estimated in this case from the decrease in fluorescence upon NBS oxidation. As a first approximation, the 48% decrease in fluorescence corresponds to a Q_f of 0.459 for each of the first 2 tryptophans oxidized given $Q = 0.159$ for the total protein. Thus, the upper limit on E in eq 1 can be set at 0.541 at this quantum yield.

When κ^2, Q_D, and E are permitted to vary, the calculated range of possible minimal distances between the Cu(II) atom and tryptophan suggest that these two atoms cannot be in contact. If κ^2 is set at its lower limit value of ⅓ at $E = 0.541$, the minimal R calculated is 15 Å. Moreover, large errors in Q_D do not markedly alter this estimate. If the fluorescence contributions of the two NBS-reactive residues were in error by 20%, i.e., their total contribution was only 30%, the calculated minimal value of R would be 12 Å.

Discussion

Fluorescence data substantiate the postulate that one or more tryptophan residues, the Cu(II) atom, and the sugar substrate mutually interact within native galactose oxidase. The reduction in fluorescence intensity upon incorporation of the Cu(II) is consistent with the red-shifted, higher-intensity absorption spectrum of the apoenzyme (Ettinger, 1974). Given a molecule with 18 tryptophans, the apparent influence of the single copper atom on fluorescence is unexpectedly large. This is consistent with the disproportionately large contribution to total fluorescence by the first 2 residues oxidized by NBS as the enzyme is inactivated; i.e., the tryptophan(s) within the

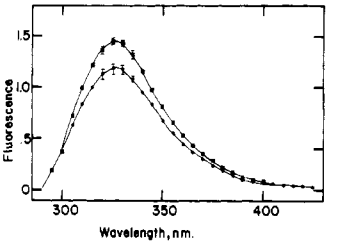

FIGURE 6: Corrected fluorescence emission spectra of galactose oxidase, the enzyme with 2.2 tryptophans modified by NBS (▲) and the modified apoenzyme (■). Spectra were recorded in 0.1 M potassium phosphate buffer, pH 7.0, after reaction was performed in 5 mM sodium acetate buffer, pH 4.15. The concentration of protein was ~0.07 mg/mL and error bars represent the standard deviation of time-averaged recordings.

active-site locus has a relatively large quantum yield. Since the relatively large influence of the single copper atom on fluorescence is not associated with any marked difference in overall protein conformation between the apo- and holoenzymes (Ettinger and Kosman, 1974; Kosman et al., 1974), these effects on fluorescence appear to reflect effects on a tryptophan residue(s) within the active-site locus of the enzyme. The energy-transfer calculations suggest that the copper effects on tryptophan fluorescence are environmental via localized conformation changes, rather than direct quenching or intersystem crossing effects due to contact interaction between a heavy metal atom and tryptophan. Experimental support for this inference is that cyanide (Giordano et al., 1974), which binds within the inner coordination sphere of the Cu(II) atom, does not affect fluorescence. An inner-sphere ligand would have large effects on fluorescence if the Cu(II) atom was exerting a classic heavy metal effect.

The substrate, dihydroxyacetone, and products, *galacto-hexodialdose* and hydrogen peroxide, perturb the enzyme's copper optical activity, tryptophan optical activity, and ESR hyperfine splitting parameters (Ettinger and Kosman, 1974). However, galactose interaction with the copper atom is not detected by ESR, nor with tryptophan residues by CD. Fluorescence proves to be sensitive enough to detect a specific tryptophan interaction with the sugar substrate. The lack of a demonstrable effect of oxygen on fluorescence is of interest in view of the fact that oxygen also does not appear to interact directly with the copper atom in galactose oxidase (Ettinger, 1974). Kinetic and spectral data suggest that oxygen binds after galactose to the enzyme is a sequential scheme (Kwiatkowski and Kosman, 1977). The fact that substrate binding

is detectable by fluorescence in 100 mM acetate in both modified and unmodified enzyme is in itself surprising, since the copper site is apparently saturated with acetate anion at this concentration (Kosman et al., 1977a). The possibility of substrate binding outer sphere to acetate has been suggested (Kosman et al., 1977b).

The marked decrease in quantum yield associated with the oxidation of the first 2 tryptophans suggests that they are in hydrophobic environments; i.e., they must be partially shielded from external solvent. As has been found with papain (Steiner, 1971; Lowe and Whitworth, 1974), reaction with NBS is not precluded by such an environment. Quenching of a fluorescence contribution from a residue in a relatively hydrophobic environment is expected to result in a reduction in quantum yield coupled to a red-shifted fluorescence spectrum (Van Duuren, 1961). However, blue-shifted fluorescence spectra are associated with the reductions in relative quantum yield associated with sugar-substrate binding and the NBS modification. Analogous blue-shift reduction in quantum yield combinations have remained difficult to rationalize in other systems (Steiner, 1971). Presumably, the direction of spectral shift reflects additional specific interactions of either the perturbed residues and/or the remaining unmodified residues.

