MODIFICATION OF LACCASE TRYPTOPHAN RESIDUES WITH 2-HYDROXY-5-NITROBENZYL BROMIDE

J. D. CLEMMER, J. CARR, D. B. KNAFF and R. A. HOLWERDA*
Department of Chemistry, Texas Tech University, Lubbock, TX 79409, USA

Received 3 May 1978

1. Introduction

Rhus vernicifera laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) catalyzes the four-electron reduction of molecular oxygen by polyphenols. The enzyme contains four copper atoms, distributed in type 1, type 2, and type 3 (2 Cu/molecule) sites. Mechanistic studies of the reduction of the laccase type 1 (blue) copper atom by mono- and disubstituted hydroquinones have shown that electron transfer rate constants are strikingly insensitive to the thermodynamic driving force for the oxidation of the substrate to the corresponding semiquinone [2,3]. The electron transfer reactivity of the laccase blue copper atom therefore appears to be controlled largely by protein-dependent activation requirements rather than by the oxidizability of the substrate. It has been suggested that the activation process may be dominated by a protein structural change that permits type 2 Cu(II) – bound monoionized hydroquinones to attack the buried blue copper atom [2,3]. Consistent with this hypothesis are Goldberg and Pecht's observations that tryptophan fluorescence enhancements accompanying reduction of *Rhus* laccase type 1 Cu(II) may be linked to a protein conformational change [4].

We report here studies of the modification of laccase tryptophan residues with Koshland's reagent, 2-hydroxy-5-nitrobenzyl bromide (HNBB). Our goal in these studies was to introduce an environment-sensitive chromophore into the metalloprotein and use changes in the chromophore spectrum to monitor the rate of any protein conformational changes. A comparison of the rates of conformational changes detected in this manner with the rate of blue copper reduction would clearly be useful in evaluating the role of conformational changes in the enzymatic mechanism.

The UV-visible spectrum of the 2-hydroxy-5-nitrobenzyl (HNB) chromophore is known to be sensitive to the ionization state of the phenolic hydroxyl group, suggesting that HNB might be useful as a polarity probe [5,6]. In neutral or acidic solutions, HNBB selectively modifies tryptophan residues in protein which contain no accessible sulfhydryl residues [5,6]. This reagent therefore is useful in the quantitative determination of tryptophan in proteins [7] and in studies of the relative accessibilities of these residues under various conditions [8].

2. Experimental

Rhus vernicifera laccase was isolated from lacquer acetone powder and purified by the method of Reinhammer [9]. Sigma Grade II 2-hydroxy-5-nitrobenzyl bromide and sodium ascorbate were used as received, as was Eastman reagent grade hydroquinone. Other materials and methods used in preparing anaerobic solutions of laccase and reducing agents for use in spectroscopic studies were identical with those described previously [10]. Modification of laccase tryptophan residues was achieved through the addition of 50- to 100-fold excesses of HNBB to stirred, unbuffered solutions of the native protein

*To whom correspondence should be addressed

(20–40 μM). The pH was held constant through manual additions of dilute NaOH. The modified protein (HNB-L) was isolated following adsorption onto a CM Sephadex C-50 cation exchange column (2.5 X 30 cm) equilibrated with 0.01 M, pH 6 potassium phosphate buffer (5°C). The column was washed with 0.01 M buffer to remove excess reagent, and HNB-L subsequently was eluted with 0.2 M, pH 6 buffer. Before analysis, the modified protein routinely was dialyzed against two changes of cold triply distilled water.

Analyses of modified laccase samples were performed as follows: (1) The protein concentration was evaluated from a total copper analysis by the spectrophotometric biquinoline method [11], assuming 4 Cu/laccase molecule. These determinations were found to be in excellent agreement with concentrations calculated from 614 nm absorbance values, using the extinction coefficient reported for the native protein (ϵ_{614} = 5700 $M^{-1} \cdot cm^{-1}$) [12]. (2) The number of tryptophan residues modified by HNBB was determined by reading the 410 nm absorbance (ϵ_{410} = 18 000 $M^{-1} \cdot cm^{-1}$) [7] after adjusting the pH of HNB-L solutions to greater than 10 with 1 M NaOH.

Average ionization constants and extinction coefficients for protonated and ionized forms of laccase-bound HNB were determined by recording UV-visible spectra of modified protein solutions in ionic strength 0.1 M sodium phosphate buffers covering the range pH 5–10. A similar procedure was followed for 2-hydroxy-5-nitrobenzyl alcohol, prepared by allowing HNBB to fully hydrolyze. Spectra of fully reduced HNB-laccase samples were obtained by using a Hamilton gas-tight syringe to introduce excess deoxygenated reductant into a serum-capped 1-cm spectrophotometer cell containing the protein under an N_2 atmosphere.