What is perhaps most surprising about the fluorescence properties of the NBS-inactivated enzyme is that substrate and the copper atom still markedly affect the fluorescence of an unmodified tryptophan residue after the modification. Since oxidation of the first two residues profoundly affects both enzymatic activity and copper-spectral properties, it appears likely that one of these residues makes a contribution to the observed effects of both the copper atom and substrate on the fluorescence of the native enzyme. Furthermore, it is this tryptophan(s) that is accessible to NBS in the native enzyme that is critical to the integrity of the entire active site (vide infra). Hence, it would appear that the unmodified tryptophan residue, which apparently still interacts with the Cu(II) atom and substrate after NBS inactivation, is either also within the active site of the native enzyme or moves to the active site as a consequence of the modification. This residue may, of course, be outside the active-site locus and be affected by both the copper atom and sugar substrate indirectly. In any event, this tryptophan is inaccessible to NBS.

A postulate has been made that a tryptophan residue may contribute to the unusual spectral properties of the blue-copper proteins, stellacyanin and azurin (from most species) (Finazzi-Agró et al., 1973; Morpurgo et al., 1972). Tryptophan is placed in juxtaposition to the copper atom by virtue of the large fluorescence quenching effect that the metal has in both azurin and stellacyanin. Moreover, metal replacement and phosphorescence results suggest that any influence that the tryptophan may have on the copper atom in type 1 Cu(II) systems also involves vicinal environment influences rather than metal–tryptophan contact (Finazzi-Agró et al., 1970, 1973). Results with galactose oxidase reveal that at least one tryptophan residue has a profound influence on the activity of the enzyme by virtue of its apparent role in maintaining the active conformation of the Cu(II) chelate within the enzyme. It is the correlation of inactivation by NBS oxidation with large changes in copper spectral properties which most conclusively places at least one of these residues within the active-site locus of the enzyme (Kosman et al., 1977a). That at least one of the first two tryptophans modified is within the active-site locus is also suggested by the fact that alkylation of the single, active-site histidine residue is also associated with a relatively large change in the fluorescence (17% increase) of the enzyme (Kosman et al., 1977b). Furthermore, while the native enzyme is also specifically inactivated by alkylation of a single, active-site histidine, prior tryptophan oxidation prevents the alkylation reaction. Thus, the integrity of much of the active site in galactose oxidase is markedly influenced by at least one tryptophan within a hydrophobic area at least 12 Å from the Cu(II) atom. These results illustrate the critical roles that nonligand protein groups can have on the structure–function properties of metal active sites.

References

Bannister, W. H., Dalgleish, D. G., Bannister, J. V., and Wood, E. J. (1972), *Int. J. Biochem. 3,* 560.

Chen, R. F. (1965), *Science 150,* 1593.

Chen, R. F. (1967), *Anal. Biochem. 20,* 339.

Ettinger, M. J. (1974), *Biochemistry 13,* 1242.

Ettinger, M. J., and Kosman, D. J. (1974), *Biochemistry 13,* 1247.

Finazzi-Agró, A., Giovagnoli, C., Avigliano, L., Rotilio, G., and Mondovi, B. (1973), *Eur. J. Biochem. 34,* 20.

Finazzi-Agró, A., Rotilio, G., Avigliano, L., Guerrieri, P., Boffi, V., and Mondovi, B. (1970), *Biochemistry 9,* 2009.

Förster, T. (1959), *Discuss. Faraday Soc. 27,* 7.

Giordano, R. S., Bereman, R. D., Kosman, D. J., and Ettinger, M. J. (1974), *J. Am. Chem. Soc. 96,* 1023.

Holmes, L. G., and Robbins, F. M. (1974), *Photochem. Photobiol. 19,* 361.

Imoto, T., Forster, L. S., Rupley, J. A., and Tanaka, F. (1971), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1151.

Kosman, D. J., Ettinger, M. J., Bereman, R. D., and Giordano, R. S. (1977a), *Biochemistry 16* (preceding paper in this issue).

Kosman, D. J., Ettinger, M. J., Weiner, R. E., Giordano, R. S., and Bereman, R. D. (1977b), *Arch. Biochem. Biophys.* (submitted).

Kosman, D. J., Ettinger, M. J., Weiner, R. E., and Massaro, E. J. (1974), *Arch. Biochem. Biophys. 165,* 456.

Kwiatkowski, L., and Kosman, D. J. (1977), *Arch. Biochem. Biophys.* (submitted).

Lowe, G., and Whitworth, A. S. (1974), *Biochem. J. 141,* 503.

Maradufu, A., Cree, G. M., and Perlin, A. S. (1971), *Can. J. Chem. 49,* 3429.

Melhuish, W. A. (1962), *J. Opt. Soc. Am. 52,* 1256.

Morpurgo, L., Finazzi-Agró, A., Rotilio, G., and Mondovi, B. (1972), *Biochim. Biophys. Acta 271,* 292.

Parker, C. A., and Rees, W. T. (1960), *Analyst 85,* 587.

Steiner, R. F. (1971), *Biochemistry 10,* 771.

Teale, F. W. J. (1960), *Biochem. J. 76,* 381.

Van Duuren, B. L. (1961), *J. Org. Chem. 26,* 7954.

Weber, G. (1961), *Nature (London) 190,* 27.

Wu, C.-W., and Stryer, L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1104.