UV-visible spectra were obtained using Cary 17, Aminco DW-2a, and Bausch and Lomb Spectronix-710 spectrophotometers. A Metrohm/Brinkman Model 103 pH-meter, calibrated against pH 4, 7 and 9 standard buffers, was used to make pH measurements.

3. Results and discussion

The results of tryptophan modification studies at various pH's and temperatures are summarized in table 1. Laccase samples modified at room temperature for 0.5–1 h invariably were eluted from CM Sephadex C-50 columns in a single broad band. Analysis of the samples indicated that less than one tryptophan residue per laccase molecule was modified in each case, with the number of residues modified increasing from 0.30 at pH 6.95 to 0.90 at pH 3.00. By contrast, elution of product mixtures obtained after incubation at pH 3.30, 33°C yielded two overlapping bands. The first band (blue) contained laccase with 0.91 HNB/laccase molecule, while the second fraction (blue green) contained more extensively modified protein (2.41 HNB/laccase molecule). Rechromatography of these two separated fractions on 2.5 X 30 cm CM C-50 columns showed each to be homogeneous with respect to HNB content.

Results of spectrophotometric titrations of various

Table 1
Modification of laccase tryptophan residues with 2-hydroxy-5-nitrobenzyl bromide

pH of reaction	Excess HNBB used	Reaction time (h)	Reaction temperature	Moles HNB/ moles protein
6.95	50-fold	0.5	ambient	0.30
6.50	50-fold	0.5	ambient	0.26
6.00	50-fold	0.5	ambient	0.41
5.00	50-fold	0.5	ambient	0.47
4.00	50-fold	0.5	ambient	0.58
3.50	100-fold	1.0	ambient	0.90
3.00	100-fold	1.0	ambient	0.90
3.30	110-fold	1.5	ambient	2.39
3.30	100-fold	1.5	33°C	0.91 2.41

Table 2
Average ionization constants and extinction coefficients for HNB-laccase derivatives[a,b]

Species	pK_a	ϵ_{410} ($M^{-1} \cdot cm^{-1}$)[c]	ϵ'_{410} ($M^{-1} \cdot cm^{-1}$)[c]
0.38 HNB/molecule oxidized	7.20 (0.07)	7.37 (0.29) × 10^3	1.67 (0.03) × 10^4
0.38 HNB/molecule reduced[d]	7.38 (0.07)	5.13 (0.29) × 10^3	1.41 (0.03) × 10^4
0.49 HNB/molecule oxidized	7.37 (0.08)	4.63 (0.20) × 10^3	1.59 (0.24) × 10^4
0.91 HNB/molecule oxidized	7.29 (0.07)	3.19 (0.27) × 10^3	1.44 (0.04) × 10^4
2.39 HNB/molecule oxidized	6.92 (0.13)	2.74 (0.31) × 10^3	9.36 (0.38) × 10^3
2.39 HNB/molecule reduced[e]	6.81 (0.12)	2.04 (0.35) × 10^3	9.48 (0.38) × 10^3
HNB-OH	6.83 (0.01)	0	1.93 (0.01) × 10^4

[a] 25°C, 0.1 M ionic strength sodium phosphate buffers
[b] Standard deviations are given in parentheses
[c] Extinction coefficients expressed in terms of HNB concentration, ϵ_{410} and ϵ'_{410} refer to the protonated and ionized forms of HNB, respectively
[d] Reduction with sodium ascorbate
[e] Reduction with hydroquinone

HNB-laccase samples and of 2-hydroxy-5-nitrobenzyl alcohol (HNB-OH) are presented in table 2. In each case the absorbance at 410 nm (λ_{max} for the ionized HNB chromophore) was read for a series of buffered solutions (pH 5–10) containing equal concentrations of the HNB moiety. Spectrophotometric titration curves for HNB-OH and for both the oxidized and reduced forms of HNB-laccase are given in figs.1 and 2, respectively. A non-linear least squares fit of the spectrophotometric data to the equation:

$$A_{410} = \left[\frac{\epsilon_{410}(H^+) + \epsilon'_{410} K_a}{(H^+) + K_a}\right] (HNB)_{tot}\, \ell$$

was performed, where K_a is the ionization constant of the HNB phenolic-OH group, $(HNB)_{tot}$ is the total concentration of the chromophore, and ℓ is the spectrophotometric path length; ϵ_{410} and ϵ'_{410} are molar extinction coefficients of the protonated and ionized HNB-modified species. The pK_a values in table 2 reveal that the acidity of the HNB label in laccase is only slightly less than that exhibited by HNB-OH in aqueous solution. More importantly, in light of the original aim of this investigation, pK_a values for the oxidized and fully reduced forms of HNB-laccase are essentially identical. The differences between the pK_a values of the HNB group in the

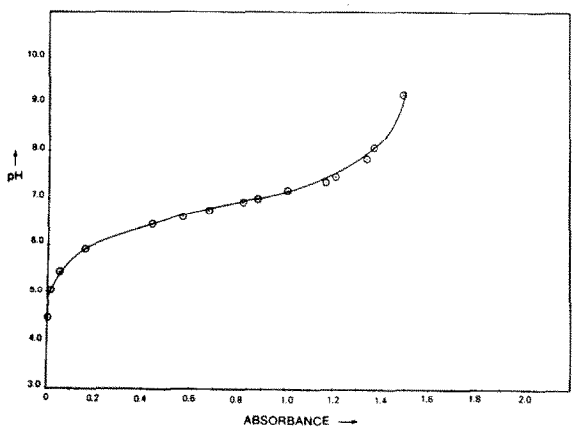

Fig.1. Spectrophotometric titration curve fore 2-hydroxy-5-nitrobenzyl alcohol; λ = 410 nm, μ = 0.1 M (phosphate), (HNB-OH) = 7.64 × 10^{-5} M.

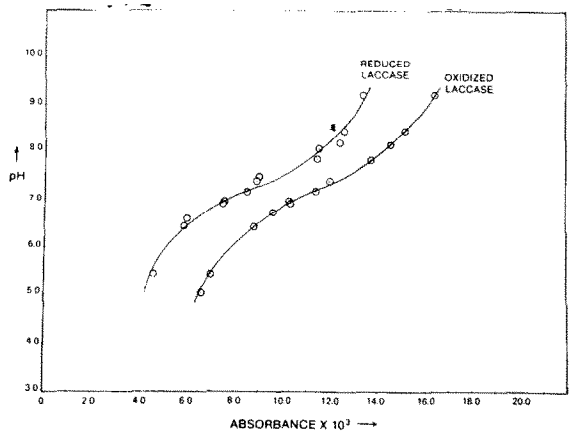

Fig.2. Spectrophotometric titration curves for oxidized and reduced HNB-laccase (0.38 HNB/molecule); λ = 410 nm, μ = 0.1 M (phosphate), $(laccase)_{tot}$ = 2.5 × 10^{-6} M.

oxidized and reduced protein never exceed 0.2 units.

The similarity between pK_a values for the HNB group in laccase containing 1–2-modified tryptophan residues and in aqueous HNB-OH suggests that the laccase-bound chromophore is in an essentially aqueous environment. The two tryptophans (of the total of six present in laccase [9]) most accessible to the solvent thus appear to be the first that are attacked by HNBB. That the pK_a of HNB-OH is in fact sensitive to the polarity of its environment is demonstrated by spectroscopic data presented in fig.3. The visible spectrum of 2-hydroxy-5-nitrobenzyl alcohol in water adjusted to pH 11.2 with NaOH shows only the intense 410 nm absorption maximum characteristic of the phenoxide anion, while the spectrum of HNB-OH in water acidified to pH 2.3 with HCl exhibits a single band at 320 nm characteristic of the protonated phenol. Addition of dioxane to the pH 11.2 HNB-OH solution causes A_{410} to decrease, while A_{320} steadily

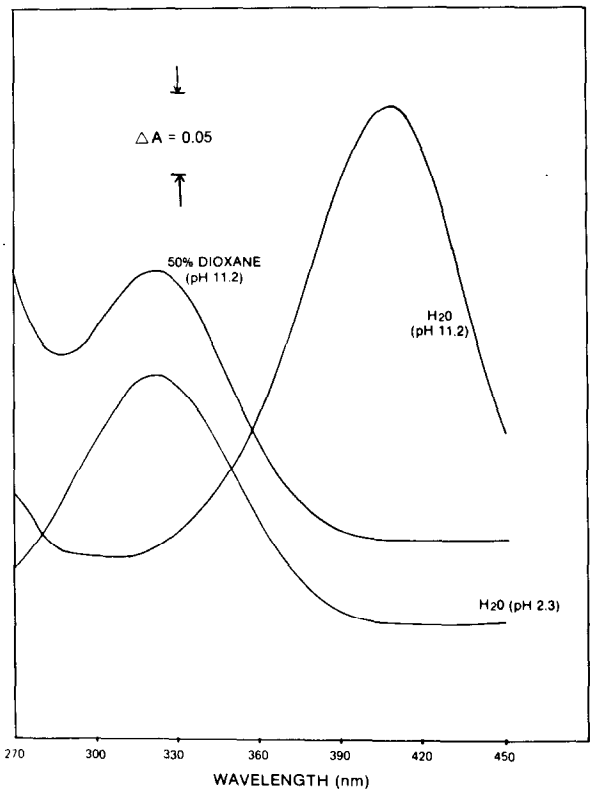

Fig.3. Absorption spectra of 2-hydroxy-5-nitrobenzyl alcohol; (HNB-OH) = 3.32×10^{-5} M.

increases. The spectrum of HNB-OH at pH 11.2 in a 50% dioxane/water mixture exhibits only the band due to the unionized phenol. The pK_a value thus is shifted from that of 6.83 observed in aqueous solution to a value greater than 11 in the mixed solvent system with lower dielectric constant. It seems clear therefore that the HNB group potentially is a good reporter group for protein conformational changes when bound to a tryptophan residue whose environment undergoes a significant change in polarity.

The small change in the pK_a value of laccase-bound HNB found upon reduction of the enzyme clearly does not provide a good basis for the spectrophotometric detection of protein conformational movement. Reduction of laccase with ≤ 1 HNB/molecule does, however, result in substantial decreases in the 410 nm extinction coefficients of both the protonated and ionized forms of the HNB chromophore (table 2). The $\Delta\epsilon_{410}$ (oxidized-reduced) value for the native protein (ca. 400 $M^{-1} \cdot cm^{-1}$) is very small compared with the values of 2.2×10^3 and 2.6×10^3 $M^{-1} \cdot cm^{-1}$ determined for the protonated and ionized forms of HNB-laccase (0.38 HNB/laccase molecule), respectively. These large extinction coefficient differences do provide, in principal, a basis for determining the rate at which the environment of the modified tryptophan residue changes. Presumably either the absorbance of the fully ionized ($pH >> pK_a$) or fully protonated ($pH << pK_a$) HNB chromophore could be followed.

Preliminary kinetic studies of the reaction of ascorbic acid with HNB-L (< 1 HNB/laccase molecule) have shown that the blue copper reduction rate is essentially identical with that observed for the native enzyme (J. D. Clemmer and R. A. Holwerda, unpublished.) Modification of only one tryptophan residue, therefore, probably does not substantially perturb the conformation of the enzyme, consistent with the observation that HNB-L (< 1 HNB/molecule) cannot be separated from the native enzyme either by chromatography or electrophoresis. In contrast, laccase modified with > 1 HNB/molecule can be separated from the native protein by chromatography, suggesting an alternation in conformation that probably renders the more extensively modified laccase unsuitable as a tool for studying the mechanism of the native protein.

An intriguing aspect of the data presented in table 2

is the fact that HNB-OH groups linked to laccase tryptophan residues absorb much more strongly at 410 nm than the free alcohol, which is essentially non-absorbing at this wavelength. It should also be noted that the ϵ_{410} value for both L-HNB-OH and L-HNB-O^- decreases systematically as the number of tryptophan residues modified per molecule increases. At present it is not clear whether these alternations in extinction coefficient result from interaction of the HNB chromophores with its local environment in the protein or are simply associated with tryptophan – HNB adduct formation. It is clear though that the pK_a of HNB-OH can, in general, serve as a sensitive indicator of local environment. Furthermore, modification of the most accessible tryptophan residue in laccase introduces a reporter group that is potentially capable of following the kinetics of protein conformational changes.

Acknowledgements

This work was supported by grants from the National Science Foundation (PCM 76-24131, to D.B.K.) and Research Corporation (to R.A.H.)

References

[1] Malkin, R. (1973) in: Inorganic Biochemistry (Eichhorn, G. L. ed) vol. 2, pp. 689–709, Elsevier, New York.
[2] Holwerda, R. A., Clemmer, J. D., Yoneda, G. S. and McKerley, B. J. (1978) Bioinorg. Chem. 8, 255–265.
[3] Clemmer, J. D., Gilliland, B. L., Bartsch, R. A. and Holwerda, R. A. submitted for publication.
[4] Goldberg, M. and Pecht, I. (1971) Proc. Natl. Acad. Sci. 71, 4684–4687.
[5] Koshland, D. E., Karkhanis, Y. D. and Latham, H. G. (1964) J. Am. Chem. Soc. 86, 1448–1450.
[6] Horton, H. R. and Koshland, D. E. (1965) J. Am. Chem. Soc. 87, 1126–1132.
[7] Barman, T. E. and Koshland, D. E. (1967) J. Biol. Chem. 242, 5771–5776.
[8] Oza, N. B. and Martin, C. J. (1967) Biochem. Biophys. Res. Commun. 26, 7–13.
[9] Reinhammer, B. (1970) Biochim. Biophys. Acta 205, 35–47.
[10] Holwerda, R. A. and Gray, H. B. (1974) J. Am. Chem. Soc. 96, 6008–6022.
[11] Broman, L., Malmström, B. G., Aasa, R. and Vänngård, T. (1962) J. Mol. Biol. 5, 301–310.
[12] Malmström, B. G., Reinhammer, B. and Vänngård, T. (1970) Biochim. Biophys. Acta 205, 48–57.